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Bioavailability of Iron from Wheat *Aegilops* Derivatives Selected for High Grain Iron and Protein Contents

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ABSTRACT: A coupled in vitro digestion/Caco-2 model was employed to assess iron bioavailability from wheat *Aegilops* derivatives selected for high iron and protein contents. The iron content in wheat genotypes used in this study correlated to a great extent with both protein (r = 0.80) and phytate (r = 0.68) contents. The iron bioavailability was based on Caco-2 cell ferritin formation from cooked digests of these derivatives (relative to WL711 control) and correlated positively with dialyzable iron (r = 0.63) and total iron content (r = 0.38) but not with the phytate content. The apparently decreased phytate/iron molar ratios, however, correlated negatively (r = −0.42) with the iron bioavailability, justifying the utilization of these parameters in biofortification programs. Iron bioavailability in the derivatives increased up to 1.5-fold, corresponding to a 1.5−2.2-fold increase observed in iron content over control. These data suggest that biofortification for iron proportionately leading to higher iron bioavailability will be the most feasible and cost-effective approach to combat micronutrient deficiency.

KEYWORDS: biofortification, iron bioavailability, *Aegilops kotschyi*, *Aegilops peregrina* derivatives, Caco-2 cell ferritin, dialyzable iron

INTRODUCTION

More than half of the world's population depending largely on staple foods is affected by hidden hunger, that is, deficiency of iron and zinc micronutrients. Marginal intake of micronutrients and protein contributes to increased morbidity and mortality rates in infants, children, adolescents, and child-bearing women, thus adversely affecting their health and capacity to work.[1] Biofortification of cereal grains employing a combination of conventional and molecular breeding methods appears to be the most feasible, cost-effective, and sustainable approach among the different interventions being currently adopted to overcome micronutrient and protein deficiency.[2]

Wheat is the primary staple food for almost one-third of the world's population. It is expected to assume still greater importance as a source of micronutrients and protein as compared to the other cereals in the future.[3] However, the lack of sufficient genetic variation in grain micronutrient content and grain protein content (GPC) in the cultivated wheat germplasms has limited the ability of plant breeders to improve the micronutrient content as well as GPC.[4,5] An interesting approach for increasing wheat micronutrient as well as protein content lies in the use of related wild wheat germplasms, namely, *Triticum* and *Aegilops* species. These possess a distinct genetic system for wheat biofortification. The wild germplasm of wheat has been reported and reviewed as a rich source of useful variability for grain micronutrients and GPC for the improvement of cultivated wheat.[6,7] *Triticum aestivum* L. and *Triticum turgidum* L. ssp. *durum* (Desf.) are reported to be important sources of dietary protein.[7] The wild wheat germplasm, however, may contain both favorable traits as well as undesirable features such as low yield, fragile spikes, and lodging susceptibility, which may affect the organoleptic properties and thus make them unacceptable for technological as well as commercial applications. Advanced breeding programs have adopted backcross populations as a method for the identification and introgression of useful genes from wild relatives. These possess the potential to improve the agronomic performance of elite cultivated lines.[8] A large number of wheat-alien addition, substitution, and translocation lines have been developed and utilized for wheat improvement as reported and reviewed earlier.[9,10] Introgression of genes responsible for high micronutrient content belonging to the group 2 and 7 chromosomes of *Aegilops kotschyi* [9] and group 4 and 7 chromosomes of *Aegilops perigrina* [10] was observed in their respective derivatives. A series of wheat *Aegilops* amphiploids were reported to have high total iron and zinc concentrations and could also be used as an immortal source of variability for wheat biofortification.[11] Grain iron quality trait loci (QTL) have been mapped on chromosomes 2 and 7 in *Triticum boeticum* × *Triticum monococcum* recombinant inbred (RIL) populations.[12]

Introgression analysis of derivatives, though, indicates an additive effect of the genes/QTLs for high micronutrient content; this may, however, not be translated to corresponding increase in the micronutrient bioavailability, which is defined as the proportion of the total amount of mineral element that is potentially absorbable in a metabolically active form.[13] Iron bioavailability in turn is determined by an array of dietary and host-related factors. Dietary factors contributing to the reduced bioavailability of iron from cereal-based diets include the presence of several inhibitors of micronutrient bioavailability (phytic acid, polyphenols, food fibers, etc.) [2,14] and/or the absence of iron absorption enhancers (muscle proteins, ascorbic acid, inulin, onion, garlic, etc.).[14−16]

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Table 1. Pedigree, Morphological Characteristics, Chromosome Number, and Introgressed Alien Chromosomes of the Selected High Grain Iron and Protein Content BC_2F_2 and BC_1F_3 Derivatives

ID	pedigree	leaf and spike waxiness	1000 kernel weight (g)	harvest index (%)	chromosome no.	introgressed *Aegilops* chromosome(s)[a]
T. aestivum cv. WL711	recepient/control	waxy	41.7	36.5	42	
T. aestivum cv. CS (*PhI*)	recepient	waxy	34.2	32.7	42	
T. aestivum cv. PBW343	recepient	waxy	43.6	35.2	42	
Ae. kotchyi 396	donor parent	nonwaxy	11.4	NR	28	
Ae. kotchyi 3790	donor parent	nonwaxy	13.9	NR	28	
Ae. peregrina 3519	donor parent	nonwaxy	12.5	NR	28	
Ae. peregrina 1155-1-1	donor parent	waxy	13.4	NR	28	
Ae. peregrina 13772	donor parent	waxy	14.7	NR	28	
BC_2F_2 48-41-6	CS/*Ae. kotschyi* 396//PBW343-3///PBW373-41-6	waxy	35.9	17.2	42	$2S^{Ktr}$, $7S^{K}$
BC_2F_2 49-1-11	CS/*Ae. kotschyi* 396//PBW343-3///UP2425-1-11	nonwaxy	42.3	31.8	42	1^{K}, $2S^{K}$, $7S^{K}$, $7U^{K}$
BC_2F_2 49-1-73	CS/*Ae. kotschyi* 396//PBW343-3///UP2425-1-73	nonwaxy	36.7	23.9	43	1^{K}, $2S^{K}$, $7S^{K}$
BC_1F_3 75-1-4	CS/*Ae. kotschyi* 396//UP2382-1-1-4	nonwaxy	40.2	35.3	44	$1U^{K}$, $2S^{K}$, $7S^{K}$
BC_2F_2 54-3-12	CS/*Ae. kotschyi* 396//UP2425-2///PBW373-3-12	waxy	39.4	36.4	44	$2S^{K}$, $7S^{K}$
BC_1F_3 77-36-6	CS (*PhI*)/*Ae. kotschyi* 3790//UP2338-2R-36-6	nonwaxy	38.5	35.0	42	$2S^{K}$, $7S^{K}$
BC_1F_3 77-50-15	CS (*PhI*)/*Ae. kotschyi* 3790//UP2338-2R-50-15	nonwaxy	41.7	36.4	41	$2S^{K}$, $7S^{K}$
BC_2F_2 17-1-3-38	CS (*PhI*)/*Ae. peregrina* 3519//WL 711-3///WL 711-1-3-38	waxy	40.5	28.3	42	$7U^{P} + 4^{P} + 5U^{P}$
BC_2F_2 17-1-2-2	CS (*PhI*)/*Ae. peregrina* 3519//WL 711-1///WL 711-1-2-2	waxy	50.8	28.5	43	$7S^{P} + 4^{P}$
BC_2F_2 1-1-7-18	CS (*PhI*)/*Ae. peregrina* 1155-1-1//PBW 373-1///WL 711-1-7-18	waxy	45.9	25.6	42	$7S^{P}$
BC_2F_2 14-1-8-18	CS (*PhI*)/*Ae. peregrina* 13772//WL 711-1///WL 711-1-8-18	waxy	50.5	26.5	41	$4S^{P}$

