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Selection of Bread Wheat for Low Grain Cadmium Concentration at the Seedling Stage Using Hydroponics versus Molecular Markers

Caixia Liu, Mary J. Guttieri, Brian M. Waters, Kent M. Eskridge, and P. Stephen Baenziger*

ABSTRACT

The excessive accumulation of Cd in harvested crops grown on high-Cd soils has increased public concerns for food safety. Due to the high consumption of bread wheat (*Triticum aestivum* L.) per capita, high concentrations of Cd in wheat grain can significantly affect human health. Breeding is a promising way to reduce grain Cd concentration. However, a lack of efficient selection methods impedes breeding for low grain Cd concentration in bread wheat. In this study, a recombinant inbred population segregating for grain Cd concentration was used to assess the efficacy of two selection methods for decreasing grain Cd concentration in bread wheat: a hydroponic selection method used shoot Cd concentration in 2-wk-old seedlings growing in Cd-containing medium, and a marker-based selection method using markers linked to *heavy metal transporting P1B-ATPase 3 (HMA3)*, the gene underlying *Cdu1*. Both methods effectively selected low-Cd lines. The *HMA3*-linked marker-based selection was superior to hydroponic selection in terms of both simplicity and response to selection. The *HMA3*-linked markers explained 20% of the phenotypic variation in grain Cd concentration with an additive effect of 0.014 mg kg⁻¹. The hydroponic selection and marker-based selection may target two different and independent processes controlling grain Cd accumulation, and they had no effect on grain Zn and Fe concentrations. The *ALMT1-UPS4* marker associated with Al tolerance was not associated with grain Cd concentration but increased grain Zn and Fe concentrations. The 193-bp allele of the *Rht8*-associated marker, *GWM261*, was associated with increased grain Cd concentration.

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Abbreviations: ICP–MS, inductively coupled plasma–mass spectrometry; KASP, Kompetitive Allele-Specific Polymerase Chain Reaction; LOQ, limit(s) of quantitation; PCR, polymerase chain reaction; QTL, quantitative trait locus; RIL, recombinant inbred line; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; STS, sequence-tagged site.

EXCESSIVE ACCUMULATION of Cd in agricultural soil (Zimakowska-Gnoińska et al., 2000; Han et al., 2002; Liu et al., 2005b; Rodríguez et al., 2009; Sayadi, 2014), which is the source of Cd to the plant, has increased concerns for food quality and safety. Exposure to Cd is associated with increased cancer mortality (Nishijo et al., 2006; Akesson et al., 2008; Menke et al., 2009). Estimated from a Swedish cohort, 80% of the dietary Cd intake was from cereals and vegetables (Akesson et al., 2008). Bread wheat (*Triticum aestivum* L.) is widely consumed by humans, with global consumption of 67 kg per capita in 2016–2017 (FAO, 2016). Thus, the content of toxic elements in wheat grain can significantly affect human health. To protect consumers from excessive exposure of dietary Cd, the maximum level of Cd in wheat grain is regulated at 0.2 mg kg⁻¹ in Europe (The European Commission, 2006), and 0.1 mg kg⁻¹ in China (USDA Foreign Agricultural Service, 2014), New Zealand, and Australia (Food Standards Australia New Zealand, 2016). Therefore, wheat breeders need to produce new cultivars to meet the changes in environmental conditions and customers' requirements (Collard and Mackill, 2008). Grain Cd concentration is affected largely by soil characteristics, yet among a set of cultivars, those with high

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or low grain Cd concentration were consistently high or low across environments (Oliver et al., 1995; Clarke et al., 2002; Stolt et al., 2006; Guttieri et al., 2015). Therefore, it is possible to select cultivars that genetically accumulate less Cd in grain.

Although the importance of bread wheat in the human diet and the concerns about Cd exposure are well known, little effort has been made to breed bread wheat cultivars for low grain Cd concentration, in part because previous studies indicated bread wheat accumulated less Cd in grain than durum wheat (*Triticum durum* Desf.; Zook et al., 1970). Lack of low-Cd germplasm, lack of knowledge on the genetic architecture of grain Cd accumulation, and the inability to efficiently select low-Cd lines are the major impediments to breeding low-Cd cultivars of bread wheat. Historically, the selection of low-Cd lines in bread wheat was done using mature grain Cd concentration. To do so, breeders first needed to plant all the lines on high-Cd soil and harvest them at maturity. Then, grain Cd concentration was determined by expensive analytical instruments preceded by the digestion of grain samples using strong inorganic acid treatment (Seravalli, 2012). The disadvantages of this selection system are (i) it requires a large research field that is contaminated with Cd to conduct the experiment; (ii) because the grain Cd concentration is largely affected by environmental factors, each genotype needs to be replicated within and across sites to get an accurate estimate of the genetic potential; (iii) the selection has to be made after harvesting and completed before the next planting, which is a narrow time window in many areas (e.g., in Nebraska, about 8 to 10 wk); and (iv) the selection process, which involves sample digestion and mineral analysis, is hazardous, time and labor consuming, and needs expensive equipment and trained analytical experts. Thus, an efficient selection method is needed to facilitate the breeding of low-Cd cultivars.

Grain Cd concentration at maturity is positively correlated with the shoot Cd concentration after anthesis in rice (*Oryza sativa* L.) (Liu et al., 2005a, 2007), durum wheat (Chan and Hale, 2004; Greger and Löfstedt, 2004; Harris and Taylor, 2004, 2013; Wiebe et al., 2010; Arduini et al., 2014), and bread wheat (Guttieri et al., 2015). One objective of this study was to test the feasibility and efficiency of selecting low-Cd bread wheat lines using shoot Cd concentration in 2-wk-old seedlings in a hydroponic system. If it is feasible and efficient, breeders could use this system to eliminate the high-Cd lines before planting to better allocate the testing resources to high-priority, low-Cd lines. A hydroponic system could be a good replacement for a field experiment because (i) it can be done in a laboratory, where a large number of lines can be planted in limited space, making it more flexible and scalable than a field experiment; (ii) it needs fewer replications to remove the confounding effect of environment; and (iii) it does not need a high-Cd field site to make selections, though

it still would need a small field contaminated with Cd to validate the hydroponic selection. Hydroponic systems have been widely used by scientists to study the uptake, translocation, and allocation of Cd in wheat (Chan and Hale, 2004; Van der Vliet et al., 2007; Wiebe et al., 2010; Harris and Taylor, 2013; Vergine et al., 2017).

