VALIDATION OF MOLECULAR MARKERS ASSOCIATED WITH GRAIN CADMIUM IN DURUM WHEAT (*TRITICUM TURGIDUM* L. VAR. DESF.)

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Title

Validation of Molecular Markers Associated with Grain Cadmium in Durum Wheat

(*Triticum turgidum* L. var. *durum* Desf.)

By

Evan Salsman

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Durum wheat is capable of accumulating cadmium, a toxic heavy metal, in the grain at levels that have been deemed unsafe. Previous studies have identified genetic variation in durum wheat that can be exploited to create low Cd cultivars. In this study, six KASP markers were validated on 4,178 durum wheat samples from preliminary and advanced yield trials grown in 2013 and 2014 at Langdon, Minot, and Williston, North Dakota. One marker on chromosome 5B was polymorphic in all crosses between high and low Cd parents and had r^2 values ranging from 0.38-0.85. Two other markers on the same chromosome predicted similar levels of variation in many trials; however these were not polymorphic in all populations. Two markers linked to the grain Cd locus on chromosome 5B are suitable for marker assisted selection due to the more widely shared polymorphism of one and the closer linkage distance of the other.

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INTRODUCTION

Cadmium (Cd) is a heavy-metal element that is commonly found in many soils throughout North America, with some soils containing higher concentrations of the element than others. Certain plant species have the undesirable potential to collect Cd when abundant in the soil and store it in the edible portions of the plant. When found in high concentrations in food products, the effects can be deleterious to human health (Codex, 2011). Durum wheat (*Triticum turgidum* L. var. *durum* Desf.) has demonstrated the ability to accumulate Cd (Zook et al., 1970) and can exceed the proposed limit of 0.2 mg/kg grain concentration (Codex, 2011). Although grain Cd concentrations can be measured, the procedure is costly and may not be economically viable when examining a large number of samples. With the use of marker assisted selection, however, the progeny that carry alleles associated with low Cd (Penner et al., 1995) can be more efficiently incorporated into durum breeding programs. The objective of this study was to validate six markers, previously found to be predictive of grain Cd concentration, within the diverse range of lines in our 2013 and 2014 durum wheat yield trials.

LITERATURE REVIEW

Durum Wheat

Durum wheat is a tetraploid wheat species composed of genomes from two species in the Poaceae family named *Triticum urartu* and *Aegilops speltoides*, which contributed the A and B genomes, respectively (Dvorak et al., 1993; Akhunov et al., 2005). This polyploid event is thought to have occurred slightly less than 0.5 million years ago (Huang et al. 2002). The initial AABB result created what is today known as wild emmer wheat (*Triticum dicoccoides*), which produces a grain very different from modern durum wheat and far less suited for agriculture.

Approximately 9-10 thousand years ago, human selection of wild emmer slowly gave rise to domesticated emmer (*Triticum dicoccum*), the progenitor of durum wheat and the A and B genomes of bread wheat (*Triticum aesitivum*) (Willcox, 2005; Tanno and Willcox, 2006). One of the most significant traits selected for in this process was the non-brittle rachis, which prevented shattering prior to harvest and is controlled by loci on chromosomes 3A and 3B (Nalam et al., 2006). In the transition from domesticated emmer to durum wheat, at least one allele responsible for free-threshing grain, the *Q* allele on chromosome 5A, became fixed (Muramatsu, 1986; Faris et al., 2006; Jantasuriyarat et al., 2004). The successful assemblage of many other alleles contributing to traits associated with domestication syndrome such as yield, kernel weight, and flowering time is a likely reason durum wheat is still widely grown today (Elias et al., 1996; Peng et al., 2003).

Durum wheat was first brought to the US by the United States Department of Agriculture in 1850 (Joppa and Williams, 1988). Although farmers were not quick to adopt durum due to different milling properties compared with bread wheat, superior rust resistance eventually made growing durum an appealing option. Today, durum wheat produced in the US is mainly used for

pasta products. Durum is often grown due to its high price per bushel and increased drought tolerance compared to other wheats. In 2015, durum wheat acreage has been estimated at 1.95 million in the US, with approximately 56% of the total acreage in North Dakota (National Agricultural Statistics Service, 2015).

Cadmium in the Food Chain

Cadmium occurs naturally in soils and often exists at low levels that are not of agronomic importance. However, the mining and processing of non-ferrous elements, such as zinc, and the use of Cd in products, such as batteries, has increased the amount of Cd available to plants (Nriagu and Pacyna, 1988). These human influences coupled with high levels of naturally occurring Cd in certain areas have made some soils capable of producing high Cd products when Cd accumulating crops are sown. Crops such as non-oilseed sunflower (*Helianthus annuus* L.), flax (*Linum usitatissimum* L.), and durum wheat have demonstrated the potential to contain more grain Cd than most other crops (Li et al., 1997). This problem can be partially alleviated if genetic variability for Cd uptake exists within a species. In durum wheat, at least one major gene controlling Cd uptake exists, which when utilized will allow for the creation of low Cd cultivars that ultimately produce safer end-products.

Effect of Cadmium on Human Health

High dietary levels of Cd can affect human health in a variety of detrimental ways. In the kidneys, Cd amounts tend to increase with age and can lead to renal failure (Codex, 2011). Cadmium has a long half-life of 15-30 years and urinary excretion often does not match dietary intake (Codex, 2011; Berglund et al., 2000). Several urinary markers indicative of renal dysfunction have been found to increase in individuals with high levels of urinary Cd (Trzcinka-Ochock et al., 2004). The disruption of renal tubular function is believed to indirectly affect

bone health as well. Aoshima et al., (2003) found correlations between Cd induced renal tubular dysfunction and several biochemical markers associated with bone formation and resorption. In rats, Cd is also believed to directly interact with the formation of bone tissue by interfering with cell types involved with bone maintenance (Chen et al., 2009). Zhu et al. (2004) found an association between increased urinary Cd and reduced forearm bone mineral density in both men and women. Post-menopausal women are of particular concern due to naturally larger decreases in bone mineral density with age than men (Kazantzis, 2004). In Japan and China, high Cd in rice (*Oryza sativa* L.) grain has been linked with Itai-itai disease in humans. This disease is caused by severe Cd exposure and is characterized by reduced bone density and many symptoms that are similar to osteoporosis. Like rice, durum wheat can be a major component of an individual's diet, which makes low Cd durum cultivars crucial to human food safety.

Though Cd is clearly detrimental to human health, it is not entirely clear whether it is a carcinogen. Several studies have examined carcinogenicity of Cd in European populations exposed to abnormally high levels of Cd (Sorahan and Esmen,2004; Nawrot et al., 2006; Akesson et al., 2008). Both Nawrot et al. (2006) and Akesson et al. (2008) found positive correlations between increased Cd exposure and cancer, though Sorahan and Esmen (2004) did not find significant results. Possible confounding factors in these studies are the forms of Cd participants were exposed to, smoking, and diet, among others. Although it can be difficult to strongly identify Cd as a carcinogen in humans due to the large sample sizes and accurate health records needed, many studies have investigated the effects of Cd administration in other mammals. Oral Cd administration was found to increase tumor incidence in rats (Waalkes and Rehm, 1992) while subcutaneous administration did the same in both mice and rats (Waalkes

and Rehm, 1994; Waalkes et al., 2000). Results from multiple species lead to the classification of Cd as a carcinogen by the International Agency for Research on Cancer (1993).

Environmental Influences on Cd Uptake

Several studies have been conducted pertaining to environmental influences on Cd uptake. Soil Cd and chloride (Cl) content have been associated with high grain Cd (Norvell et al., 2000). In durum grown on a diverse range of soils near Langdon, North Dakota, the model that best predicted grain Cd used soil extractable Cd and Cl as the variables. Although soil zinc (Zn), pH, and salinity were also significantly associated with grain Cd, the addition of these to the best stepwise two variable model did not significantly increase the R^2 values. Cl is believed to increase the solubility of Cd which releases more Cd into the soil solution and enhances plant uptake (Bingham et al., 1984).

Soil pH is also believed to influence the availability of Cd to plants. A study conducted by Adams et al. (2004) using paired wheat and soil samples found the factors that most reliably predicted grain Cd in wheat were soil Cd concentration and pH. When these two factors were combined, approximately 49% of the grain Cd variation was explained. In general, low soil pH and high soil Cd are considered conducive to high grain Cd (Iretskaya and Chien, 1999) though it is important to note that in some instances, total soil Cd can be misleading and an extraction method predicting plant available Cd may produce more reliable results (Mench et al., 1997). This is especially concerning since soil acidification tends to increase with nitrogen fertilizer use (Guo et al., 2010; Schroder et al., 2010), which when coupled with high soil Cd could increase the amount of Cd in the food chain.