[a] Based on GISH and molecular marker analysis described previously.[9,10] K and P represent introgressed chromosome from *Ae. kotschyi* and *Ae. perigrina* respectively, and tr represents an introgressed chromosome with small terminal wheat translocation in the short arm from *Ae. kotschyi*. NR, data not recorded.

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Preliminary screening tools are widely employed for assessing mineral bioavailability to meet the current demand for foods with increased bioavailable mineral content. This goal may be achieved by increasing the mineral content as well as by selectively modifying the amounts of naturally occurring components that inhibit or enhance mineral absorption. One of the major determinants of bioavailability of minerals is the proportion that is absorbed from the gastrointestinal tract. Among the available methods to assess bioavailability, Caco-2 cell model in combination with simulated in vitro digestion is considered to be a relatively rapid and inexpensive method for the prediction of bioavailability of iron as well as zinc and is widely employed for ranking selected genotypes of plant foods.[17−19]

The present study was aimed to analyze the bioavailability of iron in wheat derivatives from *Aegilops* selected for high iron and protein contents using a coupled in vitro digestion/Caco-2 model. The genotypes were ranked on the basis of ferritin formation from Caco-2 cells in response to cooked digests of derivatives relative to the cultivar WL711, which was considered as the control in this study. The derivatives with enhanced bioavailable iron were identified, and their use in future wheat biofortification programs was discussed.

MATERIALS AND METHODS

Seed Material and Field Experiments. The development of wheat addition and substitution lines following interspecific crossing of high grain micronutrient containing *Ae. kotschyi* (acc. 396 and 3790) and *Ae. peregrina* (acc. 3519, 1155-1-1, and 13772)[6] with a bread wheat line 'Chinese Spring' with Ph^1,[20] a suppressor of the wheat Ph^1 gene followed by backcross of the F1 hybrids with elite wheat cultivars WL711, UP2338, PBW343, or 373 has been described in detail by us previously.[9,10] Further characterization of selected derivatives with high micronutrient content for morphology, cytology, microsatellite markers, genomic in situ hybridization (GISH), and fluorescent in situ hybridization (FISH) was also described.[9,10] Table 1 shows a summary of the pedigree and a few important morphological traits from our previous studies,[9,10] which may affect the micronutrient content and bioavailability along with the chromosome number and introgressed alien chromosomes in the selected derivatives.

Chemicals, Enzymes, and Hormones. Unless otherwise stated, all of the chemicals were purchased from Sigma-Aldrich (USA).

Analysis of Total Iron Content. Whole-grain samples from the selected wheat derivatives were washed briefly with 0.1 N HCl followed by Milli-Q water and dried in a hot air oven at 80 °C until constant weight. Grain samples (0.5 g) taken in triplicates were digested in a 2:1 mixture of nitric acid and perchloric acid as per the standard procedure described previously.[6] Digestion was continued until a white residue was obtained. The required volume was adjusted after the completion of the digestion process, and digests were analyzed by atomic absorption spectrophotometry (GBC-Avanta Garde M, Australia).

Measurement of Phytate Contents. The phytate contents of all the wheat genotypes used in this study were quantified and calculated according to an earlier described method.[21] Briefly, 50 mg of the wheat flour was extracted in 1 mL of 0.65 N HCl at room temperature overnight (~12 h) by placing the vials on a rocker. The supernatants were separated by centrifugation, and the phytate content in the supernatant was quantified in a 96-well plate following the modified Wade assay as described previously.[21]

Analysis of Total Protein Content. Total grain proteins were extracted from whole wheat flour of the selected derivatives using urea buffer (2 M urea, 10% glycerol, 65 mM DTT, and 20 mM Tris, pH 8.0) according to an earlier described method[22] and analyzed using the Bradford assay with BSA as the standard. The total protein content was also estimated using a Perten single-kernel near-infrared (SKNIR) system analyzer (Perten Instruments, Inc., Springfield, IL) in 100 grains of each derivative in two replications.

Qualitative Evaluation of Grain Hardness. The grain hardness of the selected derivatives was evaluated qualitatively on the basis of the strength required to crush the seeds manually. The genotypes were ranked as hard, medium hard, medium soft, and soft relative to the WL711 control. The hardness index score of WL711 was previously recorded as 91.20 ± 3.46 (mean ± SEM) analyzed using the Single Kernel Characterization System (SKCS model 4100).

Iron Dialyzability and Bioavailability Assay. *Cell Culture.* Caco-2 cells were procured from the National Centre for Cell Sciences (Pune, India) at passage 27 and were used in the experiments at passage 30−35. Cells were seeded at a density of about 5×10^4 cells/mL in Corning CellBIND surface six well plates (Corning). The cells were grown in Dulbecco's modified Eagle medium high glucose (GIBCO) with phenol red, 25 mM HEPES, and nonessential amino acids supplemented with 10% v/v fetal bovine serum (GIBCO, BRL, Inchinnan, U.K.), 100 units of penicillin, 100 μg of streptomycin, and 250 ng of amphotericin B (GIBCO) per milliliter of medium and maintained in a humidified 5% CO_2 incubator at 37 °C. The medium was changed every alternate day, and the cells were used for the iron uptake experiments at 13 days post seeding.