Marker-assisted selection is preferred for traits that are difficult and expensive to phenotype and has been extensively used in plant breeding to greatly increase the efficiency of selection compared with selection based only on phenotypic data (Collard et al., 2005). An effective marker-assisted selection requires reliable markers tightly linked to quantitative trait loci (QTLs) with large effects on the trait of interest. In durum wheat, grain Cd concentration is controlled by a major dominant gene, *Cdu1* (Clarke et al., 1997). Three *Cdu1* co-segregating markers explained >80% of the variation in grain Cd accumulation and were used to assist the selection of durum wheat for low grain Cd concentration (Wiebe et al., 2010). A set of single nucleotide polymorphisms (SNPs) associated with grain Cd concentration in bread wheat were identified in a region homoeologous to the *Cdu1* locus in durum wheat (Guttieri et al., 2015). These markers together explained 12 to 19% of the phenotypic variation in grain Cd concentration in the association mapping panel where they were first identified (Guttieri et al., 2015). Based on alignment to the International Wheat Genome Sequencing Consortium reference sequence (IWGSC RefSeq v1.0), two of these SNPs were selected that span a 0.2-Mb region of chromosome 5AL, which includes the heavy metal transporting P1B-ATPase 3 (*HMA3*) (accession KF683296.1: 581,139,389–581,140,334 bp). *HMA3* has been associated with major QTLs for the variation of grain Cd accumulation in rice (Ueno et al., 2010; Miyadate et al., 2011). OsHMA3 mediates Cd efflux into root vacuoles and thus modulates root-to-shoot translocation in rice. The *HMA3*-flanking SNPs were successfully converted to Kompetitive Allele-Specific Polymerase Chain Reaction (KASP) markers. The KASP genotyping technology has been used across different fields because of its speed and flexibility (He et al., 2014). One objective of this study was to validate the effect of selection for the *HMA3*-linked markers on grain Cd concentration in a recombinant inbred line (RIL) population segregating for grain Cd concentration.

In this study, replicated selections were done in a bread wheat RIL population at seedling stage independently using two selection methods. One selection method used shoot Cd concentration of 2-wk-old seedlings grown in a hydroponic system. The other selection method used *HMA3*-linked markers. The main objectives of this study were to assess the efficiency of each selection method on reducing mature grain Cd concentration and develop a breeding protocol for low-Cd bread wheat cultivars. The procedure for measuring Cd concentration also measured

the concentration of other minerals (e.g., As, Ca, Cu, Fe, Mg, Mn, Ni, P, and Zn). Thus, the correlations of grain Cd concentration with the grain concentrations of other minerals also were evaluated in the RIL population. Interestingly, the RIL population also segregated for an Al-activated malate transporter 1 (*ALMT1*)-associated sequence-tagged site (STS) marker, *ALMT1-UPS4*, and a *reduced height-8* (*Rht8*)-associated simple sequence repeat (SSR) marker, *GWM261*. Both the Al tolerance allele and reduced height allele were contributed by the high-Cd parent, ‘Wesley’. Considering the association of *TaALMT1* with Al³⁺ tolerance as a consequence of malate efflux from roots (Pineros et al., 2008), and the association of plant height with grain Cd concentration (Perrier et al., 2016), we tested the associations of these marker loci with grain mineral concentrations.

MATERIALS AND METHODS

Plant Material

A population of 105 RILs ($F_{4:6}$) derived from the cross between ‘Panhandle’ as female and ‘Wesley’ as male was used in this study. Panhandle consistently had significantly lower grain Cd concentration than Wesley (Guttieri et al., 2015). The $F_{4:5}$ RIL population initially had 120 lines and was randomly divided into three sets of 40 lines for seed increase in Yuma, AZ, but some of the lines were lost during the growing process. This resulted in 31 $F_{4:6}$ RILs in Set 1, 37 $F_{4:6}$ RILs in Set 2, and 37 $F_{4:6}$ RILs in Set 3. Later, selections were performed in these three sets independently to test the reproducibility of the selection methods.

Selection Methods

Selection Using Shoot Cadmium Concentration in Two-Week-Old Seedlings

The two parents were included in evaluations of all three sets of RILs. The selection using shoot Cd concentration in 2-wk-old seedlings in a hydroponic system (hereafter referenced as hydroponic selection) was performed for each set independently so that the selection procedure was replicated.

Eight seeds were randomly taken from each line (RILs and two parents). The seeds were surface sterilized for 20 min with 1.2% sodium hypochlorite (6% HOCl, The Clorox Company) and then rinsed with ultrapure water and soaked for 2 h in a solution of 2 mM CaCl₂ + 0.001% Dividend (7.73% difenoconazole, 1-({2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl)methyl}-1,2,4-triazole, 1.93% mefenoxam, methyl (2R)-2-[N-(2-methoxyacetyl)-2,6-dimethylanilino]propanoate, Syngenta Crop Protection). The seeds were then germinated for 2 d on germination paper well soaked with 2 mM CaCl₂ + 0.001% Dividend solution at room temperature in the dark. Four germinated seeds from each line, which gives 98% possibility to capture the heterogeneous lines, were transferred to 1.5-mL centrifuge tubes (one seed in each tube) with the cap removed. The centrifuge tubes were cut open 2 to 3 mm from the bottom and filled with perlite beforehand. The plants were supplied with 3 L of chelator-buffered nutrient solutions for 2 wk in four containers, with the RILs and two