Another environmental factor that can influence Cd uptake is the amount of available Zn present in the soil. In several species such as flax, durum, lettuce (*Lactuca sativa* var. *longifolia*

cv. Paris Island), and spinach (*Spinacia oleracea* L. cv. Vienna), an antagonistic effect on Cd uptake has been observed (Jiao et al., 2004, Hart et al., 2002, McKenna et al., 1993). This is consistent with the finding that durum wheat plants deficient in Zn accumulate higher amounts of grain Cd (Oliver et al., 1994). Although the reasoning behind this phenomenon is still unclear, the addition of Zn can partially alleviate Cd uptake. A possible explanation could be decreased membrane functionality in zinc-deficient plants (Cakmak and Marschner, 1988).

Fertilization can also affect Cd uptake in crops several different ways. Fertilizer sources of phosphorus (P) can naturally contain Cd, which when applied frequently can increase soil Cd available for uptake (Williams and David, 1973). Animal manures can contain especially high levels of Cd, particularly when the source animals are given mineral supplements (Wu et al., 2012). Wu et al. (2012) found soils applied with pig manure treatments for 16 years had at least a 4-fold increase in soil extractable Cd compared to the control. Interactions between nutrients and Cd uptake are also known to exist. Increases in leaf and grain Cd concentrations were observed in flax and durum wheat with increasing commercial and reagent grade P treatments (Jiao et al., 2004). No noticeable differences between P treatments were due to Cd contamination in the commercial grade P treatment, indicating that in this instance the P was responsible for the increase in grain Cd. Mitchell et al. (2000) found increased grain Cd concentration with higher nitrogen (N) supply. The reason for this was suggested to be a combination of increased transpiration and changes in the soil solution. Anions accompanying nutrients of interest can increase the availability of Cd as well. Using different potassium (K) treatments, Zhao et al. (2003) found the form of K significantly influenced shoot Cd concentration in wheat. Though Cd uptake can be partially controlled through fertilizer

management, this may not be practical due to the positive correlations found with N and P, implying a reduction in fertilizers may be required to achieve the desired Cd level.

Physiology of Cd in Wheat

Physiological differences in Cd translocation are known to exist between and within different species of wheat. Compared to common bread wheat (*Triticum aestivum* L.), durum wheat has the potential to accumulate more Cd in the grain (Zook et al., 1970). Though bread wheat can accumulate much higher root and shoot Cd, translocation to the grain is greatly reduced when compared to durum wheat (Hart et al., 1998). Increased apoplasmic binding of Cd in bread wheat was suggested for the total plant increase; however, the mechanism for decreased grain levels in bread wheat remains unclear. In durum wheat, high shoot Cd can be used as an indicator of high grain Cd, though the effectiveness depends largely on the duration of Cd exposure (Archambault et al., 2001; Hart et al., 2006). Gregor and Lofstedt (2004) concluded that root Cd cannot be used as an indicator of grain Cd since both high and low accumulating lines contained similar root concentrations. Hart et al., (2006) used two near-isogenic lines differing in grain Cd accumulation to determine that reduced xylem loading of Cd is likely responsible for the low Cd phenotype. This has led to the belief that shoot Cd is translocated from the leaves to the grain during the filling stage in durum wheat.

Since Cd is a nonessential element chemically similar to zinc, it has been suggested that some membrane transporters cannot completely distinguish between Zn and Cd. Hart et al., (2002) concluded that Zn and Cd concentration in the leaves of bread and durum wheat were largely controlled by one transporter. Studies of other species have found that once Cd is in the cell, Cd can bind to certain proteins that interact with other essential metals, though by binding

with Cd, their intended function is presumably not performed (De Filippis and Ziegler, 1993; Brzoska and Moniuszko-Jakoniuk, 2001).

Marker Assisted Selection in Plant Breeding

Classical markers have aided plant breeders long before the existence of molecular marker techniques. When a gene of interest has a close linkage distance with a gene controlling a visual trait, selection of the visual trait increases the probability of inheriting the gene of interest. In durum wheat, an example of this is selection of white glumes, which is controlled by a gene residing closely to a quantitative trait locus (QTL) partially controlling gluten strength (Leslie et al., 1981). Use of these markers is limited however, since an easily identifiable morphological trait co-segregating with a trait of interest is rare and selection upon morphological traits is not phenotype neutral (Tanksley, 1989). Today, molecular markers have largely supplanted visual markers due to the vastly increased chances of discovering closely linked markers.

One of the first molecular marker techniques used in plants was Restriction Fragment Length Polymorphism (RFLP) (Tanksley et al., 1989). Several crop species such as tomato (*Lycopersicon esculentum* L.) and maize (*Zea mays* subsp. *mays* L.) have had genetic linkage maps constructed using this system (Helentjaris et al., 1986; Zhang et al., 2002; Schön et al., 1994). RFLP markers have several shortcomings that have led to a decline in use, the most notable being high cost and the necessity for radioactive material (Rafalski and Tingey, 1993). The first widely available molecular marker technique commonly used for plant improvement was the Randomly Amplified Polymorphic DNA (RAPD) technique due to the low cost and abundance of markers, although the low repeatability of this technique was a concern that limited its acceptance.

Next generation sequencing allows for the detection of polymorphisms on an enormous scale. These marker systems use high throughput sequencing to identify thousands of single nucleotide differences that can be utilized for selection in plant breeding programs. Screening with numerous molecular markers allows for the selection of very tightly linked markers at the loci of interest. Another advantage of this method is the ability to screen a large number of samples simultaneously, which makes association mapping studies including hundreds of individuals feasible. The numerous advantages of this method of sequencing have made it one of the most widely used marker identification systems today.

With the advent of next generation sequencing, marker validations using non-gel techniques became possible. Kompetitive Allele Specific Polymerase Chain Reaction (KASP) is one such method (LGC Ltd., Teddington, UK). This system allows for high throughput screening of previously identified markers and is highly suited to plant breeding applications (Semagn et al., 2014). The main attraction of this method is the reduced cost of screening genotypes with a low number of single nucleotide polymorphisms (SNP), making it significantly cheaper when a large number genotypes are involved.

Use in Marker Assisted Selection for Reducing Cd Concentration in Durum Wheat

A study conducted by Penner et al. (1995) revealed the presence of *Cdu1*, a dominant gene associated with low grain Cd accumulation. This locus was identified using the RAPD marker system. The primer that detected the polymorphism and was most closely linked to the low Cd allele was designated 'OPC-20'. A further examination of this marker within their germplasm found 'OPC-20' to effectively screen all adapted cultivars and 18 out of 20 exotic lines. Later studies conducted by Clarke et al. (1997) and Knox et al. (2009) determined that the expression of *Cdu1* was highly heritable and that the gene is located on chromosome 5B,

respectively. Since *Cdu1* is simply inherited it can easily be incorporated into existing germplasms if a suitable marker is used.

The discovery of this variation has led to the search for useful genetic markers to aid in the selection of low Cd durum cultivars in other germplasms. A polymorphic marker that resides in the majority of low Cd durum lines and is closely linked to the Cd uptake locus would be the most useful. With a close linkage distance, the likelihood of a break between the marker and the low Cd allele would be lower and the possibility for selection errors would be reduced. The markers used in this study were identified by Wesam AbuHammad in biparental and association mapping studies using a custom-designed 9k Illumina iSelect BeadChip platform and the Illumina Infinium Assay protocol (AbuHammad 2013; Steemers et al., 2006). The population in the biparental mapping study consisted of recombinant inbred lines (RILs) created from the parents Grenora and Haurani. Grenora is a high Cd accumulating cultivar developed by the North Dakota Agricultural Experiment Station (Elias and Manthey 2007c) and Haurani is low Cd accumulating landrace selected by the International Center for Agricultural Research in Dry Areas (ICARDA) located in Syria. The screening of this population lead to the identification of an SNP linked to a gene responsible for high Cd accumulation from Grenora that controlled approximately 54% of the phenotypic variation. A second RIL population was used to validate this marker using the parents Strongfield (Clarke et al. 2005) and Alkabo (Elias and Manthey 2007a). In this population, Strongfield was the source of low Cd accumulation with its low Cd allele descended from Nile, which was obtained through the International Center for Agricultural Research in Dry Areas (ICARDA) (Clarke et al., 1997). Alkabo is a cultivar released by the North Dakota Agricultural Experiment Station which generally accumulates high amounts of Cd (Elias and Manthey 2007a). This created a second population segregating for the grain Cd

uptake locus. Results indicated that the marker could effectively identify low Cd lines from a different parentage.

AbuHammad, (2013) also conducted an association mapping study that revealed four potentially useful markers related to Cd uptake. The mapping population entailed two collections of low and high Cd durum lines. Of the four markers discovered, two identified a locus similar to the one found in the study conducted by Penner, (1995). The markers on chromosome 5B are of primary importance since the two were found to explain 33.7% and 27% of the phenotypic variation, respectively. Markers found in this study have not been validated yet.