The growth medium was changed to minimum essential medium (MEM, no. 41500; GIBCO) at pH 7.4 supplemented with 10 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES), 100 units of penicillin, 100 μg of streptomycin, and 250 ng of amphotericin B per milliliter of medium, hydrocortisone (4 mg/L) (Sigma catalog no. H0888), insulin (5 mg/L) (Sigma catalog no. I6634), sodium selenite (5 μg/L) (Sigma catalog no. S5261), tri-iodo thyronine (34 μg/L) (Sigma catalog no.T6397), and epidermal growth factor (20 μg/L) (Sigma catalog no. E4127) 1 day before as well as on the day of the digestion.

Processing of Wheat Samples for Dialyzability and Bioavailability Studies. The wheat grains from the selected derivatives were collected and washed briefly with 0.1 N HCl, followed by Milli-Q water to remove any extraneous contamination, then dried, and milled. The dough was then prepared using Milli-Q water and was cooked in the form normally consumed, that is, made into bread using a microwave oven. During the entire processing, utmost care was taken to avoid iron contamination from any source. The cooked samples were then homogenized, frozen, and lyophilized to dryness. About 0.5 g of the sample was used, and ascorbic acid was added to the samples at a 10:1 molar ratio of ascorbic acid to iron for reasons described previously.[23]

In Vitro Digestion. The in vitro digestion and the treatment of cells with the intestinal digests were performed according to the previously described method with slight modifications.[23] Briefly, a two-chamber system was created by inserting sterilized bicameral inserts fitted with a 12−15 kDa cutoff dialysis membrane into the wells with the Caco-2 cell monolayers with freshly replaced 1 mL of MEM. For gastric simulation, 0.5 g of the cooked wheat samples was treated with Chelex-treated pepsin (Sigma catalog no. P-7000) at pH 2.0 and 37 °C followed by raising the pH to 6.7 and addition of Chelex-treated bile−pancreatin (Sigma catalog no. B-8631 and P1750) solution to a final volume of 15 mL. Two milliliter aliquots of the intestinal digests of each sample were added to the upper chambers of four wells selected randomly from different plates. The plates were then covered and incubated in an incubator shaker for cell culture at 50 rpm/min at 37 °C for 2 h. This was followed by termination of the intestinal digestion stage by removing the insert rings with the digests. An additional 1 mL of fresh MEM was added to the wells, and the plates were incubated for a further 22 h, after which time the cells were harvested for analysis.

For the determination of iron that was dialyzable during the intestinal digestion stage, plates without cells were used and treated identically to those with cells for each replication of the experiment.

Table 2. Total Iron Concentration, Percent of Iron Relative to Wheat Cultivar WL711 Control and Iron Content per Seed in the Cultivars as well as Selected BC_2F_2 and BC_1F_3 Derivatives

	total iron		
ID	concentration mean[a] ± SD (mg/kg)	% of WL711	content[a] (μg/seed)
T. aestivum cv. WL711	26.5 ± 1.1		1.1
T. aetivum cv. Chinese Spring	23.5 a ± 0.5	88.6	0.8 a
T. aestivum cv. PBW343	26.7 ± 0.4	100.7	1.2
Ae. kotschyi 396	65.6 e ± 1.4	247.5	0.7 a
Ae. kotschyi 3790	62.8 e ± 1.6	236.9	0.9
Ae. peregrina 3519	58.4 d ± 1.0	220.3	0.7 a
Ae. peregrina 1155-1-1	60.1 de ± 0.3	226.7	0.8 a
Ae. peregrina 13772	65.8 e ± 0.5	248.2	1.0
BC_2F_2 48-41-6	42.1 bc ± 1.5	158.8	1.5 b
BC_2F_2 49-1-11	53.5 d ± 1.3	201.8	2.3 d
BC_2F_2 49-1-73	44.8 bc ± 2.4	169.0	1.6 b
BC_1F_3 75-1-4	58.3 de ± 0.4	219.9	2.3 d
BC_2F_2 54-3-12	42.9 bc ± 1.2	161.8	1.7 b
BC_1F_3 77-36-6	48.5 c ± 1.6	182.9	1.9 bc
BC_1F_3 77-50-15	45.3 c ± 2.4	170.9	1.9 bc
BC_2F_2 17-1-3-38	55.7 d ± 0.6	210.1	2.3 d
BC_2F_2 17-1-2-2	44.4 c ± 0.5	167.5	2.3 d
BC_2F_2 1-1-7-18	39.6 b ± 0.8	149.4	1.8 bc
BC_2F_2 14-1-8-18	39.7 b ± 0.8	149.8	2.0 c

[a] Different letters represent significant difference from control (WL711), a−d respectively at $p < 0.05$.

Measurement of Dialyzable Iron. The entire volumes of dialysates from the plates without cells were collected for measurement of dialyzable iron at the end of the intestinal digestion period. The dialyzable iron was quantified according to a previously described method.[24] Briefly, the protein precipitant solution (10% trichloroacetic acid, 10% hydroxylamine hydrochloride, and 10% concentrated HCl) was added to equal volumes of dialysates as well as standard aliquots ranging from 0.05 to 1 μg/mL iron (iron standard for AAS 1 mg/mL), at a ratio of 1:2, protein precipitant/dialysates, or standard aliquots and thoroughly mixed. The mixture was heated in a boiling water bath for 20 min and then centrifuged. An aliquot of the clear supernatant was transferred to a clean test tube, and the chromogen solution (25 mg bathophenanthroline sulfonate (Sigma catalog no. B1375) dissolved in 100 mL of 2 M sodium acetate) was added with thorough mixing at a ratio of 2:1 supernatant/chromogen. The formation of Fe^{2+}−bathophenathroline sulfonate was measured spectrophotometrically at 535 nm using a Cary Win 50 UV−vis spectrophotometer (Varian, Inc.) after 10 min against a reagent blank. The level of Fe^{2+} was quantified on the basis of the standard curve.

Harvesting of Cells. The cells were harvested 24 h after the start of the intestinal digestion period. The cell monolayers were washed twice with phosphate-buffered saline (PBS) at pH 7.4, scraped, and finally lysed in 400 μL of lysis buffer (40 mM Tricine (pH 7.8), 50 mM NaCl, 2 mM EDTA, 1 mM $MgSO_4$, 5 mM DTT, 1% Triton X-100). The cell lysates were frozen immediately and stored at −20 °C for protein and ferritin analysis.