parents randomized within each container once. The nutrient solution (Wiebe et al., 2010) was prepared with ultrapure water and contained 1.0 mM Ca(NO₃)₂, 0.3 mM Mg(NO₃)₂, 0.3 mM NH₄NO₃, 0.25 mM KNO₃, 0.1 mM K₂HPO₄, 0.1 mM K₂SO₄, 50 μM KCl, 100 μM Fe(NO₃)₃, 10 μM H₃BO₃, 0.2 μM Na₂MoO₄, 10 μM ZnSO₄, 2 μM CuSO₄, 1 μM MnSO₄, 5 μM CdCl₂, 0.1 μM NiCl₂, 143 μM N-(2-hydroxyethyl) ethylenediaminetriacetic acid (HEDTA), 1.42 mM KOH, and 2 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 6.0). The pH of the nutrient solution was adjusted to the target value of 6.0 every 2 d. After 2 wk, the four plants from each line were carefully removed from their tubes and harvested in bulk. Their roots were washed using ultrapure water for 5 min, soaked in 2 mM CaCl₂ solution for 10 min, and then washed using ultrapure water for another 5 min. The roots and shoot were separated by cutting at approximately 1 mm below and 2 mm above the seed. Harvested tissues were placed in labeled envelopes and dried in an oven at 70°C for 2 d.

After drying, the tissues were digested and the mineral concentrations were measured using the protocol as described by Guttieri et al. (2015). Briefly, samples were digested in concentrated HNO₃ overnight at room temperature, followed by 1.5 h at 100°C under refluxing conditions, 1.5 h at 125°C, and 2 h at 150°C after adding H₂O₂. Finally, the samples were evaporated to dryness at 165°C. The dried residue was resuspended in 1% (w/w) HNO₃. Mineral concentrations in the resuspended digest were measured using an Agilent 7500cx inductively coupled plasma-mass spectrometry (ICP-MS, Agilent Technologies) with Ar carrier and a He collision cell at the University of Nebraska Redox Biology Center Proteomics and Metabolomics Core in Lincoln, NE. Within each set, lines were ranked by shoot Cd concentration from lowest to highest. Ten lines with the lowest rank were classified as low Cd lines (hereafter referenced as hydroponic low-Cd lines), and 10 lines with the highest rank were identified as high Cd lines (hereafter referenced as hydroponic high-Cd lines) (Supplemental Fig. S1). The natural log of shoot Cd concentrations was used for statistical analysis to improve the normality of the data.

HMA3-Linked Marker Assays

Selection using Cd-associated *HMA3*-linked SNP markers (Guttieri et al., 2015) (hereafter referenced as *HMA3*-linked marker-based selection) also was conducted for each set independently. DNA was isolated and purified from the leaves of four seedlings at 2 wk after germination for each set of RILs described above (total of 105 $F_{4:6}$ lines) plus two parents using BioSprint 96 DNA Plant Kits (Qiagen). The seedlings used for *HMA3*-linked marker-based selection were different subsamples of the inbred lines used for hydroponic selection. The DNA was diluted to 10 ng μL⁻¹. The 105 lines were screened using two *HMA3*-linked markers (IWB43741 and IWA7579; detailed information is listed in Supplemental Table S1) derived from the 90K iSelect array (Guttieri et al., 2015) and converted to KASP, with primers obtained from IDT (Integrated DNA Technologies, Coralville, IA). Each reaction included 2.5 μL of DNA template, 2.5 μL 2× master mix (LGC), and 0.07 μL 72× assay mix. The assay mix (72×) was prepared such that the concentration of each forward primer was 12 μM, and the concentration of the reverse primer was 30 μM. The thermal

cycling was performed on an Eppendorf Mastercycler Pro 384. The thermal cycling program was designed according to KASP genotyping chemistry user guide (<https://www.lgcgroup.com/LGCGroup/media/PDFs/Products/Genotyping/KASP-genotyping-chemistry-User-guide.pdf>). The KASP assay for each SNP marker included one reaction cell for each line, three reaction cells for each parent, five reaction cells for simulated heterozygous DNA samples, and three reaction cells for nontemplate controls. The nontemplate controls used distilled water to substitute for the DNA template. Simulated heterozygous DNA samples were prepared by mixing equal amounts of DNA from each of the two parents. After polymerase chain reaction (PCR), plates were cooled to room temperature and read by FLUOstar Omega (BMG LABTECH). The SNP calling and genotype clustering were performed using the KlusterCaller data analysis software package (LGC Genomics, 2017). Lines clustered with Panhandle, Wesley, and heterozygous DNA samples were considered as low-Cd lines (hereafter referenced as *HMA3*-linked marker-based low-Cd lines), high-Cd lines (hereafter referenced as *HMA3*-linked marker-based high-Cd lines), and heterogeneous lines, respectively (Supplemental Fig. S2). No recombination between the two *HMA3*-linked markers was observed in this RIL population. Therefore, they produced the same selection result.

ALMT1-UPS4 Marker Screening

To assess the potential effects of *TaALMT1* on grain mineral concentrations, we screened our RIL population using an associated STS marker, ALMT1-UPS4. Each PCR reaction was conducted with 5 μ L of GoTaq green master mix (Promega), 0.3 μ L of each primer (10 μ M) (Integrated DNA Technologies, Supplemental Table S1), 1 μ L DNA template (20 ng μ L⁻¹), and 3.4 μ L nuclease-free water (Thermo Fisher Scientific). The detail of the thermal cycling program is listed in Supplemental Table S2. The PCR products were resolved by 0.9% agarose gel electrophoresis and visualized by ethidium bromide staining using FOTO/Analyst FX (Fotodyne).

GWM261 Marker Screening

To test the association of *Rht8* with grain mineral concentrations, particularly Cd, we screened our RIL population using a *reduced height-8* (*Rht8*)-associated SSR marker, GWM261. Each PCR reaction was conducted in a volume of 10 μ L with 2 μ L of 5 $\times$ GoTaq Flexi Buffer (Promega), 1 μ L MgCl₂ (25 mM), 0.2 μ L deoxynucleotide mix (10 mM), 0.05 μ L forward tailed primer (10 μ M), 0.08 μ L reverse primer (10 μ M), 0.03 μ L M13 dye (FAM)-labeled primer (10 mM), 0.04 μ L GoTaq enzyme (5 U μ L⁻¹), and 6.6 μ L autoclaved nanopore water. The primer sequences were listed in Supplemental Table S1. The detail of the thermal cycling program is listed in Supplemental Table S2. The PCR products were diluted 10-fold with water, and an aliquot was added to Hi-DI-formamide (Invitrogen) containing a labeled molecular weight ladder. After denaturation at 95°C for 5 min, PCR products were electrophoresed on an Applied Biosystems Incorporated (ABI) capillary electrophoresis system. The ABI output was processed by GeneMarker (SoftGenetics; Hulce et al., 2011).