A genetic marker closely linked to the Cd uptake gene could bring benefits to several areas of the industry. The North Dakota State University (NDSU) Durum Breeding Program could benefit from the more cost-effective method of incorporating low Cd into their existing germplasm. From a grower's perspective, durum could be grown in fields that formerly produced unsafe levels of grain Cd when using susceptible cultivars. Also, with grain Cd being controlled mostly genetically, expensive management costs such as zinc fertilization may be unnecessary to produce low Cd grain. Lastly, pasta companies and consumers may have the most to gain due to increased product safety.

MATERIALS AND METHODS

Plant Materials

In this experiment, breeding lines and checks in preliminary (PYT), advanced (AYT), and elite yield trials (EDA) and the Uniform Regional Durum Nursery (URDN) were used. This provided information on a broad spectrum of lines and cultivars used as checks. Trials were grown in Langdon and Williston in 2013 and Langdon, Williston, and Minot in 2014. Due to the high number of selfing generations ($F_{4:6}$ to $F_{4:9}$), it is unlikely many of the lines will be segregating for the Cd uptake gene. A total of 1,130 and 3,048 samples were phenotyped in 2013 and 2014, respectively.

Grain Sample Preparation

Subsamples were first collected from each plot at Langdon, Minot, and Williston, ND. The subsamples consisted of eight hand harvested spikes that were cut using stainless steel knives in order to avoid cadmium contamination. Once cut, the spikes were stored in envelopes and placed in a drier if the moisture content was above 13%. The remaining grain from each plot was harvested with a combine. This seed was not used to determine grain Cd concentration since contamination may have occurred from parts within the combine.

The eight hand-harvested spikes of each line were used for genotypic and phenotypic data. A specialized grain thresher equipped with leather parts was used to thresh the grain in order to avoid contamination that may originate from non-stainless steel metal. Once threshed, four seeds were chosen from each line and sown in Sunshine Mix Complete potting soil for the purpose of testing the genetic markers.

Genotype Analysis

A 2-inch leaf tissue sample was chosen from the seedlings of each line at the three to four leaf stage for genotyping. While harvesting the leaf tissue, tools were wiped with a 95% ethanol solution between samples to prevent DNA cross-contamination. The leaf tissue samples were then placed in plates that contained 96 cells, with seven cells in each plate used for checks and one empty cell used as a control. The checks Strongfield (Clarke et al., 2005), Haurani, and D041735 had the low Cd allele and the checks Alkabo, Carpio (Elias et al., 2014), Divide (Elias and Manthey 2007b), Grenora (Elias and Manthey 2007c), and Joppa (Elias and Manthey 2016) had the high Cd allele. D041735 was developed at the North Dakota State University Durum Breeding Program by Dr. Elias.

Once all tissue samples were collected, the plates were submitted to the USDA-ARS Cereal Crops Genotyping Laboratory in Fargo, ND for analysis in collaboration with Dr. Shiaoman Chao. After a drying period of several weeks, the DNA was extracted and tested with six different genetic markers previously found to be associated with grain Cd (Table 1). All markers except Cad 5B were found by Abuhammad (2013) in either bi-parental or association mapping studies. Cad 5B was developed from a RAPD marker discovered by Penner et al. (1995) and later converted for use in the marker system used in this study by Dr. Shiaoman Chao at the USDA Small Grains Genotyping Laboratory. The position of Ex_c1775 within a 10 cM region of chromosome 5B, from the consensus map of tetraploid wheat constructed by Maccaferri et al. 2015, is presented in Figure 1.

The Kompetitive Allele Specific PCR (KASP) genotyping system was used (LGC Ltd., Teddington, UK). Previously discovered nucleotide sequences explaining allelic variation of grain Cd were sent to LGC to develop the KASP markers. PCR amplification was performed

with the Rouche Lightcycler480 (Rouche Diagnostics, Indianapolis IN). Thermal cycling conditions were 94 ºC for 15 minutes, 10 cycles at 94 ºC for 20 sec and 61-55 ºC for 60 sec dropping 0.6 ºC per cycle, and 26 cycles at 94 ºC for 20 sec and 55 ºC for 60 sec. The Rouche Lightcycler480 software version 1.5 was used to determine the allele present for each marker in each sample (Figure 2).

Table 1. Markers associated with Cd accumulation in durum wheat.

SNP marker	Chromosome	Position	r^2 %	Abbreviation in this paper
Ex_c1343_2570756†	5B	82.9	54.3	Ex_c1343
Ex_c17754_26503892‡	5B	165.7	33.7	Ex_c1775
Ex_c20019_29052512‡	5B	178.3	27.0	Ex_c2001
Cad 5B§	5B	?	?	Cad 5B
Ex_c1996_3754393‡	2B	7.25	3.04	Ex_c1996
Ra_rep_c106727_90434958‡	2B	7.25	3.04	Ra_rep_c1067

† Discovered in bi-parental mapping study by Abuhammad (2013)
‡ Discovered in association mapping study by Abuhammad (2013)
§Marker originally discovered by Penner et al., (1995) and converted to KASP marker system by Dr. Shiaoman Chao at the USDA Small Grains Genotyping Laboratory

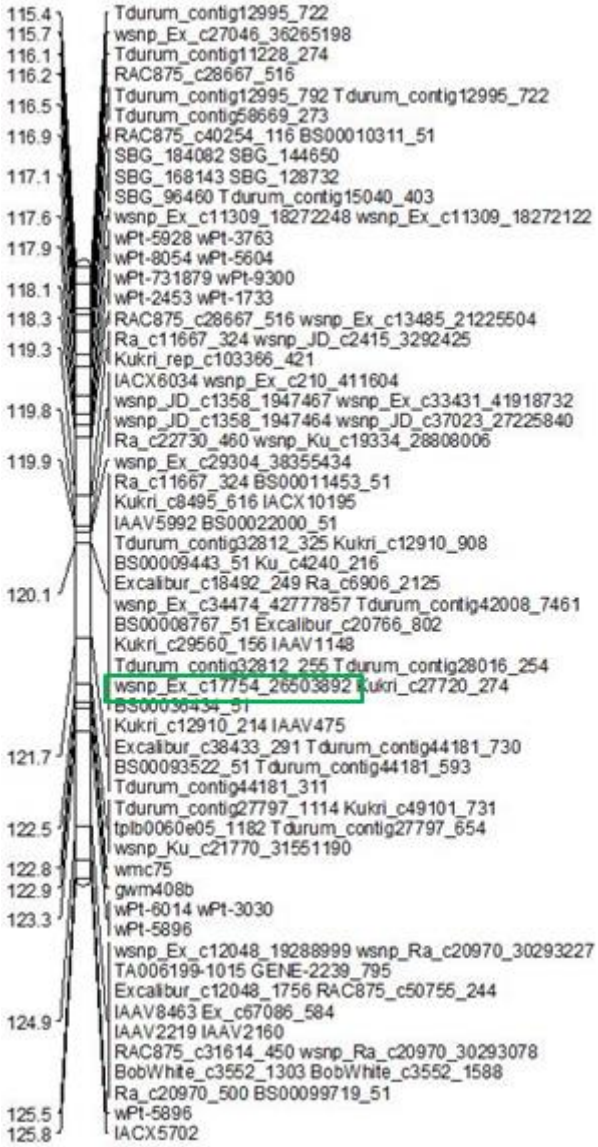

Figure 1. Location of Ex_c1775 within a 10 cM region from a consensus map of the long arm of chromosome 5B (Maccaferri et al. 2015).

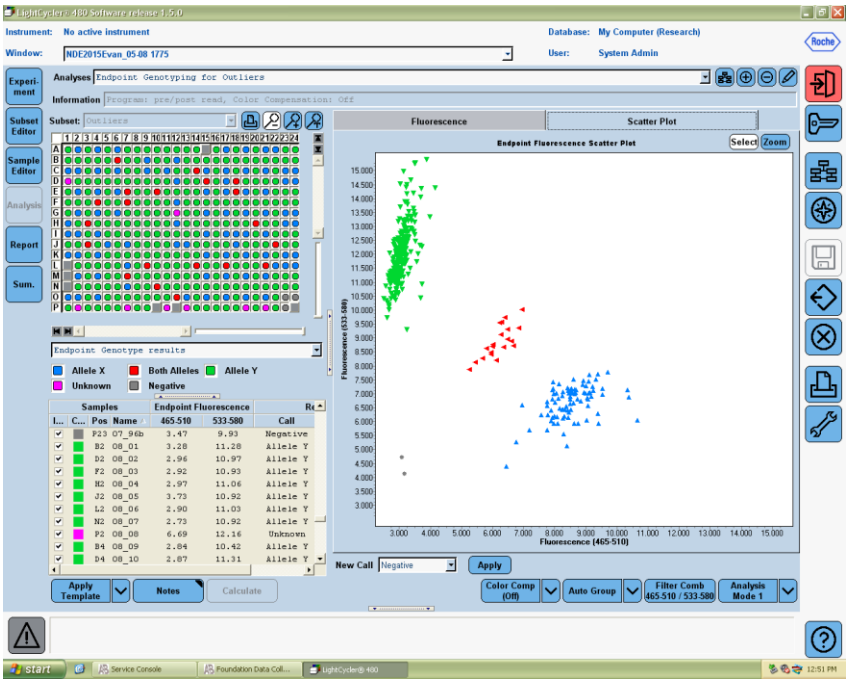

Figure 2. Genotype call in Lightcycler480. Clusters represent the allele present in a line or cultivar.