Protein and Ferritin Analyses. The protein from the cell lysates was measured using a QuantiPro BCA Assay Kit from Sigma (USA), and ferritin was determined using a Ferritin ELISA Kit from Calbiotech, Inc. (Springvalley, CA). For this assay 25 μL aliquots of each lysates were used in triplicate for ferritin analyses. Protein was read at 562 nm and ferritin at 450/630 nm in an ELISA plate reader (Oasys, Austria). The ferritin formation by the cells in each replicate was normalized to nanograms per milligram of protein.

Statistical Analysis. The values were expressed as the mean ± SEM. The statistical package used was GraphPad Prism 5 (GraphPad Software, Inc., San Diego CA) for the calculation of Pearson correlations, r for total iron, phytate content, grain protein content, iron dialyzability, and iron bioavailability. The same software package was also used for the evaluation of the statistical significance of the results by one-way ANOVA at a 5% level of significance.

RESULTS

Analysis of Total Iron. Table 2 shows the total concentration of iron in the grains (as well per grain) of the selected derivatives used in this study along with the donors and recipient varieties with respect to the control wheat cultivar WL711.

All of the accessions of *Ae. kotschyi* and *Ae. peregrina* had nearly 2−2.5-fold higher iron content than the wheat cultivars. The iron content per seed in these wild varieties was, however, significantly lower than that of the cultivars, and this could possibly be attributed to the smaller size of the seeds and a very low TKW (Tables 1 and 2). The selected derivatives had significantly higher total iron content as well as higher iron content per seed as the seeds were as bold as or even bolder than the cultivar seeds. The TKW of these derivatives was almost equal to or greater than that of the wheat cultivars used in this study. The maximum TKW of almost 51 g was recorded for the *Ae. peregrina* derivative BC_2F_2 17-1-2-2 (Table 1), showing an almost 20% increase in TKW over the WL711 control. The selected derivatives showed almost 1.5−2.2-fold increases in total iron concentration relative to WL711 control. The BC_1F_3 75-1-4 derivative of *Ae. kotschyi* showed the maximum iron content in the selected derivatives followed by the BC_2F_2 17-1-3-38 derivative of *Ae. peregrina*.

Grain Protein and Phytate Analysis. The total grain protein and phytate contents were analyzed and are summarized in Table 3 along with the phytate/iron molar ratios. The wild accessions were found to have very high grain protein content, with the accessions of *Ae. kotschyi* with comparatively higher GPC than that of *Ae. peregrina* used in this study. *Ae. kotschyi* acc. 3790 showed the highest GPC, which was >2-fold higher than the WL711 control, followed by *Ae. kotschyi* acc. 396 (1.9-fold). All of the accessions of *Ae. peregrina* showed around 1.5−1.6-fold higher GPC over control. A significant increase in the grain protein content was observed in most of the derivatives selected in this study. The *Ae. peregrina* derivative BC_2F_2 17-1-2-2 showed a maximum increase in the protein content among the derivatives, that is, about 47% over the control WL711, followed by the *Ae. kotschyi* derivative BC_2F_2 49-1-11, depicting an increase of about 30%. Increase in protein content was, however, not observed for the BC_2F_2 48-41-6 *Ae. kotschyi* derivative. The correlation between the total iron and protein contents is depicted in Figure 1. A significant positive correlation (r = 0.80) was observed between the grain iron and protein concentrations in the selected derivatives.

All of the accessions of *Ae. kotschyi* and *Ae. peregrina* were recorded to have nearly 1.3−1.5-fold higher phytate content than the cultivars. The phytate/iron molar ratios for these wild germplasms were, however, found to be low because of the

Table 3. Grain Protein Contents and Phytate Contents in Selected BC_2F_2 and BC_1F_3 Derivatives Relative to WL711 Control along with the Phytate/Iron Molar Ratios

ID	protein[a] (%)	protein (% of control)	phytate[a] (%)	phytate (% of control)	phytate/Fe molar ratio[a]
T. aestivum cv. WL711	13.26 ± 0.98		0.78 ± 0.013		24.9
T. aetivum cv. Chinese Spring	12.33 ± 1.11	93.0	0.75 ± 0.004	96.0	24.2
T. aestivum cv. PBW343	13.91 ± 0.87	104.9	0.75 ± 0.006	95.9	23.7
Ae. kotschyi 396	24.80 d ± 1.25	187.0	1.00 ± 0.036	129.1	13.0 c
Ae. kotschyi 3790	27.91 e ± 1.63	210.5	1.02 ± 0.031	131.5	13.0 c
Ae. peregrina 3519	20.05 c ± 1.09	151.2	1.09 ± 0.042	139.3	15.8 b
Ae. peregrina 1155-1-1	21.12 c ± 1.18	159.3	1.17 ± 0.054	150.7	16.6 ab
Ae. peregrina 13772	21.56 c ± 0.92	162.6	1.03 ± 0.033	132.6	13.3 c
BC_2F_2 48-41-6	13.46 ± 1.31	101.5	0.90 a ± 0.012	115.2	18.1 a
BC_2F_2 49-1-11	17.21 a ± 0.66	129.8	1.06 b ± 0.017	136.3	16.8 ab
BC_2F_2 49-1-73	17.65 a ± 0.85	133.1	0.95 ab ± 0.025	121.7	18.0 a
BC_1F_3 75-1-4	16.47 a ± 0.87	124.2	0.96 ab ± 0.027	122.7	13.9 c
BC_2F_2 54-3-12	15.82 a ± 1.25	119.3	0.93 ab ± 0.023	119.7	18.4 a
BC_1F_3 77-36-6	16.14 a ± 0.96	122.8	0.91 ab ± 0.010	116.9	15.9 b
BC_1F_3 77-50-15	16.26 a ± 0.96	122.6	0.80 ± 0.022	102.0	14.9 bc
BC_2F_2 17-1-3-38	16.64 a ± 0.82	125.5	0.67 a ± 0.021	84.2	10.0 d
BC_2F_2 17-1-2-2	19.53 b ± 0.90	147.3	0.73 ± 0.014	93.0	13.8 c
BC_2F_2 1-1-7-18	16.50 a ± 0.89	124.4	0.84 a ± 0.049	108.0	18.0 a
BC_2F_2 14-1-8-18	16.97 a ± 0.72	128.0	0.78 ± 0.016	99.8	16.6 ab

[a] Different letters represent significant difference from control (WL711), a−d respectively at $p < 0.05$.