Experimental Design and Phenotypic Evaluation

Field trials were conducted during the 2015–2016 growing season at the University of Nebraska–Lincoln research farms near Mead and Clay Center, NE. Soil test data are given in Supplemental Table S3. All 105 RILs along with two parents and three other checks ('Overland', 'Freeman', and 'Ruth') and 15 bulks were randomized in two complete replicates with nested incomplete blocks of five plots in each location as an α -lattice design. The randomization was done in AGROBASE Generation II (Mulitze, 2004). The experimental unit was a four-row plot of 1.5-m width. Plot length was measured at grain maturity and ranged from 103 to 138 cm in Mead and from 110 to 134 cm in Clay Center. The field management followed the standard agricultural practices for both locations, and each plot was sprayed with fungicide to control fungal disease. Our previous study showed that the application of fungicide has no effect on grain Cd concentration (C. Liu et al., 2018).

Mature grains were analyzed for mineral concentrations (Guttieri et al., 2015), after drying for 2 d in an oven at 70°C. Limits of quantitation (LOQ) were calculated as three times the standard deviation of the concentrations of the reagent blanks, expressed on a 2.5-g grain basis (Masson et al., 2010). The concentration of Ca, Cd, Cu, Fe, Mg, Mn, Ni, P, and Zn in grain samples were always above the LOQ. Arsenic concentration was above the LOQ for 89% of the grain samples.

Statistical Analysis

Field data were analyzed by mixed effects analysis of variance for each trait using PROC GLIMMIX in SAS 9.4 (SAS Institute, 2013). Genotype, location, and genotype $\times$ location were tested as fixed effects. Block(location) and incomplete block(block, location) were tested as random effects. The effect of genotype was partitioned to the effect of checks and bulks and the effect of RILs by including a classification variable in the model, where checks and bulks were nested in Level 0 of the classification variable, and RILs were nested in Level 1 of the classification variable.

The genetic variance of the RIL population (σ_{RIL}^2) and the genetic $\times$ environment variance of the RIL population ($\sigma_{\text{RIL} \times \text{env}}^2$) were estimated by fitting RIL and RIL $\times$ location as random effects. The broad-sense heritability on entry-mean basis was calculated as

$$H^2 = \frac{\sigma_{\text{RIL}}^2}{\sigma_{\text{RIL}}^2 + \frac{\sigma_{\text{RIL} \times \text{env}}^2}{2} + \frac{\sigma_{\text{error}}^2}{4}}$$

where σ_{error}^2 is the residual variance. The effect of repeated selection, each selection method, and their interactions on grain Cd concentration were tested using ANOVA using PROC GLIMMIX in SAS 9.4 by expanding the previous model. The 104 df of RILs was partitioned to 2 df for set (Set 1, Set 2, and Set 3), 2 df for hydroponic selection (hydroponic high-, medium-, and low-Cd group), 2 df for *HMA3*-linked marker-based selection (*HMA3*-linked marker-based high-Cd, heterozygotes, and *HMA3*-linked marker-based low-Cd group), 4 df for the set $\times$ hydroponic selection, 4 df for set $\times$ *HMA3*-linked marker-based selection, 4 df for hydroponic selection $\times$ *HMA3*-linked

marker-based selection, and 6 df for set $\times$ hydroponic selection $\times$ *HMA3*-linked marker-based selection, and 80 df for RILs within set $\times$ hydroponic selection $\times$ *HMA3*-linked marker-based selection. The 104 df of RIL $\times$ location was partitioned in the same way.

RESULTS AND DISCUSSION

The Effects of Genotype, Location, and Genotype $\times$ Location Interaction

Analysis of variance was initially conducted for each location separately. The ratio of the residual variances from two locations (larger variance/smaller variance) was <5 for all the tested traits, except As concentration. Therefore, data from the two locations was combined for the ANOVA for all the traits, except As concentration. The RIL $\times$ location interaction was statistically significant for grain Cd, Cu, Ni, K, and Zn concentration, plant height, grain volume weight, and grain yield (Table 1). However, the rank correlations of genotypic least square means between Clay Center and Mead were significant ($p < 0.05$), the broad-sense heritabilities (H^2) were >0.5 , and the $\sigma^2_{\text{RIL}}/\sigma^2_{\text{RIL} \times \text{env}}$ ratios were >1 for all these traits (Supplemental Fig. S3). Thus, these traits had high genetic repeatability in this study, and RIL $\times$ location interaction mainly changed the genotype means by magnitude, not significantly by rank. Therefore, genotypic least square means across locations were calculated for each trait. Genotypic least square means within locations were also calculated and used as appropriate.

When planted in Mead and Clay Center, respectively, 3 and 24% of the RILs had grain Cd concentration higher than the Codex regulatory limit of 0.2 mg kg^{-1} (Fig. 1). The Cd concentration in agricultural soils in some parts of the United States could be much higher than that in our research site (Holmgren et al., 1993), and some released cultivars accumulated more Cd in grain than the high-Cd parent of our RIL population (Guttieri et al., 2015). Therefore, it is likely that grain Cd concentration of some

released cultivars could exceed the regulatory limit when grown on high-Cd soil. Thus, it is necessary to breed low-Cd wheat, especially for areas contaminated with Cd.