Cadmium Content Estimation

Grain samples were sent to Dr. Michael A. Rutzke, College of Agriculture and Life Sciences Nutrient Analysis Laboratory, Cornell University, NY for Cd analysis. A representative flour sample of up to 0.5 g from each genotype was digested in 4 mL of mix (40% of concentrated nitric acid and 60% of perchloric acid) and an extra 1 mL perchloric acid. The sample and acid were placed in a fluorocarbon vessel. The open vessel was heated on a hot plate unit. After cooling, the vessel contents were allowed to settle and then were diluted to a 20 mL volume. Analysis was performed with the appropriate SW-864 method (EPA Method No. 3051, 3050, 3052).

Soil Sampling

Previous results have shown that soils in Langdon and Williston are capable of producing grain samples with enough Cd to distinguish between low and high Cd accumulating lines

(AbuHammad 2013). In 2014, soil samples were taken from each location to facilitate a further understanding of circumstances conducive to high Cd translocation to the grain. These soil samples were taken to a depth of 15.24 cm. Mean comparisons were performed on Cd, Zn, Manganese (Mn), and Sodium (Na) soil concentrations between locations using the GLM procedure in SAS 9.3 (SAS Institute, Cary NC). Means separation was performed using a *t*-test at α=0.05.

Experimental Design and Statistical Analysis

In 2013, a simple lattice design was used for all experiments with the exception of EDA, PYT13, and PYT14, the first of which was a randomized complete block design (RCBD) and the latter two augmented designs. For the augmented designs, a total of 80 experimental lines were tested with five checks, with every check replicated in all four blocks. The AYT lattice designs had seven to nine sub-blocks while all PYTs had ten sub-blocks. In 2014, lattice designs were used for all trials except the URDN, which was a RCBD. SAS 9.3 (SAS Institute, Cary NC) was used to obtain means, with PROC LATTICE used on all lattice designs and PROC GLM used on the augmented and RCBD designs.

The data were analyzed using SAS 9.3 (SAS Institute, Cary NC). Regression using PROC GLM was first performed on the LS means for Cd from the experimental design results, with Cd concentration as the dependent variable and the molecular markers as the independent variable. Genotype information was coded as 0, 1, or 2, depending on the allele present. R^2 values for each marker were obtained using single variable models. Means were separated using *F*-protected LSD at p=0.05 and p=0.01. Correlations were also performed on Cd concentrations and markers in each trial.

Analysis of variance was performed on trials grown in two locations. Homogeneity of error variances among locations was determined by the ratio of the largest error variance to the smallest. If the ratio was less than 10, the variances were considered homogeneous and the data was combined. In this analysis, both locations and lines were considered random effects. *F*-tests were considered significant at $p<0.05$. Regression and correlation analyses were performed on the results to relate the Cd concentrations to the marker data. Broad sense heritability was estimated using genetic and genetic X location variance estimates from the analysis of variance across locations. Standard error of heritability was calculated according to Knapp et al. (1985).

A mixed model was conducted using PROC MIXED for analysis across years and locations. Data from the years 2013 and 2014, all locations, and all trials except the two with augmented designs were used. Outliers were first identified within trials and values greater than three standard deviations from the mean were removed. After removal, least squared means were obtained and used in a combined data set. Heterozygotes were removed and genotype information was coded as 0 or 2, corresponding to homozygous dominant and recessive alleles. Cd was set as the dependent variable and molecular marker, genotype, location, year, and various two-way interactions were set as explanatory variables. Only genotype, genotype X year and genotype X location interactions were considered random effects.

RESULTS

Soil Test Results

Total soil Cd concentrations were not significantly different between locations (Figure 3) using a t-test, though plant available Cd was not measured and may have been partially responsible for differences in grain Cd (Mench et al., 1997). Mean grain Cd levels of PYT4 grown in Langdon and Willison were 79.6 and 142.5 µg/kg respectively. In AYT7, mean grain Cd levels were 139 and 113 µg/kg for Minot and Williston respectively, making total soil Cd unable to explain the large grain Cd differences of lines grown in Langdon to lines grown in Minot and Williston. Levels of several other cations did have significant differences. The Langdon soil was significantly higher in both calcium and sodium than the Minot and Williston soils. Langdon also had significantly higher levels of zinc than Williston (Figure A1), which could partially explain the lower grain Cd levels due to competitive inhibition between ions (Jiao et al., 2004, Hart et al., 2002, McKenna et al., 1993). Though Langdon did have slightly higher Zn levels than Minot, the differences were not significant. Since Minot produced higher grain Cd and had higher soil Zn levels than Williston, it is clear that Zn is not the only factor influencing grain Cd.

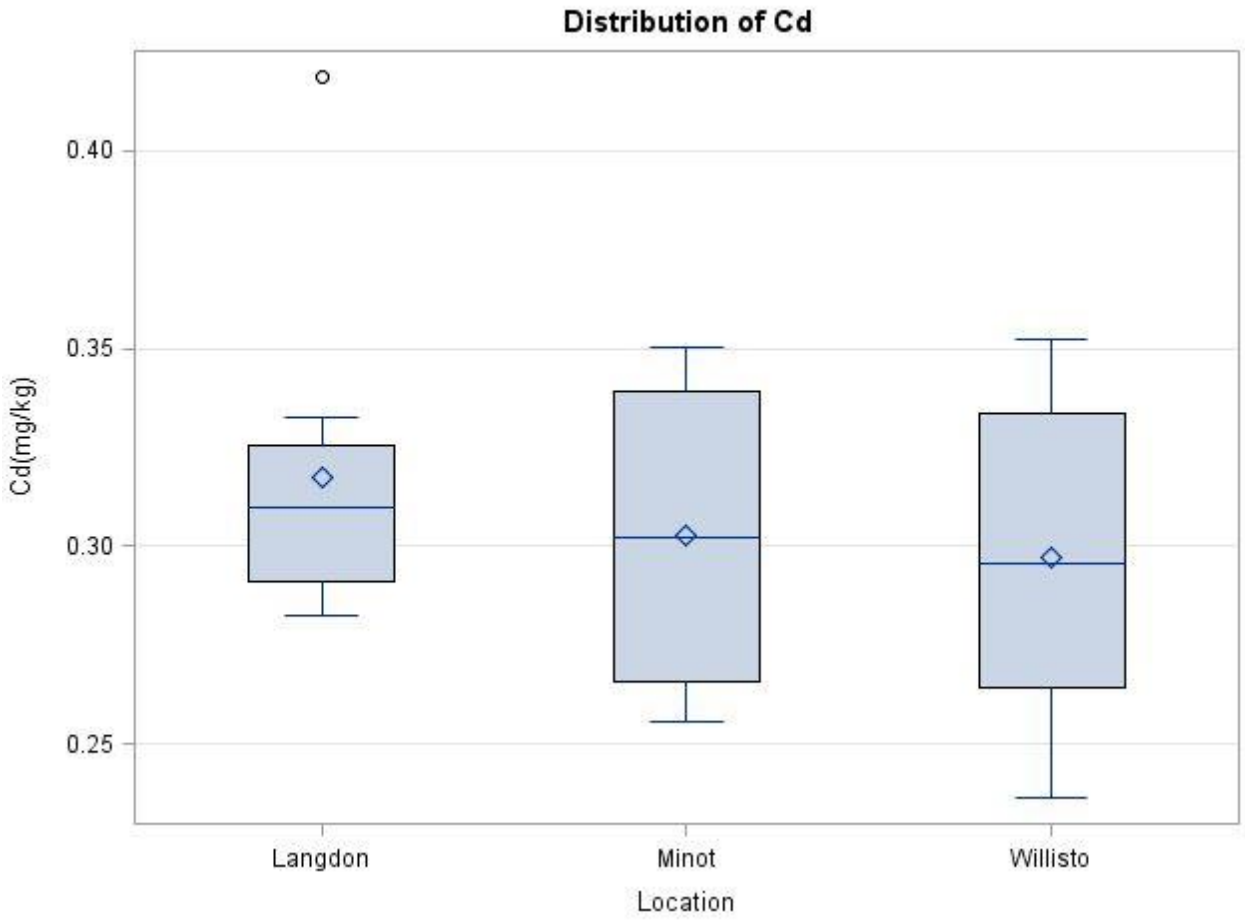

Figure 3. Soil Cd (mg/kg) levels in Langdon, Minot, and Williston with 10, 4, and 8 soil samples, respectively.