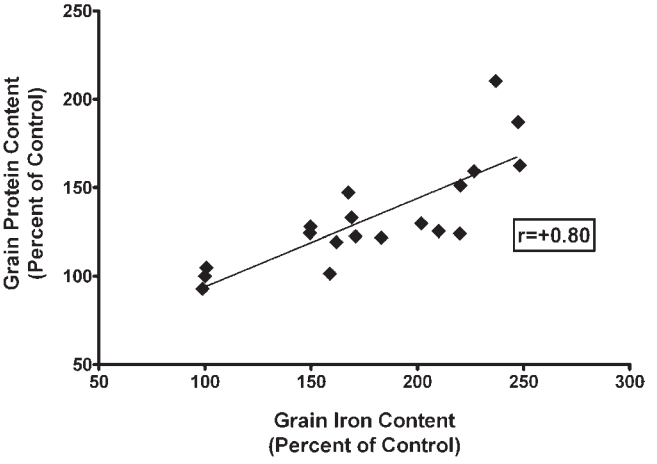

Figure 1. Correlation of total iron and protein content from wheat cultivars and derivatives of *Aegilops* expressed as percent relative to the cultivar WL711 (considered as control).

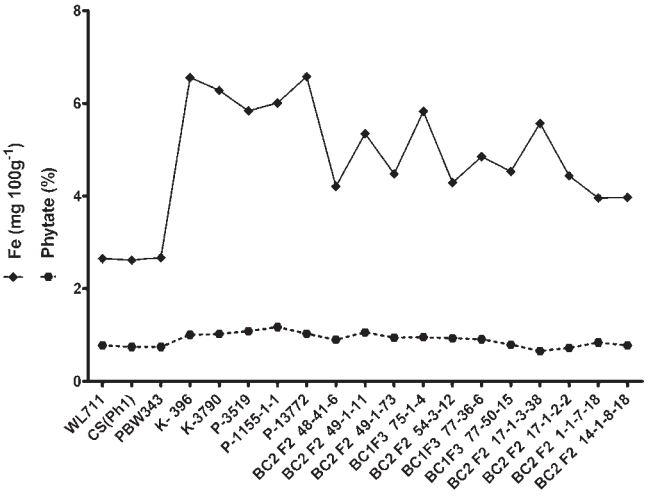

Figure 2. Comparison of variability in total iron and phytate contents from wheat cultivars and derivatives of *Aegilops*. Results are expressed as the mean, $n = 6$.

invariably higher iron concentrations of these accessions (Table 3). The wheat cultivars and derivatives included in this study, however, showed minimum variation in phytate contents. As shown in Figure 2, the grain phytate content correlated significantly with the total iron content ($r = 0.68$). The derivatives of *Ae. kotschyi* appeared to have slightly higher phytate content except for the derivative BC_1F_3 77-50-15 which had a phytate content almost equal to that of the control WL711. The *Ae. peregrina* derivatives had phytate contents almost equal to that of the wheat cultivars used in this study except for the derivative BC_2F_2 17-1-3-38. which showed an almost 16% decrease relative to the WL711 control. Furthermore, the phytate/iron molar ratio obtained for this derivative was also minimum, which could be attributed to lower phytate and enhanced iron contents respectively. All of the other derivatives also showed a reduced ratio of phytate/iron, which was significant at $p < 0.05$. (Table 3). The grain phytate content showed a significant positive correlation with the grain protein content among all of the wheat genotypes used in this study ($r = 0.61$).

Grain Hardness Evaluation. The grain hardness of the selected derivatives was evaluated qualitatively relative to the cultivar WL711 control. The *Ae. perigrina* derivative BC_2F_2 17-1-2-2 appeared to be harder than WL711. The grain hardness of the derivatives BC_2F_2 17-1-3-38 and BC_1F_3 77-36-6 of *Ae. kotschyi* and *Ae. perigrina*, respectively, were found to be close to that of

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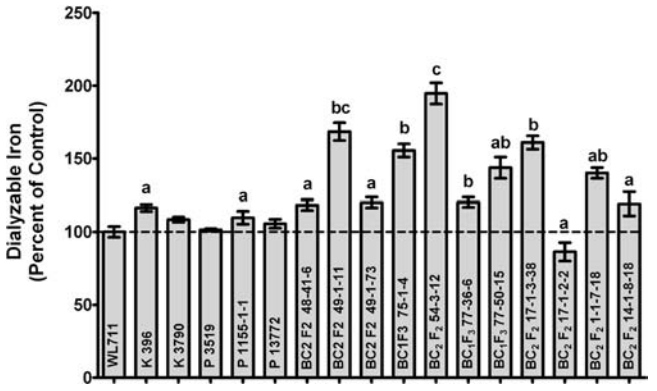

Figure 3. Dialyzability of iron from wheat derivatives of *Aegilops* expressed as percent relative to the cultivar WL711 (control). Results are expressed as the mean ± SEM, $n = 12$. Different letters indicate the significant level of difference in the mean values compared to the control, a and b respectively at $p < 0.05$.

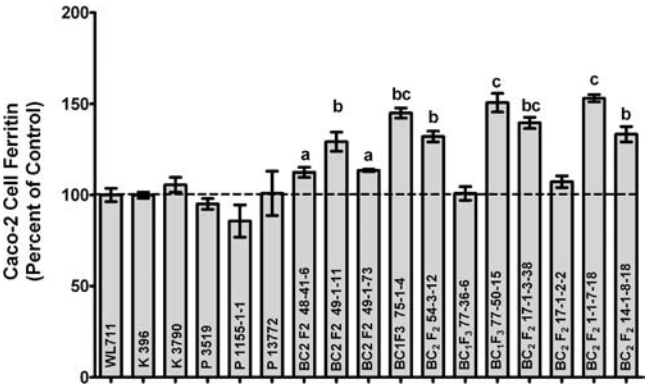

Figure 4. Iron bioavailability from wheat derivatives of *Aegilops* expressed in terms of Caco-2 cell ferritin response, represented as percent relative to the wheat cultivar WL711 (control). Different letters indicate significant level of difference in the mean values compared to the control, a and b respectively at $p < 0.05$.

the WL711 control. The *Ae. kotschyi* derivatives BC_2F_2 48-41-6 and BC_2F_2 49-1-73 and the derivative BC_2F_2 1-1-7-18 of *Ae. perigrina* were ranked as medium hard followed by the derivatives BC_2F_2 49-1-11, BC_1F_3 75-1-4, BC_2F_2 54-3-12, and BC_1F_3 77-50-15 of *Ae. kotschyi* along with the *Ae. perigrina* derivative BC_2F_2 14-1-8-18, which were ranked as medium soft.