The higher soil Cd concentration in Mead (0.18 mg kg^{-1}) than in Clay Center (0.15 mg kg^{-1}) did not explain the lower grain Cd concentration in Mead than in Clay Center. To explore potential factors causing this deviation from our expectation, the Mead trial was divided into a south section (1st–15th tiers), which was waterlogged during flowering due to the high precipitation (Supplemental Table S3) and was the lower end of the field bordered by a road that acted as a dam, and a north section (16th–24th tiers). The north and south sections of the trial had a similar concentration of DTPA-extractable soil Cd before planting. Grain harvested from the south section of the Mead trial (waterlogged) had significantly lower (0.11 vs. 0.18 mg kg^{-1}) Cd concentration but higher As concentration (0.003 vs. 0.001 mg kg^{-1}) than grain harvested from north part of the Mead trial (less waterlogged) (Supplemental Fig. S4), which might be a result of the waterlogging condition in the south part of the field during flowering. Excess water could create a reducing soil environment, where SO_3^{2-} is reduced to H_2S and may react with Cd to form insoluble CdS (Bingham et al., 1976). Although the reducing soil condition appears to have decreased bioavailable Cd in soil, it likely increased bioavailable As by reducing arsenate to arsenite, which is more soluble (Reynolds et al., 1999). Flooding significantly decreased grain Cd concentration and increased grain As concentration in rice (Arao et al., 2009; Sun et al., 2014), where 2 to 3 wk before and after flowering were the most effective periods for reducing grain Cd concentration by flooding. Grain Cd concentration in rice was reduced by five- to sixfold by flooding during 3 wk before heading to 3 wk after heading (Arao et al., 2009), and it was decreased by 84% by flooding from 15 d before heading to 25 d after heading (Inahara et al., 2007). Grain Cd concentration was negatively correlated with grain As concentration on plot basis ($r = -0.54$, $p < 2.2 \times 10^{-16}$; Supplemental Fig. S5) but

Table 1. *F* values from the ANOVA for the effects of location (L), classification variable (CLV), CLV $\times$ location (CLV $\times$ L), checks [nested in level 0 of CLV, C(0)], check $\times$ location [C(0) $\times$ L], recombinant inbred line [nested in Level 1 of CLV, RIL(1)], and RIL $\times$ location [RIL(1) $\times$ L] on grain mineral (Ca, Cd, Cu, Fe, Mg, Mn, Ni, P, and Zn) concentration, plant height (hgt), grain volume weight (vw), and grain yield (yld).

Effect	NumDF†	DenDF‡	F value												
			Ca	Cd	Cu	Fe	K	Mg	Mn	Ni	P	Zn	hgt	vw	yld
L	1	2	36.8*	1.4	0.15	7.8	5.5	2.53	615**	0.61	2.3	57*	6.1	1.4	2.9
CLV	1	2	0.03	2.1	2	0	0.3	0.37	2.1	0.06	1.2	1.1	14	0.26	12
L $\times$ CLV	1	2	0.28	0.01	0.2	0.12	0.24	0	0.58	0.07	0.2	1.1	0.99	0.08	3.6
C(0)	19	149	2.9***	7.7***	0.77	3***	0.69	1.2	1.4	4***	1.6	1.5	5***	5.2***	6.1***
C(0) $\times$ L	19	149	0.69	0.7	0.55	0.85	1	1.3	1	1.2	0.98	0.45	0.87	1.2	1.2
RIL(1)	104	149	3.1***	6.5***	3.1***	2.6***	3.5***	1.3	2.4***	6.8***	1.8***	2.8***	6.3***	11***	4.9***
RIL(1) $\times$ L	104	149	1.26	1.93***	1.4*	1.3	1.5*	1	1	2.3***	1.13	1.5*	1.5**	3.4***	2.3***

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

† NumDF, numerator df.

‡ DenDF, denominator df.

Fig. 1. Cumulative frequency of the least square means of grain Cd concentration of 105 recombinant inbred lines harvested from Mead (salmon) and Clay Center (blue). The value on the x axis of “P,” “O,” “R,” “W,” and “F” present the grain Cd concentration for Panhandle, Overland, Ruth, Wesley, and Freeman, respectively, in Mead (salmon) and Clay Center (blue).

not on entry basis ($p = 0.6$) (Supplemental Fig. S6) in the Mead trial, which suggested an opposite effect of environment on grain Cd concentration and grain As concentration. The factor that caused this opposite effect might be waterlogging. Correlation analyses also were performed in Clay Center (Supplemental Fig. S5 and S6) to evaluate the relationship of grain Cd and As concentration in the absence of waterlogging. Although still significant, the negative plot basis correlation between grain Cd concentration and grain As concentration was much weaker in Clay Center ($r = -0.16$, $p = 0.012$) than in Mead ($r = -0.54$, $p < 2.2 \times 10^{-16}$). Therefore, waterlogging of the south part of the Mead trial might be a potential explanation for the lower grain Cd concentration in Mead than in Clay Center despite the higher soil Cd concentration prior to planting. Because of the significant effect of soil variation on grain Cd concentration, it is important to replicate genotypes within a location to remove the effect of field variation and obtain accurate estimates of genotype effects when the trial is not homogeneous. Despite the significant location effect on grain Cd concentration, the rank correlation of genotypic least square means between Mead and Clay Center and the heritability for grain Cd concentration was high ($r = 0.61$, $p < 0.001$, $H^2 = 0.72$). This high repeatability of grain Cd concentration agrees with other studies (Kubo et al., 2008; Gao et al., 2011), and this suggests the possibility of breeding consistently low-Cd wheat cultivars across environments.

Effect of Sets on Selection Results

The selection process was replicated using three sets of RILs to test if the effect of selection is reproducible for

different sets of lines. The ratio of the number of *HMA3*-linked marker-based low-Cd lines to the number of heterogeneous lines to the number of *HMA3*-linked marker-based high-Cd lines (*HMA3*-linked marker-based low:heterogeneous:*HMA3*-linked marker-based high) was 16:3:12 in Set 1, 16:7:14 in Set 2, and 17:5:15 in Set 3. Thus, the RILs segregated appropriately for the SNP markers as a single locus within each set ($p > 0.1$).

To test whether our replicated selection protocols were equally effective, both selection by set interaction and set had no significant ($p > 0.05$) effect on grain Cd concentration (Supplemental Table S4). Therefore, the effect of selection on grain Cd concentration was reproducible with different sets of lines. Because our replicated selection protocols were equally effective in three sets, the RILs from all three sets were combined to better assess the effect on grain Cd concentration for both hydroponic selection and *HMA3*-linked marker-based selection.