Marker Results

Mean grain Cd levels across years were highest in Minot (130µg/kg) and lowest in Langdon (40µg/kg). Overall mean Cd levels for lines with each marker allele are presented in Table 2. When polymorphic, markers residing on chromosome 5B consistently explained more variation than those on chromosome 2B. In all trials, the low Cd check Strongfield was more than one $LSD_{0.05}$ lower than the high Cd checks Divide and Carpio. When included, the low Cd check Haurani was at least one $LSD_{0.05}$ lower than the high Cd checks in all trails except PYT3 (Table 18).

Different introgressions have provided three main sources of low Cd in North American durum wheat. In our germplasm, the line D041735 was used as a source of low Cd in much of the 2013 yield trials. D041735 was identified in the breeding program to have low levels of FHB (Fusarium head blight caused by the fungus *Fusarium graminearum* Schwabe (telomorph *Gibberella zeae* (Schw.) Petch.) susceptibility from Sumai 3, which may also be the source for of low grain Cd. Haurani, a low grain Cd landrace obtained through ICARDA, was the second source in our breeding program. Nile, a low Cd landrace also from Syria, was used as a source of low Cd in the Canadian cultivars Strongfield and Transcend, both of which were used as parents for several populations in our study (Clarke et al. 1997; Clarke et al. 2005; Singh et al., 2012).

Table 2. Mean grain Cd concentration of experimental lines with each marker.

Marker (Chromosome)	**Allele (L=Low,H=High)**	**Mean (µg/kg)**	**Number of lines with allele**
CAD (5B)	L	57.7	923
	H	92.5	947
Ex_c1343_2570756 (5B)	L	54.9	1253
	H	124.0	539
Ex_c17754_26503892 (5B)	L	54.8	1187
	H	117.2	594
Ex_c20019_29052512 (5B)†	L	81.0	215
	H	76.0	1576
Ex_c1996_3754394 (2B)	L	65.7	893
	H	84.0	858
Ra_rep_c106727_90434958 (2B)	L	67.7	100
	H	75.7	1709

† This marker was fixed with the high Cd allele in several trials grown in Langdon, where soils generally produce lower levels of grain Cd than Minot and Williston. See Table 28 for a more accurate estimate of the diagnostic properties of this marker.

Single Trial Analysis

Of the four markers hybridizing with chromosome 5B, Cad 5B, Ex_c1343, and Ex-c1775 explained the most grain Cd variation and often had very similar r^2 values, suggesting they reside closely to the same QTL controlling grain Cd variation. Regression results from the marker on Cd concentrations in each trial are presented in Tables 3-20 and A1-A6 in the Appendix. In Tables 3, 11, 12, 14, 17, 19, 20, and A4, the markers Cad 5B, Ex_c1343, and Ex_c1775 had R^2 percentages no more than 5-percentage units apart. In most instances the r^2 values for these three differed by 0.5-0.8 percentage units, though there were a few exceptions due to the low Cd parental lines used to create different populations. Markers Ex_c1996 and Ra_rep_c1067 on chromosome 2B explained much less variation in most trials and results concerning the performance of these markers are condensed in a later section. For correlation coefficients of each marker with grain Cd concentration, see Table A7 in the Appendix.

Results from trials grown in Langdon and Williston in the 2013 growing season are presented in Tables 3-9. Separate LSDs were necessary in PYT13 and PYT14 (Tables 7 and 9) for check vs. check and check vs. line comparisons due to the augmented design employed in these trials. Mean grain Cd levels for Langdon and Williston in 2013 were 40 µg/kg and 99 µg/kg respectively. In the trial EDA1 (Table 3) grown in Langdon, the single variable regression models for all four markers residing on chromosome 5B had *p*-values less than 0.01. Of these four, markers Cad 5B, Ex_c1343, and Ex_c1775 were able to separate the five highest grain Cd lines from the five lowest. The low grain Cd check Strongfield was less than one $LSD_{0.05}$ from the high Cd check Joppa; however, this was the only instance the two were not significantly different at p=0.05.

Table 3. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial EDA1 grown in Langdon, North Dakota in 2013.

Genotype	**Marker Name**						**Cd Concentration (µg/kg)**
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	1067[‡‡]	
Lowest Cd Lines							
D101132	+[§§]	+	+	-	-	+	26.3
D101232	+	+	+	+	-	+	26.6
D101871	+	+	+	+	-	+	28.6
D101545	+	+	+	.	+	+	28.9
D101076	+	+	+	+	-	+	31.3
Highest Cd Lines							
D101787	-	-	-	-	-	+	118.3
D10924	-	-	-	-	+	+	116.6
D101047	-	.	-	-	.	+	112.5
D10582	-	-	-	-	-	+	111.6
D101543	.	.	.	+	+	+	107.1
Checks							
JOPPA	-	-	-	-	-	+	54.6
CARPIO	-	-	-	-	-	+	84.8
DIVIDE	-	-	-	-	+	+	73.0
STRONGFIELD	+	+	+	+	+	+	36.0
Statistics							
r^2	0.56*	0.52*	0.57*	0.19*	0.00	0.04	
$LSD_{0.05}$							31.9
$LSD_{0.01}$							42.4
CV%							24.3%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] Abbreviation of marker Ra_rep_c106727_90434958 on chromosome 2B.

[§§] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively.

* Indicates $p<0.01$.

In the trials with results presented in Tables 4-8, the low grain Cd line D041735, with its source of low Cd possibly descended from Sumai 3, was used as a parent in most of the populations. The markers Cad 5B and Ex_c20019 were not polymorphic in any of the lines derived from D041735, and subsequently were only diagnostic of low grain Cd in the check Strongfield and the few lines with low grain Cd descended from Nile. While markers Ex_c1343 and Ex_c1775 predicted grain Cd variation comparably to most other trials, Cad 5B had a *p*-value less than 0.01 in only one trial (Table 7) and neither Cad 5B or Ex_c2001 had r^2 values above 0.10 in any of the regression models for these trials.

PYT13 and PYT14 (Tables 7 and 9) contained several lines created using the low grain Cd parent CDC Verona, which had the high Cd alleles for Ex_c1775 and Ex_c2001. This resulted in lower r^2 values for these markers since both high and low grain Cd phenotypes were present with fixed high Cd marker alleles. The non-diagnostic nature of Ex_c1775 in CDC Verona was unexpected since the low Cd allele of this marker is present in both Strongfield and Transcend, which originate from the same Canadian durum wheat breeding program and presumably have the same source of the low Cd as the cultivar Nile.

Table 4. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT10 grown in Langdon, North Dakota in 2013.

Genotype	Marker Name						Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	1067[‡‡]	
Lowest Cd Lines							
D12940	-[§§]	+	+	-	-	+	16.8
D12915	-	+	+	-	+	+	19.7
D12912	-	+	+	-	-	+	19.9
D12952	-	+	+	-	+	+	20.4
D12931	-	+	+	-	+	+	21.4
Highest Cd Lines							
DIVIDE	-	-	-	-	+	+	106.5
ALKABO	-	-	-	-	-	+	85.5
D121010	-	-	-	-	+	+	84.7
D12989	-	-	-	-	+	+	80.0
D12966	-	-	-	-	+	+	74.1
Checks							
JOPPA	-	-	-	-	-	+	64.0
CARPIO	-	-	-	-	-	+	71.1
DIVIDE	-	-	-	-	+	+	106.5
ALKABO	-	-	-	-	-	+	85.5
STRONGFIELD	+	+	+	+	+	+	38.9
Statistics							
r^2	0.01	0.70*	0.70*	0.00	0.02	0.00	
$LSD_{0.05}$							17.7
$LSD_{0.01}$							23.5
CV%							21.6%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] Abbreviation of marker Ra_rep_c106727_90434958 on chromosome 2B.

[§§] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively.

* Indicates $p<0.01$.

Table 5. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT11 grown in Langdon, North Dakota in 2013.

Genotype	Marker Name						Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	1067[‡‡]	
Lowest Cd Lines							
D121053	-[§§]	+	+	-	-	+	13.8
D121070	-	+	+	-	-	+	14.9
D121069	-	+	+	-	-	+	15.1
D121051	-	+	+	-	-	+	15.2
D121061	-	+	+	-	-	+	16.0
Highest Cd Lines							
D121029	-	-	-	-	+	+	88.9
D121027	-	-	-	-	+	+	86.7
D121104	-	-	-	-	-	+	81.3
D121034	-	-	-	-	+	+	77.8
D121020	-	-	-	-	+	+	76.7
Checks							
JOPPA	-	-	-	-	-	+	46.7
CARPIO	-	-	-	-	-	+	75.0
DIVIDE	-	-	-	-	+	+	65.2
ALKABO	-	-	-	-	-/+	+	56.0
STRONGFIELD	+	+	+	+	+	+	20.7
Statistics							
r^2	0.01	0.76*	0.76*	0.00	0.07*	0.00	
$LSD_{0.05}$							19.6
$LSD_{0.01}$							25.9
CV%							26.8%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] Abbreviation of marker Ra_rep_c106727_90434958 on chromosome 2B.

[§§] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively.

* Indicates $p<0.01$.