Dialyzable Iron. Dialyzable iron from all of the wild accessions and derivatives used in this study was determined by estimating the amount of iron in the bottom chamber at the end of the intestinal digestion period and is represented relative to the WL711 control in Figure 3. The wild accessions did not show any significant increase in the dialyzable iron over control except for *Ae. kotschyi* acc. 396 and *Ae. perigrina* acc. 1155-1-1, both of which showed a very low 16 and 18% increase in dialyzability over WL711 control, respectively. The dialyzable iron from the derivatives of wheat used in this study varied extensively. The *Ae. kotschyi* derivatives, namely, BC_2F_2 49-1-11, BC_1F_3 75-1-4, BC_2F_2 54-3-12, and BC_1F_3 77-50-15, and the derivatives from *Ae. perigrina* BC_2F_2 17-1-3-38 and BC_2F_2 1-1-7-18 showed considerably enhanced dialyzability corresponding to an increase of 40−95% over WL711 control. The maximum dialyzability was, however, observed for the derivative BC_2F_2 54-3-12, which showed an almost 2-fold increase in dialyzability over the control. The dialyzable iron from the *Ae. kotschyi* derivatives BC_2F_2 48-41-6, BC_2F_2 49-1-73, and BC_1F_3 77-36-6 and the *Ae. perigrina* derivative BC_2F_2 14-1-8-18 also showed about 20% increase in dialyzability over the control, which was significant at $p < 0.05$. The *Ae. perigrina* derivative BC_2F_2 17-1-2-2 showed a very low but significant decrease ($p < 0.05$) in dialyzability over the WL711 control.

The dialyzable iron in the case of wheat *Aegilops* derivatives showed a significant positive correlation with the total iron content ($r = 0.5$), significant at $p < 0.05$, and a negative correlation with the phyate/iron molar ratios ($r = -0.26$) to a certain extent.

Caco-2 Cell Ferritin synthesis. The iron bioavailability in the selected derivatives used in this study was ranked on the basis of Caco-2 cell ferritin synthesis in response to cooked wheat digests as a percent relative to that from the cultivar, WL711 taken as control, and is summarized in Figure 4. The wild accessions of *Aegilops* did not show any increase in the bioavailability of iron. Except for *Ae. kotschyi* derivative BC_1F_3 77-36-6 and the *Ae. perigrina* derivative BC_2F_2 17-1-2-2, all of the other derivatives showed a significant increase in the bioavailability over the WL711 control as indicated by the increased ferritin response. The maximum bioavailability was observed for *Ae. perigrina* derivative BC_2F_2 1-1-7-18 (153%) followed by *Ae. kotschyi* derivatives BC_1F_3 77-50-15 (151%) and BC_1F_3 75-1-4 (145%) relative to the WL711 control. A substantial 30−40% increase in iron bioavailability over the WL711 control was observed from the derivatives BC_2F_2 49-1-11 and BC_2F_2 54-3-12 of *Ae. kotschyi* as well as BC_2F_2 17-1-3-38 and BC_2F_2 14-1-8-18 of *Ae. perigrina*. The *Ae. kotschyi* derivatives BC_2F_2 48-41-6 and BC_2F_2 49-1-73 showed a marginal 10−15% increase over the WL711 control, which was, however, significant at $p < 0.05$.

The bioavailable iron from the derivatives showed a significant positive correlation to a considerable extent with the dialyzable iron ($r = 0.63$) and to a certain extent with the total iron content as well ($r = 0.38$). A very low nonsignificant positive correlation was also observed between the bioavailability and grain protein content ($r = 0.16$), but the phytate content did not correlate with the bioavailability. The iron bioavailability, however, apparently correlated negatively with the phytate/iron molar ratios ($r = -0.42$) for the derivatives.

DISCUSSION

A large number of wheat-alien addition, substitution, and translocation lines have been developed and utilized for wheat improvement as reported and reviewed earlier.[9,10,25] The present study revealed considerably enhanced dialyzability as well as bioavailability for iron among most of the selected fertile derivatives of wheat from *Ae. kotschyi* and *Ae. perigrina* addition and substitution lines. This provides unequivocal proof of the concept that the related wild germplasm species possess a distinct genetic system for wheat biofortification.[6,9,10]

The selected derivatives had bolder seeds, harvest index comparable to the cultivars, higher iron content as well as GPC, and lower phyate/iron molar ratios than the elite wheat cultivars. The strong correlation observed between the iron content and GPC ($r = 0.80$) in the wild accessions of *Aegilops* and the derivatives used in this study indicated that the genes controlling iron concentration and GPC in many plants including wheat are possibly associated as reported earlier.[26] Furthermore, the grain

proteins are supposed to be colocalized with the micronutrients in the protein storage vacuoles (PSVs) of the aleurone layer as well as in the embryos as globoid crystals and thus act as a sink for the micronutrients and are expected to increase concomitantly.[27] Comprehensive morphological and molecular analysis of the selected derivatives used in this study had revealed introgression of group 2 and 7 chromosomes in *Ae. kotschyi* derivatives and group 4 and 7 chromosomes in derivatives from *Ae. perigrina*, thus contributing to their enhanced micronutrient content.[9,10] Furthermore, the introgressed genes belonging to group 1 chromosomes as observed in a few of the *Ae. kotschyi* derivatives used in this study are suggested to have the greatest effect on increasing total iron content according to our unpublished results. Continuous variation in GPC is generally exhibited due to polygenic control and the influence of environmental factors, mainly nitrogen and water availability, temperature, and light intensity. Papers reviewed previously indicate the location of the factors influencing protein concentration in cultivars on all chromosomes.[28] Thus, the increase in the GPC in the selected derivatives may be attributed to the additive affect from genes of the alien chromosomes. However, the waxiness of leaves and low GPC of *Ae. kotschyi* derivative BC_2F_2 48-41-6 was supposed to be the result of a small translocation in the S^K chromosome in its short arm according to our unpublished results suggesting that most of the QTL for high GPC and the locus with waxiness were localized at the telomeric end of group 2 chromosomes. The substantial increase in the GPC of *Ae. kotschyi* derivatives was thus attributed to the addition of $2S^K$, which was supposed to be orthologous to grain protein QTL on the short arm of group 2 chromosomes. Although the GPC was found to exhibit considerable correlation with the total iron ($r = 0.80$), a nonsignificant weak correlation was observed between the bioavailability of iron and GPC content ($r = 0.16$) for these derivatives.