Effect of Hydroponic Selection on Grain Cadmium Concentration

When grown in the field following selection, hydroponic high Cd lines had significantly ($p < 0.01$) higher average grain Cd concentration than the hydroponic low-Cd lines (0.165 vs. 0.144 mg kg⁻¹, Fig. 2). However, there was overlap between the two groups for grain Cd concentration of individual field-grown lines (Fig. 2). Thus, the lines selected as low-Cd lines using the hydroponic system at seedling stage tended to, but did not always, have lower grain Cd concentration in the field at maturity than the lines selected as high Cd lines. Hydroponic selection had

Fig. 2. Average grain Cd concentration in the low- and high-Cd groups selected using hydroponic selection (left panel), *HMA3*-linked marker-based selection (middle panel), and the combination of hydroponic and *HMA3*-linked marker-based selections (right panel). **, ***, and **** denote that the difference between the two groups is significant with $p < 0.01$, 0.001 , and 0.0001 , respectively. SNP, single nucleotide polymorphisms.

no significant effect on other tested traits, including grain Fe and Zn concentration (Supplemental Table S5).

The selection performance may be improved by optimizing the hydroponic system. The experience from this study is that shoot Cd concentration separated the high- and low-Cd lines better than the root Cd concentration (data not shown), and that the shoot is much easier to sample than the roots. Therefore, breeders may not need to harvest the roots for making selections. Two-week-old seedlings were used for selection in this study because the time window for making selections between winter wheat harvest and subsequent planting is narrow, and in our preliminary study, shoot Cd concentration in 2-wk-old seedlings growing under $5 \mu\text{M}$ Cd solution effectively separated two known low Cd cultivars (Panhandle and Overland) from two known high-Cd cultivars (Wesley and Freeman). Although this system distinguished the two high-Cd cultivars well from the two low-Cd cultivars, it may not be the optimum system for making selections in large segregating populations with continuous variation. The populations used in breeding programs are generally large, ranging from hundreds to thousands of lines. Therefore, the hydroponic system could likely be further standardized and optimized using large populations to ensure its scalability and repeatability before being routinely used as a selection tool in breeding programs. Another concern with the use of hydroponic system in breeding programs is that the selection will need a large volume of nutrient media containing $5 \mu\text{M}$ Cd, which requires careful disposal.

Effect of *HMA3*-Linked Marker-Based Selection on Grain Cadmium Concentration

When grown in the field after selection, the *HMA3*-linked marker-based high-Cd lines had significantly ($p < 0.0001$) higher average grain Cd concentration than the *HMA3*-linked marker-based low-Cd lines (0.169 vs.

0.142 mg kg^{-1} , Fig. 2). Average grain Cd concentration in the heterogeneous RILs (for the *HMA3*-linked markers) was significantly higher than the average grain Cd concentration in the *HMA3*-linked marker-based low-Cd RILs (0.171 vs. 0.142 mg kg^{-1} , $p < 0.0001$), whereas it was not significantly different from the average grain Cd concentration in the *HMA3*-linked marker-based high Cd RILs (0.170 vs. 0.169 mg kg^{-1} , $p > 0.05$). Thus, the effect of the QTL linked to the *HMA3*-linked markers on grain Cd concentration is likely dominant, with the high-Cd allele being the dominant allele. The additive effect of the *HMA3*-linked markers on grain Cd concentration was 0.014 mg kg^{-1} , which is $0.47\sigma_p$ of the grain Cd concentration in the RIL population across two locations, where σ_p is the phenotypic standard deviation. This agrees with the additive effect of 0.0008 to $0.0259 \text{ mg kg}^{-1}$ reported in the genome-wide association study where this set of markers was first identified (Guttieri et al., 2015). The *HMA3*-linked markers explained 20% of the phenotypic variation in grain Cd concentration in the RIL population. This implies the QTL linked to these markers has a large effect on grain Cd concentration, but in addition to this QTL, there may be other loci controlling grain Cd concentration in this RIL population. Except for the effect on grain Cd concentration, *HMA3*-linked marker-based selection had no significant effect on other tested traits, including grain Fe and Zn concentration (Supplemental Table S5).

Given the large effect of the *HMA3*-linked markers and the presence of other unknown QTLs controlling grain Cd concentration, a genome-wide selection model with the *HMA3*-linked marker fitted as fixed effect and the remaining markers as random effects (Bernardo, 2014) may be a useful approach to more accurately predict grain Cd concentration. Another advantage of genome-wide selection over using single marker only is that the genome-wide selection could improve other traits concurrently

within the population over generations, whereas the single-marker approach would be used for one generation to select lines, which will be fixed at the target locus after one generation of selection with no information on other target regions. Hence, we recommend using the *HMA3*-linked markers for early generation selection to enrich the population for marker-based low Cd lines and genome-wide selection in later generations.

Comparison of Hydroponic and Marker-Based Selection for Selection Efficiency

As both hydroponic selection and *HMA3*-linked marker-based selection were capable of distinguishing the high- and low-Cd lines, we next compared the two selection methods for their efficiency to reduce grain Cd concentration. Four selection strategies of keeping hydroponic low-Cd lines, discarding hydroponic high-Cd lines, keeping *HMA3*-linked marker-based low-Cd lines, and discarding *HMA3*-linked marker-based high-Cd lines were compared (Supplemental Table S6). Among the four selection strategies, the two strategies of keeping hydroponic low-Cd lines and keeping *HMA3*-linked marker-based low-Cd lines significantly ($p < 0.05$) reduced grain Cd concentration in the population (Supplemental Table S6). These two selection strategies resulted in a similar mean and level of variation for grain Cd concentration, though keeping *HMA3*-linked marker-based low-Cd lines had a slightly greater response. In terms of simplicity, the *HMA3*-linked marker-based selection method takes <1 mo for one person to screen the RIL population (105 RILs + two parents), whereas the hydroponic selection method takes nearly 3 mo for one person to screen the RIL population (105 RILs + two parents) with continuous work. Furthermore, many breeding programs use markers for many traits, so the only additional cost might be running the KASP assay for Cd accumulation genes. Although there were advantages of the *HMA3*-linked marker-based selection method, the hydroponic system will remain a powerful substitution to field study for identifying low-Cd lines at the seedling stage, especially for species where breeder-friendly markers are not available or where the markers cannot explain a large proportion of the variation in grain Cd concentration. The increasing understanding of genetic architecture and physiological processes controlling grain Cd concentration and the development of technologies will help to continuously improve the efficiency of selecting low-Cd bread wheat.