Table 6. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT12 grown in Langdon, North Dakota in 2013.

Genotype	Marker Name						Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	1067[‡‡]	
Lowest Cd Lines							
D121117	-[§§]	+	+	-	+	+	16.7
D121120	-	+	+	-	-	+	16.8
D121180	-	+	+	-	+	+	16.9
D121187	-	+	+	-	-	+	17.5
D121216	-	+	+	-	-	+	17.6
Highest Cd Lines							
D121165	-	-	-	-	-	+	85.3
D121146	-	-	-	-	+	+	83.5
CARPIO	-	-	-	-	-	+	81.8
D121177	-	-	-	-	-	+	80.7
D121220	-	+/-	+/-	-	-	+	77.1
Checks							
JOPPA	-	-	-	-	-	+	53.0
CARPIO	-	-	-	-	-	+	81.8
DIVIDE	-	-	-	-	+	+	74.7
ALKABO	-	-	-	-	-	+	47.9
STRONGFIELD	+	+	+	+	+	+	30.2
Statistics							
r^2	0.00	0.68*	0.68*	0.00	0.01	0.01	
$LSD_{0.05}$							15.3
$LSD_{0.01}$							20.3
CV%							19.4%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] Abbreviation of marker Ra_rep_c106727_90434958 on chromosome 2B.

[§§] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively.

* Indicates $p<0.01$.

Table 7. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT13 grown in Langdon, North Dakota in 2013.

Genotype	Marker Name						Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	1067[‡‡]	
Lowest Cd Lines							
D12918	-[§§]	+	+	-	-	-	6.4
D12944	-	+	+	-	.	+	9.4
D12890	+	+	+	-	+	+	11.2
D12942	-	+	+	-	-	-	12.6
D12926	-	+	+	-	-	-	13.1
Highest Cd Lines							
D12975	-	-	-	-	+	+	78.4
DIVIDE	-	-	-	-	+	+	72.5
D121017	-	-	-	-	+	+	72.2
D12994	-	-	-	-	+	+	68.5
D12804	+	+	+	-	-	+	64.1
Checks							
JOPPA	-	-	-	-	-	+	51.1
CARPIO	-	-	-	-	-	+	60.4
DIVIDE	-	-	-	-	+	+	72.5
ALKABO	-	-	-	-	-	+	60.5
STRONGFIELD	+	+	+	+	+	+	29.9
Statistics							
r^2	0.09*	0.56*	0.40*	0.00	0.00	0.02	
$LSD_{0.05}$ for checks							15.8
$LSD_{0.01}$ for checks							22.1
$LSD_{0.05}$ for checks vs. lines							24.9
$LSD_{0.01}$ for checks vs. lines							34.9
CV%							27.1%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] Abbreviation of marker Ra_rep_c106727_90434958 on chromosome 2B.

[§§] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively.

* Indicates $p<0.01$.

Table 8. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT11 grown in Williston, North Dakota in 2013.

Genotype	Marker Name						Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	1067[‡‡]	
Lowest Cd Lines							
D121092	-[§§]	+	+	-	+	+	54.2
D121063	-	+	+	-	+	+	56.4
D121068	-	+/-	+	-	+/-	+	57.6
D121099	-	+	+	-	+	+	58.2
D121074	-	+	+	-	-	+	59.1
Highest Cd Lines							
D121024	-	-	-	-	+	+	237.8
D121018	-	-	-	-	+	+	237.5
D121037	-	-	-	-	+	+	221.1
D121102	-	-	-	-	+	+	216.2
D121029	-	-	-	-	+	+	215.2
Checks							
JOPPA	-	-	-	-	-	+	166.0
CARPIO	-	-	-	-	-	+	171.1
DIVIDE	-	-	-	-	+	+	204.0
ALKABO	-	-	-	-	+/-	+	210.0
STRONGFIELD	+	+	+	+	+	+	87.1
Statistics							
r^2	0.00	0.70*	0.73*	0.00	0.05**	0.00	
$LSD_{0.05}$							50.9
$LSD_{0.01}$							67.4
CV%							21.7%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] Abbreviation of marker Ra_rep_c106727_90434958 on chromosome 2B.

[§§] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively.

* Indicates $p<0.01$.

** Indicates $p<0.05$.

Table 9. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT14 grown in Williston, North Dakota in 2013.

Genotype	Marker Name						Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	1067[‡‡]	
Lowest Cd Lines							
D12779	+[§§]	+	-	-	-	+	42.1
D12790	+	+	-	-	-	+	44.6
D12838	+	+	+	-	+	+	44.6
D12782	+	+	-	-	-	+	48.0
D12844	+	.	+	-	-	+	48.2
Highest Cd Lines							
D12900	-	-	-	-	-	.	230.0
DIVIDE	-	-	-	-	+	+	177.0
D12828	-	-	-	-	-	+	166.8
CARPIO	-	-	-	-	-	+	161.7
ALKABO	-	-	-	-	+	+	157.7
Checks							
JOPPA	-	-	-	-	-	+	153.7
CARPIO	-	-	-	-	-	+	161.7
DIVIDE	-	-	-	-	+	+	177.0
ALKABO	-	-	-	-	+	+	157.7
STRONGFIELD	+	+	+	+	+	+	75.7
Statistics							
r^2	0.49*	0.47*	0.13*	0.00	0.00	0.00	
$LSD_{0.05}$ for checks							48.4
$LSD_{0.01}$ for checks							67.9
$LSD_{0.05}$ for checks vs. lines							76.6
$LSD_{0.01}$ for checks vs. lines							107.3
CV%							34.0%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] Abbreviation of marker Ra_rep_c106727_90434958 on chromosome 2B.

[§§] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively.

* Indicates $p<0.01$.

Trials grown in Langdon, Minot, and Williston in 2014 are presented in Tables 10-20 and A1-A6 in the Appendix. The results for marker Ra_rep_c106727 were omitted from these tables since it was not polymorphic in any of the 2014 experimental lines. Similarly to 2013, mean grain Cd concentrations were lower in Langdon (62 µg/kg) than Williston (87 µg/kg). The trial grown in Minot had the highest grain Cd mean of 130 µg/kg. Populations derived from the parent D041735 were less frequent in the 2014 trials, resulting in much higher r^2 values for the marker Cad 5B.

In the trials grown in Langdon during the summer of 2014 (Tables 10-13), linkage breaks between the low Cd gene and markers Ex_c1343 and Ex_c1775 were apparent in one of the lowest and one of the highest Cd lines in the URDN and PYT6, respectively (Tables 10 and 13). This was unexpected due to the identical linkage distances associated with Ex_c1343 and Ex_c1775 in the bi-parental mapping study by Abuhammad (2013). Among these four trials, Cad 5B explained the most variation since the allele diagnostic of low grain Cd remained in coupling with the low Cd QTL. Linkage breaks did not occur between the markers Cad 5B, Ex_c1343, and Ex_c1775 and the Cd uptake QTL in trials corresponding to Tables 11 and 12, where the three markers had identical r^2 values. Ex_c2001 had a p-value ≤ 0.05 only once (Table 12) and was a poor predictor of grain Cd in these trials.

Table 10. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial URDN grown in Langdon, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D09690	+[‡‡]	+	+	.	+	16.3
D08900	+	+/-	+/-	+/-	-	19.2
STRONGFIELD	+	.	+	.	+/-	22.0
D101871	+	+	+	+	-	22.6
D101786	+	-	-	-	-	23.9
Highest Cd Lines						
D09970	-	-	-	-	-	124.8
CARPIO	-	-	-	-	.	100.3
ALKABO	-	-	-	-	+	96.1
D101795	-	-	-	+	-	90.5
JOPPA	-	-	-	-	-	90.2
Checks						
MOUNTRAIL	-	-	-	.	+/-	73.5
ALKABO	-	-	-	-	+	96.1
DIVIDE	-	-	-	-	+	72.8
TIOGA	-	-	-	-	-	80.4
CARPIO	-	-	-	-	.	100.3
JOPPA	-	-	-	-	-	90.2
STRONGFIELD	+	.	+	.	+/-	22.0
Statistics						
r^2	0.82*	0.62*	0.54*	0.12	0.05	
$LSD_{0.05}$						31.2
$LSD_{0.01}$						42.0
CV%						26.9%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Table 11. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT4 grown in Langdon, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13447	+[‡‡]	+	+	-	-	24.4
D13450	+	.	.	.	.	27.6
HAURANI	+	+	+	+	+	27.6
D13440	+	+	+	-	+	28.2
D13452	+	+	+	-	+	29.5
Highest Cd Lines						
D13224	-	-	-	-	-	150.6
D13203	-	-	-	-	-	143.3
D13190	-	-	-	-	+/-	139.4
D13221	-	-	-	-	-	135.3
ALKABO	-	-	-	.	+/-	135.1
Checks						
JOPPA	-	-	-	-	+/-	119.2
CARPIO	-	-	-	+/-	-	121.9
DIVIDE	-	.	-	+/-	+	110.7
ALKABO	-	-	-	.	+/-	135.1
STRONGFIELD	+	+	+	+	+	44.5
HAURANI	+	+	+	+	+	27.6
Statistics						
r^2	0.82*	0.82*	0.82*	0.01	0.34*	
$LSD_{0.05}$						26.9
$LSD_{0.01}$						35.6
CV%						15.9%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Table 12. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT5 grown in Langdon, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13418	+[‡‡]	+	+	-	-	28.9
D13423	+	.	+	.	-	30.7
D13404	+	+	+	-	-	31.9
D13403	+	+	+	-	-	33.0
D13415	+	+	+	.	-	33.3
Highest Cd Lines						
D13249	-	-	-	-	+	126.9
D13264	-	-	-	-	-	119.0
D13244	-	-	-	-	-	113.9
D13267	-	-	-	-	-	111.7
D13240	-	-	-	-	-	109.2
Checks						
JOPPA	-	-	-	-	+/-	75.4
CARPIO	-	-	-	-	-	107.4
DIVIDE	-	-	-	-	+	82.4
ALKABO	-	-	-	-	-	100.8
STRONGFIELD	+	+	+	+	+	44.4
HAURANI	+	+	+	+	+	40.3
Statistics						
r^2	0.69*	0.69*	0.69*	0.05**	0.22*	
$LSD_{0.05}$						29.2
$LSD_{0.01}$						38.7
CV%						21.1%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