Significant marginal correlation of GPC with grain hardness in wheat was reported earlier,[29] and our unpublished results on studies on effect of grain hardness on bioavailability of iron in a set of landraces of wheat revealed a significant impact of this parameter on iron dialyzability as well as bioavailability. Therefore, the grain hardness of the selected derivatives of wheat was evaluated qualitatively relative to the WL711 control and ranked accordingly. The *Ae. perigrina* derivative BC_2F_2 17-1-2-2, which showed a maximum increase in GPC (~1.5-fold over control) among all of the derivatives used in this study, was found to be harder than the WL711 control. Furthermore, this derivative also recorded the maximum TKW. Consequently, the bioavailability of iron from this derivative did not increase significantly relative to the WL711 control; rather, the iron dialyzability decreased. This indicated the impact of remarkably enhanced GPC and TKW with concomitant increase in grain hardness on iron dialyzability as well as bioavailability. However, the selected wheat derivatives with nominally increased yield and apparently enhanced GPC did not have any adverse effect on the bioavailability of iron from these grains as evidenced in earlier studies on iron bioavailability using rats.[30] Furthermore, molecular analysis of genes controlling grain hardness could also be an important parameter in the selection of wheat derivatives for enhanced iron bioavailability.

The apparent positive correlation observed for the total iron content with the dialyzability as well as bioavailability of iron ($r = 0.50$ and $r = 0.42$, respectively) in most of the selected *Aegilops* derivatives indicates that the increase may be attributed to the translational increase in the bioaccessible and bioavailable forms of iron rather than the increase in the absolute iron content alone. The wild accessions of *Aegilops*, however, did not show any substantial increase in dialyzability and bioavailability over the cultivar control. This may be attributed to the presence of high levels of antinutritional factors such as enzyme inhibitors and phytate in these wild germplasms, which may restrict mineral bioavailability.[31] It can be thus presumed that the introgressed alien chromosomes in most of the *Aegilops* derivatives may have genes with major influence in enhancing the bioaccessible or bioavailable forms of iron, justifying utilization of these unique derivatives in future wheat biofortification programs. Furthermore, the bioavailability and dialyzability of iron from the selected wheat derivatives also correlated positively to a great extent ($r = 0.63$) with one another, which was in accordance with some earlier results.[32]

The phytate content also correlated positively ($r = 0.68$) with the total iron content. The selected derivatives and cultivars, however, showed a low variation. No significant correlation was observed with the bioavailability of iron as reported earlier in the previous studies on 15 rice genotypes.[23] However, the phytate/iron molar ratios were found to decrease substantially in the wild germplasms as well as their derivatives from the cultivars, and in the case of derivatives the phytate/iron molar ratios were also found to correlate negatively to a significant extent with bioavailability ($r = -0.42$) as well as dialyzability ($r = -0.26$) of iron, indicating the feasibility of breeding non low-phytic acid wheat genotypes with more appropriate phytate/iron ratios. This is in line with reports from previous studies on the biofortification of maize.[33] The significant decrease in the phytate/iron molar ratios for one of the *Ae. kotschyi* derivatives, BC_2F_2 17-1-3-38, reducing to as low as 10:1 and the corresponding increase in the iron bioavailability to 1.4-fold relative to the WL711 control justify the utilization of these parameters in future wheat biofortification programs. In the wild germplasms and the derivatives used in this study, a significant positive correlation ($r = 0.61$) was also observed for the phytate content and GPC as well.

The results suggest that the selection of derivatives of wheat from wild germplasms with marginally increased yield, enhanced iron content, and apparently increased GPC along with reduced phytate/iron molar ratios and correspondingly enhanced dialyzability and bioavailability for iron can be considered as an ideal breeding source for future biofortification programmes. With an aim to further enhance the potential of these derivatives from wild germplasm, induced homologous chromosome pairing and irradiation for precise transfer of genes controlling micronutrient content, GPC and grain hardness is currently in progress. These might help in improving the nutritive values of grains for human population. It is expected that this approach would improve the mineral nutrition of human populations.

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■ ABBREVIATIONS USED

GPC, grain protein content; QTL, quality trait loci; SKCS, single-kernel characterization system; SKNIR, single-kernel near-infrared; RIL, recombinant inbred lines; GISH, genomic in situ hybridization; FISH, fluorescent in situ hybridization; MEM, minimum essential medium; BCA, bicinchonic acid; BSA, bovine serum albumin; TKW, thousand kernel weight.

■ REFERENCES

(1) Gitlin, J. D. Distributing nutrition. *Science* **2006**, *314*, 1252–1253.

(2) Zimmerman, M. B.; Hurrel, R. F. Nutritional iron deficiency. *Lancet* **2007**, *370*, 511–519.

(3) FAO. Cereals and other starch-based staples: are consumption patterns changing? Document CCP:GR-RI/04/4; Rome, Italy, 2004.

(4) Ortiz-Monasterio, I.; Graham, R. D. Breeding for trace minerals in wheat. *Food Nutr. Bull* **2000**, *21*, 392–396.

(5) Halloran, G. M. Genetic analysis of grain protein percentage in wheat. *Theor. Appl. Genet.* **1975**, *46*, 79.

(6) Rawat, N.; Tiwari, V. K.; Singh, N.; Randhawa, G. S.; Singh, K.; Chhuneja, P.; Dhaliwal, H. S. Evaluation and utilization of *Aegilops* and wild *Triticum* species for enhancing iron and zinc content in wheat. *Genet. Resour. Crop Evol.* **2009**, *56*, 53–64.

(7) Blanco, A.; Simeone, R.; Gadaleta, A. Detection of QTLs for grain protein content in durum wheat. *Theor. Appl. Genet.* **2006**, *112*, 1195–1204.

(8) Tanksley, S. D.; Nelson, J. C. Advanced backcross QTL analysis: a method for the simultaneous discovery and transfer of valuable QTL from unadapted germplasm into elite breeding lines. *Theor. Appl. Genet.* **1996**, *92*, 191–203.

(9) Tiwari, V. K.; Rawat, N.; Neelam, K.; Malik, S.; Randhawa, G. S.; Dhaliwal, H. S. Substitution of 2S and 7U chromosomes of *Aegilops kotschyi* in wheat enhances grain iron and zinc concentration. *Theor. Appl. Genet.* **2010**, *121*, 259–269.