Developing a Better Understanding of Cadmium Accumulation in Wheat Grain

After assessing the effect of each selection method on grain Cd concentration, we compared the combined effect of these two selection methods on grain Cd concentration. The χ^2 independence test indicated that the two classification criteria, *HMA3*-linked marker-based and

hydroponic-based, were independent from each other (Supplemental Table S7). There was a significant interaction between the two selection methods on grain Cd concentration (Supplemental Table S4). The two selection methods and their interactions together explained 32% of the phenotypic variation in grain Cd concentration. To further explore the interaction between the two selection methods, the RILs were grouped into four groups: hydroponic low and *HMA3*-linked marker-based low (HL_ML), hydroponic low and *HMA3*-linked marker-based high (HL_MH), hydroponic high and *HMA3*-linked marker-based low (HH_ML), and hydroponic high and *HMA3*-linked marker-based high (HH_MH). The HH_MH group had significantly higher average grain Cd concentration than the other three groups (Supplemental Table S8). The grain Cd concentrations of 21% of the RILs in the HH_MH group were >0.2 mg kg⁻¹, whereas grain Cd concentration of none of the RILs in the other three groups was >0.2 mg kg⁻¹ (Supplemental Fig. S7). Therefore, being low for either of the selection method equally and effectively regulated the accumulation of Cd in grain in this study.

Association of the ALMT1-UPS4 Marker with Grain Mineral Concentrations

Our RIL population also segregated for an STS marker, ALMT1-UPS4, designed from the promoter region of *TaALMT1* and associated with a functional ALMT1, which confers Al³⁺ tolerance (Sasaki et al., 2006; Pineros et al., 2008). The upstream region of *TaALMT1* was classified into six main types based on the presence of repeated blocks of sequence, and all these six upstream types could be identified using marker ALMT1-UPS4 (Sasaki et al., 2006). The Al tolerance allele was contributed by the high-Cd parent, Wesley. We screened our RIL population using ALMT1-UPS4 to assess the association between the functional allele of *TaALMT1* contributed by Wesley and grain mineral concentrations.

The positive control, Wesley, amplified two strong bands of length ~ 800 and ~ 1200 bp, indicating the insertion of repeated blocks in the upstream region (Sasaki et al., 2006). Another positive control, Panhandle, amplified one strong band of length ~ 450 bp, indicating the absence of repeated blocks in the upstream region (Sasaki et al., 2006). Forty-nine RILs amplified the ~ 1200 -bp band (hereafter denoted as the ALMT+ group), 46 RILs amplified the ~ 450 -bp band (hereafter denoted as the ALMT- group), and 10 RILs amplified both the ~ 1200 - and ~ 450 -bp bands ($\chi^2 = 0.9$, $p = 0.6$) (Supplemental Fig. S8). The segregation of ALMT1-UPS4 marker was independent of the *HMA3*-linked marker ($\chi^2 = 1.3$, $p = 0.8$) and our hydroponic selection result ($\chi^2 = 1.8$, $p = 0.8$). All the ALMT+ RILs produced extra weaker bands, which may be artifacts produced when primers and amplified

fragments misannealed to the repeated blocks (Sasaki et al., 2006). None of the RILs amplified a strong band of length ~800 bp, indicating the absence of this upstream type in the Wesley parent of this RIL population. One reason could be that Wesley is heterogeneous for ALMT1-UPS4 marker. Although the Wesley parent happens to be homozygous for this marker, the four Wesley plants combined as control DNA captured the heterogeneity of Wesley for this marker. Nevertheless, all the ALMT+ RILs produced longer band (~1200 bp) than the ALMT- RILs (~450 bp), indicating the presence of repeated blocks in the upstream sequence of *TaALMT1* in the ALMT+ group, and the absence of repeated blocks in the upstream of *TaALMT1* in the ALMT- group.

The ALMT+ and ALMT- RILs had similar grain Cd, but ALMT+ RILs had significantly ($p < 0.05$) higher average grain Fe, Mg, P, and Zn concentration than ALMT- RILs (Table 2, Supplemental Fig. S9). The additive effects of the ALMT1-UPS4 markers on grain Fe, Mg, P, and Zn concentrations were 0.65, 12, 36.5, and 0.5 mg kg⁻¹, respectively, which were 0.24 σ_p , 0.19 σ_p , 0.19 σ_p , and 0.22 σ_p of the grain Fe, Mg, P, and Zn concentration in the RIL population across two locations. These results suggest that the *TaALMT1* Al tolerance allele on 4DL could be potentially used in breeding to increase grain Fe and Zn concentrations without affecting grain Cd concentration. Recent work in *Arabidopsis* has indicated that ALMT underpins a malate-dependent mechanism of Fe relocation in root apical meristem that is a key to root adaptation to low-P conditions (Mora-Macías et al., 2017). The role of ALMT in Fe, Mg, and Zn uptake in wheat merits further investigation.

Association of the GWM261 Marker with Grain Mineral Concentrations

GWM261 is an SSR marker mapped 0.6 cM distal to *Rht8* (Korzun et al., 1998), which regulates cell elongation. The 192-bp allele of GWM261 is associated with shorter parenchyma cells in peduncle and internode I (Gasparini et al., 2012). Because of the association of this marker with plant height and the potential effect of plant height (Arduini et al., 2014; Perrier et al., 2016) on grain Cd concentration, we screened our RIL population using

GWM261 to assess the association between this marker and grain mineral concentrations.