** Indicates $p<0.05$.

Table 13. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT6 grown in Langdon, North Dakota in 2014.

Genotype	**Marker Name**					**Cd Concentration (µg/kg)**
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13512	+[‡‡]	+	+	-	-	24.1
D13537	+	+	+	-	-	26.6
D13547	+	+	+	-	+	28.8
D13479	+	+	+	-	+/-	29.2
D13561	+	+	+	-	+	29.9
Highest Cd Lines						
ALKABO	-	-	-	-	+	101.5
CARPIO	-	-	-	-	-	96.7
DIVIDE	-	.	-	-	.	91.2
JOPPA	-	-	-	-	-	89.6
D13495	-	+	+	-	+	88.6
Checks						
JOPPA	-	-	-	-	-	89.6
CARPIO	-	-	-	-	-	96.7
TIOGA	-	-	-	-	-	84.7
DIVIDE	-	.	-	-	.	91.2
ALKABO	-	-	-	-	+	101.5
STRONGFIELD	+	+	+	+	+	39.5
Statistics						
r^2	0.61*	0.41*	0.39*	0.00	0.00	
$LSD_{0.05}$						16.0
$LSD_{0.01}$						21.1
CV%						17.9%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

The highest overall grain Cd concentrations were from samples in AYT7 grown in Minot (Table 14), which produced 14 of the 64 least squared means with Cd levels above the proposed limit of 200 µg/kg (Codex, 2011), of which three were checks. In this trial, all markers residing on chromosome 5B predicted highly significant levels of grain Cd variation ($p<0.01$), though Cad 5B, Ex_c1343, and Ex_c1775 were more informative than Ex_c2001. The former markers perfectly separated the five highest grain Cd lines from the lowest. The difference of mean grain Cd concentrations between lines with different allelic forms of Cad 5B, Ex_c1343, and Ex_c1775 were greater than 80 µg/kg for each marker. Similarly to most trials, the low Cd checks Strongfield and Haurani had approximately half the grain Cd concentration of the high Cd checks.

Table 14. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial AYT7 grown in Minot, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D102621	+[‡‡]	+	+	+	-	46.2
D102582	+	+	+	-	-	48.5
D102681	+	+	+	+	+	48.7
D102644	+	+	+	+	+	53.0
D102684	+	+	+	-	.	55.0
Highest Cd Lines						
D102688	-	-	-	.	.	284.0
D102699	-	-	-	-	-	273.3
D102613	-	-	-	+	-	263.0
CARPIO	-	-	-	-	-	261.3
PIERCE	-	-	-	-	+	260.8
Checks						
JOPPA	-	-	-	-	-	193.1
CARPIO	-	-	-	-	-	261.3
TIOGA	-	-	-	-	-	192.4
ALKABO	-	-	-	-	+	245.6
DIVIDE	-	-	-	-	+	182.1
PIERCE	-	-	-	-	+	260.8
STRONGFIELD	+	+	.	.	+	105.6
HAURANI	+	+	+	+	+	87.1
Statistics						
r^2	0.71*	0.70*	0.68*	0.20*	0.03	
$LSD_{0.05}$						53.8
$LSD_{0.01}$						71.5
CV%						18.4%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Trials grown in Williston during the summer of 2014 are presented in Tables 15-20 and A1-A6 in the Appendix. In the trials corresponding to Tables 17, 18, 19, 20, A2, and A4, markers Cad 5B, Ex_c1343, and Ex_c1775 were in nearly perfect linkage disequilibrium in the five highest and lowest grain Cd lines. Discrepancies between these markers did exist, however, such as in AYT5 (Table 16), which included populations from the 2013 PYTs with the low Cd parent D041735 (with the high Cd allele for Cad 5B), explaining the low r^2 value in this instance. Similarly to PYTs 13 and14 (Tables 7 and 9) grown in 2013, PYT11 (Table A6) contained populations with the cultivar CDC Verona in the pedigree. In total, 33 of the 94 experimental lines included CDC Verona in the parentage, which greatly lowered the r^2 value of Ex_c1775.

In addition to parental differences between trials, PYT4 (Table 19) contained an unusually high number of lines with grain Cd above the proposed limit (Codex, 2011) compared to other trials grown in Williston the same year. AYT 7 (Table 17), which had only two lines with LS means above 200 µg/kg, had 34 total lines unanimously designated high Cd by markers Cad 5B, Ex_c1343, and Ex_c1775, while PYT4 had 25 LS means above this level and 45 lines designated high Cd by the same markers. This likely indicates PYT4 was placed in an area of the field capable of producing relatively higher concentrations of grain Cd and provides insight on differences of Cd concentrations one might expect within the same field.

The trials PYT6, 7, 8, 9, 10, and 11 (Tables A1-A6) contained very few high Cd lines due to screening with Cd related markers prior to being placed in yield trials. This resulted in a higher frequency of lines with false positives (high grain Cd QTL with low Cd markers). High Cd lines in trials corresponding to Tables A1 and A5 demonstrate such instances, where linkage breaks are present in two and one of the highest Cd lines, respectively. Interestingly, the two linkage breaks in Table A1 included all three of the most predictive markers (Cad 5B, Ex_c1343,

and Ex_c1775) while the linkage break in Table A5 only brought the low Cd alleles for markers Ex_c1343 and Ex_c1775 into coupling with the high Cd phenotype. This has led to the understanding that Cad 5B is the most closely linked marker to the Cd uptake gene on chromosome 5B; however, it appears to reside on the same side of the gene as the other markers on 5B, and consequently cannot be used as a flanking marker with Ex_c1343 and Ex_c1775.

Table 15. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial URDN grown in Williston, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D101132	+[‡‡]	+	+	.	+/-	33.9
D101537	+	+	+	-	+	39.6
D09690	+	+	+	.	+	40.2
D09557	+	+	-	-	+	46.2
D101871	+	+	+	+	-	48.0
Highest Cd Lines						
D07892	-	-	-	-	-	205.0
ALKABO	-	-	-	-	+	191.5
D06855	-	-	-	.	-	183.1
D06707	-	-	-	-	-	178.3
D10582	-	-	-	-	-	178.2
Checks						
MOUNTRAIL	-	-	-	.	+/-	138.4
ALKABO	-	-	-	-	+	191.5
DIVIDE	-	-	-	-	+	139.8
TIOGA	-	-	-	-	-	144.6
CARPIO	-	-	-	-	.	172.3
JOPPA	-	-	-	-	-	123.6
STRONGFIELD	+	.	+	.	+/-	52.4
Statistics						
r^2	0.85*	0.70*	0.62*	0.19**	0.08	
$LSD_{0.05}$						50.8
$LSD_{0.01}$						68.4
CV%						22.2%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