(10) Neelam, K.; Rawat, N.; Tiwari, V. K.; Kumar, S.; Chhuneja, P.; Singh, K.; Randhawa, G. S.; Dhaliwal, H. S. Introgression of group 4 and 7 chromosomes of *Ae. peregrina* in wheat enhances grain iron and zinc density. *Mol. Breed.* **2011**, doi: 10.1007/s11032-010-9514-1.

(11) Tiwari, V. K.; Rawat, N.; Neelam, K.; Randhawa, G. S.; Singh, K.; Chhuneja, P.; Dhaliwal, H. S. Development of *Triticum turgidum* ssp. *durum*−*Aegilops longissima* amphiploids with high iron and zinc content through unreduced gamete formation in F1 hybrids. *Genome* **2008**, *51*, 757–766.

(12) Tiwari, V. K.; Rawat, N.; Chhuneja, P.; Neelam, K.; Aggarwal, R.; Randhawa, G. S.; Dhaliwal, H. S.; Keller, B.; Singh, K. Mapping of quantitative trait loci for grain iron and zinc concentration in A genome diploid wheat. *J. Hered.* **2009**, *100* (6), 771–776.

(13) House, W. Trace element bioavailability as exemplified by iron and zinc. *Field Crop Res.* **1999**, *60*, 115–141.

(14) Fairweather-Tait, S.; Lynch, S.; Hotz, Ch.; Hurrell, R.; Abrahamse, L.; Beebe, S.; Bering, S.; Bukhave, K.; Glahn, R. P.; Hambridge, M.; Hunt, J.; Lonnerdal, B.; Miller, D.; Mohktar, N.; Nestel, P.; Reddy, M.; Sandberg, A. S.; Sharp, P.; Teucher, B.; Trinidad, T. The usefulness of *in vitro* models to predict the bioavailability of iron and zinc: a consensus statement from the harvest plus expert consultation. *Int. J. Vitam. Nutr. Res.* **2005**, *75*, 371–374.

(15) Scholz-Ahrens, K. E.; Schrezenmeir, J. Inulin and oligofructose and mineral metabolism: the evidence from animal trials. *J. Nutr.* **2007**, *137*, 2513S–2523S.

(16) Gautam, S.; Platel, K.; Srinivasan, K. Higher bioaccessibility of iron and zinc from food grains in the presence of garlic and onion J. Agric. Food Chem. **2010**, *58*, 8426–8429.

(17) Glahn, R. P.; Wien, E. M.; Van Campen, D. R.; Miller, D. D. Caco-2 cell iron uptake from meat and casein digests parallels in vivo studies: use of a novel *in vitro* method for rapid estimation of iron bioavailability. *J. Nutr.* **1996**, *126*, 332–339.

(18) Yeung, C. K.; Glahn, R. P.; Miller, D. D. Iron bioavailability from common raisin-containing foods assessed with an in vitro digestion/Caco-2 cell culture model: effects of raisins. *J. Food Sci.* **2003**, *68*, 1866–1870.

(19) Zheng, L.; Cheng, Z.; Ai, C.; Jiang, X.; Bei, X.; Zheng, Y.; Glahn, R. P.; Welch, R. M.; Miller, D. D.; Lei, X. G.; Shou, H. Nicotianamine, a novel enhancer of rice iron bioavailability to humans. *PLoS One* **2010**, *5* (4), e10190.

(20) Chen, Q.; Tsujimoto, H.; Gill, B. S. Transfer of *PhI* gene promoting homeologous pairing from *Triticum speltoides* into common wheat and their utilization in alien genetic introgression. *Theor. Appl. Genet.* **1994**, *88*, 97–101.

(21) Lorenz, A. J.; Scott, M. P.; Lamkey, K. R. Quantitative determination of phytate and inorganic phosphorus for maize breeding. *Crop Sci.* **2007**, *47*, 598–604.

(22) Hurkman, W. J.; Tanaka, C. K. Extraction of wheat endosperm proteins for proteome analysis. *J. Chromatogr., B* **2007**, *849*, 344–350.

(23) Glahn, R. P.; Cheng, Z.; Welch, R. M. Comparison of iron bioavailability from 15 rice genotypes: studies using an in vitro digestion/Caco-2 cell culture model. *J. Agric. Food Chem.* **2002**, *50*, 3586–3591.

(24) Miller, D. D.; Schricker, B. R.; Rasmussen, R. R.; Van Campen, D. An in vitro method for estimation of iron availability from meals. *Am. J. Clin. Nuir.* **1981**, *34*, 2248–2256.

(25) Oliver, R. E.; Cai, X.; Xu, S. S.; Chen, X.; Stack, R. W. Wheat-alien species derivatives: a novel source of resistance to *Fusarium* head blight in wheat. *Crop Sci.* **2005**, *45*, 1353–1360.

(26) 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.

(27) Welch, R. M. Effects of nutrient deficiencies on seed production and quality. In *Advances in Plant Nutrition*; Tinker, B., Lauchli, A., Eds.; Praeger Scientific: New York, 1986; Vol. 2, pp 205−247.

(28) Konzak, C. F. Genetic control of the content, amino acid composition and processing properties of proteins in wheat. *Adv. Genet.* **1977**, *19*, 407–582.

(29) Groos, C.; Bervas, E.; Charmet, G. Genetic analysis of hardness and bread-making related traits in a hard × hard bread wheat cross. *J. Cereal Sci.* **2004**, *40*, 93–100.

(30) House, W. A.; Welch, R. M. Bioavailability to rats of iron in six varieties of wheat grain intrinsically labeled with radioiron. *J. Nutr.* **1987**, *117*, 476–480.

(31) OECD. *Consensus Document on Compositional Consideration for New Varieties of Bread Wheat (Triticum aestivum): Key Food and Feed*

Nutrients, Anti-nutrients and toxicants; Environment Directorate, Organisation for Economic Co-operation and Development: Paris, France, 2003; ENV/JM/MONO(2003)7.

(32) Argyri, K.; Theophanidi, E.; Kapna, A.; Staikidou, C.; Pounis, G.; Komaitis, M.; Georgiou, C.; Kapsokefalou, M. Iron or zinc dialyzability obtained from a modified in vitro digestion procedure compare well with iron or zinc absorption from meals. *Food Chem.* **2011**, *127* (2), 716–721.

(33) Simic, D.; Sudar, R.; Ledencan, T.; Jambrovic, A.; Zdunic, Z.; Brkic, I.; Kovacevic, V. Genetic variation of bioavailable iron and zinc in grain of a maize population. *J. Cereal Sci.* **2009**, *50*, 392–397.