Wesley and Panhandle amplified the 193- (*Rht8*-associated) and 163-bp alleles, respectively. Forty-six RILs, forty-four RILs, and fifteen RILs amplified the 163-bp allele, 193-bp allele, and both alleles, respectively. The segregation of GWM261 marker was independent from the *HMA3*-linked marker ($\chi^2 = 0.36$) and the hydroponic selection result ($\chi^2 = 0.16$). The 193-bp allele and 163-bp allele should correspond to the commonly known 192-bp allele and 165-bp allele, respectively (Korzun et al., 1998). Hereafter, we will reference the 193-bp allele as the 192-bp allele and the 163-bp allele as the 165-bp allele.

The 192-bp allele increased the grain Cd (0.015 mg kg⁻¹, $p = 0.017$), Fe (1.2 mg kg⁻¹, $p = 0.06$), Mg (22 mg kg⁻¹, $p = 0.08$), and P (70 mg kg⁻¹, $p = 0.1$) concentrations in this RIL population across two locations (Table 2, Supplemental Fig. S10). The shorter parenchyma cells in *Rht8* genotypes may have smaller vacuole for storing minerals in the stem and result in higher Cd, Fe, Mg, and P concentration in grain. The GWM261 and the *HMA3*-linked marker affected grain Cd concentration additively without interaction (Table 3). The RILs with both the 192-bp GWM261 allele and the high-Cd *HMA3*-linked marker allele had the highest average grain Cd concentration (0.171 mg kg⁻¹), whereas the RILs with both the 165-bp GWM261 allele and the low-Cd *HMA3*-linked marker allele had the lowest average grain Cd concentration (0.133 mg kg⁻¹) (Supplemental Fig. S11). These results suggest that breeders using *Rht8* to decrease plant height and reduce lodging may need to be particularly attentive to eliminating high-Cd alleles at the *HMA3*-linked locus on 5A.

Correlation of Grain Cadmium Concentration with Other Traits

In this RIL population, grain Cd concentration was positively correlated with grain Cu, Fe, Mg, Mn, and Zn concentration and negatively correlated with plant height (Supplemental Fig. S12) and was not correlated with grain yield. Studies in durum wheat showed that reduced plant height promoted Cd accumulation in grain, which may be due to the higher biomass accumulation in roots before

Table 2. Average grain mineral concentrations and plant height in ALMT+ and ALMT- recombinant inbred lines (RILs) and in Rht8+ and Rht8- RILs and differences calculated as (ALMT+) - (ALMT-) and (Rht8+) - (Rht8-).

Class	Ca	Cd	Cu	Fe	K	Mg	Mn	Ni	P	Zn	Height
	mg kg ⁻¹										cm
ALMT+	130	0.152	2.45	33	4466	1302	46.3	0.415	4624	30.4	-
ALMT-	128	0.16	2.37	31.7	4481	1278	45.2	0.421	4551	29.3	-
Difference	2	-0.008	0.08	1.3*	-15	24*	1.1	0.006	73*	1.1 *	-
Rht8+	128	0.163	2.44	33.1	4472	1305	46.4	0.427	4636	30	97.9
Rht8-	130	0.148	2.37	31.9	4464	1282	45.5	0.404	4567	29.9	99.5
Difference	-2	0.015*	0.07	1.2	8	23*	0.9*	0.023	69*	0.1	-1.6

* Significant at the 0.05 probability level.

Table 3. Analysis of variance for the effect of *Rht8*-associated markers and the *HMA3*-linked single nucleotide polymorphism (SNP) markers on grain Cd concentration (mg kg⁻¹).

Effect	df	SS†	MS‡	F value	P value
<i>Rht8</i>	2	0.006	0.003	4.33	0.016
<i>HMA3</i> -linked SNPs	2	0.017	0.009	12	2.24 × 10 ⁻⁵
<i>Rht8</i> : <i>HMA3</i> -linked SNPs	4	0.005	0.0014	1.923	0.113
Residual	96	0.07	0.0007		

† SS, sum of squares.

‡ MS, mean square.

heading and the higher Cd allocation from root to shoot after heading in short genotypes than in tall genotypes (Arduini et al., 2014; Perrier et al., 2016). Therefore, the selection of low-Cd wheat plants may unintentionally result in taller plants. In spite of the positive correlations between grain Cd concentration and grain Fe and Zn concentrations, the concentrations of Fe and Zn, as well as other minerals in grain, were not significantly reduced by the *HMA3*-linked marker-based selection and hydroponic selection in this study (Supplemental Table S5). The *TaALMT1* Al tolerance allele on 4DL was significantly and positively associated with grain Zn and Fe concentrations but was not associated with grain Cd concentration. Therefore, Zn and Fe may be regulated differently than Cd in this RIL population in some pathways, which provides the opportunity to decrease grain Cd concentration without affecting grain Fe and Zn concentrations.

CONCLUSION

Both hydroponic selection and *HMA3*-linked marker-based selection were effective in identifying high- and low-Cd lines, but the *HMA3*-linked marker-based selection was more effective and less time and labor consuming, especially in programs already genotyping lines. In addition, the hydroponic system may need to be optimized and standardized using additional populations to ensure its scalability and repeatability before being routinely used in breeding programs. The *HMA3*-linked markers explained 20% of the phenotypic variation, which may imply the presence of other QTLs controlling grain Cd concentration in this RIL population. Therefore, it may be worthwhile to test the efficiency of genomic selection on grain Cd concentration. Grain Cd concentration in bread wheat may be controlled by multiple mechanisms and the interaction among these mechanisms. The *Rht8* allele for reduced plant height was associated with increased grain Cd concentration, which may be a significant consideration for breeders working with *Rht8* germplasm. Hydroponic selection and *HMA3*-linked marker-based selection had no significant effect on grain Zn and Fe concentrations. The Al tolerance allele of *ALMT1* contributed by Wesley was not associated with grain Cd concentration, but the Al tolerance allele increased grain Zn and Fe concentrations. It is possible to

decrease grain Cd concentration without affecting grain Fe and Zn concentrations by using specific markers for Cd, such as the *HMA3*-linked marker used in this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

Supplemental Material Available

Supplemental material is available online for this article.

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