** Indicates $p<0.05$.

Table 16. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial AYT5 grown in Williston, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D12925	.[‡‡]	+	+	-	-	47.9
D121092	-	+	.	-	+	55.3
D121089	-	+	+	-	+	55.5
D121120	-	+	+	-	-	58.1
D121171	-	+	+	-	+	65.4
Highest Cd Lines						
D12994	-	-	-	-	+	226.2
ALKABO	-	-	-	-	+	207.8
CARPIO	-	-	-	-	-	203.8
D121104		-	-	-	-	195.4
D121101	-	-	-	-	+	190.1
Checks						
JOPPA	-	-	-	-	-	161.8
CARPIO	-	-	-	-	-	203.8
ALKABO	-	-	-	-	+	207.8
DIVIDE	-	-	-	-	+	161.5
STRONGFIELD	+	+	+	+	+	74.9
Statistics						
r^2	0.01	0.85*	0.85*	0.01	0.00	
$LSD_{0.05}$						38.8
$LSD_{0.01}$						52.0
CV%						16.7%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Table 17. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial AYT7 grown in Williston, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D102621	+[‡‡]	+	+	+	-	44.4
D102576	+	+	+	-	+	44.9
D102589	+	+	+	-	+	45.8
D102610	+	+	+/-	.	-	48.7
D102619	+	+	+	-	-	51.2
Highest Cd Lines						
D102688	-	-	-	.	.	210.5
D102572	-	-	-	+	-	200.0
D102699	-	-	-	-	-	194.7
D102566	-	-	-	+	-	192.8
ALKABO	-	-	-	-	+	192.4
Checks						
JOPPA	-	-	-	-	-	140.9
CARPIO	-	-	-	-	-	175.1
TIOGA	-	-	-	-	-	174.2
ALKABO	-	-	-	-	+	192.4
DIVIDE	-	-	-	-	+	126.2
PIERCE	-	-	-	-	+	159.1
STRONGFIELD	+	+	.	.	+	89.7
HAURANI	+	+	+	+	+	88.8
Statistics						
r^2	0.77*	0.77*	0.74*	0.14*	0.01	
$LSD_{0.05}$						37.0
$LSD_{0.01}$						49.1
CV%						15.7%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Table 18. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT3 grown in Williston, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13325	+[‡‡]	+	+	+	+	61.4
D13374	+	+	+	+	+	66.6
D13338	+	+	+	-	-	68.0
D13344	+	+	+	-	.	71.7
D13341	+	+	+	-	-	75.7
Highest Cd Lines						
D13358	-	-	-	-	-	244.7
D13371	-	-	-	-	+	234.5
D13332	-	-	-	+	-	232.6
D13370	-	-	-	-	+	229.3
D13364	-	-	-	-	+	229.1
Checks						
JOPPA	+	-	-	-	-	139.6
CARPIO	-	-	-	-	-	188.5
DIVIDE	-	-	-	-	+	181.3
ALKABO	-	-	-	-	-	196.0
STRONGFIELD	+	+	+	+	+	88.4
HAURANI	+	+	+	+	+	95.3
Statistics						
r^2	0.64*	0.68*	0.60*	0.28*	0.02	
$LSD_{0.05}$						44.5
$LSD_{0.01}$						58.9
CV%						14.5%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Table 19. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT4 grown in Williston, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13462	+[‡‡]	+	+	-	+	62.0
D13447	+	+	+	-	-	62.5
D13465	+	+	+	-	+	62.8
D13452	+	+	+	-	+	63.3
D13424	+	+	+	-	-	63.6
Highest Cd Lines						
D13201	-	-	-	-	-	250.0
D13203	-	-	-	-	-	238.9
D13202	-	-	-	-	-	233.7
D13200	-	-	-	-	-	233.4
D13212	-	-	-	-	-	233.1
Checks						
JOPPA	-	-	-	-	+/-	144.4
CARPIO	-	-	-	+/-	-	212.7
DIVIDE	-	.	-	+/-	+	179.5
ALKABO	-	-	-	.	+/-	186.7
STRONGFIELD	+	+	+	+	+	85.3
HAURANI	+	+	+	+	+	81.6
Statistics						
r^2	0.78*	0.75*	0.75*	0.01	0.39*	
$LSD_{0.05}$						36.9
$LSD_{0.01}$						48.9
CV%						12.4%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Table 20. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT5 grown in Williston, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13403	+[‡‡]	+	+	-	-	48.0
D13420	+	+	+	-	+	53.5
D13398	+	+	+	+	-	59.7
D13380	+	+	+	-	+	60.2
D13390	+	+	+	-	+	61.6
Highest Cd Lines						
D13270	-	-	-	-	-	224.1
D13281	-	-	-	-	-	220.2
ALKABO	-	-	-	-	-	216.5
D13264	-	-	-	-	-	210.2
D13275	-	-	-	-	-	208.6
Checks						
JOPPA	-	-	-	-	+/-	168.4
CARPIO	-	-	-	-	-	165.4
DIVIDE	-	-	-	-	+	171.2
ALKABO	-	-	-	-	-	216.5
STRONGFIELD	+	+	+	+	+	99.8
HAURANI	+	+	+	+	+	79.2
Statistics						
r^2	0.76*	0.71*	0.71*	0.03	0.21*	
$LSD_{0.05}$						42.0
$LSD_{0.01}$						55.5
CV%						16.6%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Summary of Chromosome 5B Markers

Markers Ex_c1343 and Ex-c1775 mapped to an identical location in the bi-parental mapping study conducted by Abuhammed, (2013). In the trials corresponding to Tables 7, 9, and A6, the lines with the low Cd cultivar CDC Verona in the pedigree were not polymorphic with marker Ex_c1775 (Pozniak et al 2009). Since the population Joppa/CDC Verona contributed 6, 15, and 33 lines in these trials, respectively, the r^2 values for Ex_c1775 were greatly reduced in each case. Ex_c1775 still had *p*-values less than 0.01 in Tables 7 and 9, since smaller proportions of lines had CDC Verona as a parent. No instances were found where Ex_c1775 was polymorphic when Ex_c1343 was not; meaning marker Ex_c1343 was polymorphic in slightly more populations than Ex_c17754.

Several trials contained populations that were not polymorphic for the CAD 5B marker (Tables 4, 5, 6, 7, 8, and 16). The reason for this was due to introgression of the low-Cd line D041735 without the polymorphism detected by Penner et al (1995) with the RAPD marker 'OPC-20', later converted to CAD 5B by Dr. Shiaoman Chao. In these trials, the percentage of lines with D041735 as a parent ranged from 41% to 95%, resulting in *p*-values greater than 0.01 for all trials except PYT 13 (Table 7) and r^2 values less than 0.10 for CAD 5B in each regression model. Markers Ex_c1343 and Ex_c1775 were polymorphic in the populations where Cad 5B was not and explained grain Cd variation similarly to other trials. Though Cad 5B was not diagnostic of grain Cd in much of the 2013 material, in several of the 2014 trials it explained at least 15% more grain Cd variation than the second and third most diagnostic markers (Tables 10, 13, 15, A3, and A6). This appears to be caused by linkage breaks between CAD 5B and both Ex_c1343 and Ex_c1775. Breaks between these markers occurred once in the URDN (Tables 10 and 15) and PYT11 trials (Table A6) and four times in both the PYT6 (Table 13) and PYT8 trials

(Table A3). In most cases, CAD 5B seems to stay in coupling with the low Cd gene, which provides an explanation for the lower r^2 values of Ex_c1342 and Ex_c1775 in these instances.

In the association mapping study conducted by Abuhammad (2013), marker Ex_c2001 was estimated to reside 12.6 cM from Ex_c1775 and explained 27% of the grain Cd variation. In this analysis, Ex_c2001 had *p*-values less than 0.01 in only four trials corresponding to Tables 3, 14, 17, and 18, with the highest r^2 being 0.28. Marker data from these trials revealed Ex_c2001 was segregating with the low Cd allele in some populations; however, the farther linkage distance from the QTL controlling grain Cd uptake reduced prediction accuracy compared to Ex_c1775. Furthermore, in most trials Ex_c2001 was only polymorphic in the checks, resulting in a limited predictive capacity overall.

Summary of Chromosome 2B Markers

Markers on chromosome 2B were often not polymorphic, such as Ra_rep_c1067 in all 2014 material. In trials where Ex_c1996 was polymorphic, the r^2 values for this marker were generally below 0.10. The exceptions were in two trials grown in two locations corresponding to Tables 11, 12, 19, and 20, where Ex_c1996 had r^2 values of 0.34, 0.22, 0.39, and 0.21, respectively. The reason for these unusually high values appears to be the population structure of each trial. In PYT4 (Tables 11 and 19), Ex_c1996 appears fixed in several populations that comprise 40 of the 94 experimental lines, of which all 40 fixed lines classify as high Cd phenotypes in both locations (one $LSD_{0.05}$ greater than Strongfield). Since the rest of the experimental lines in PYT4 were segregating for Ex_c1996 and grain Cd, the r^2 values for this marker may be inflated. Similarly for PYT5 (Tables 12 and 20), only 66 of the 94 experimental lines appear to be from populations polymorphic for the marker. The 28 remaining experimental lines were fixed with the high Cd marker allele for Ex_c1996, with at least 24 of the 28 lines

classifying as high Cd phenotypes in each trial. Ex_c1996 had a *p*-value less than 0.01 in only one other trial (Table 5), though this also appears attributable to the population structure of the trial. Ra_rep_c1067 did not explain more than 10% grain Cd variation or have *p*-values below 0.01 in any trials.

Prediction Accuracy of Markers on Chromosome 5B

Prediction accuracies for markers CAD 5B, Ex_c1343, and Ex_c1775 in each trial are presented in Table 21. Noticeable decreases in accuracy occurred in trials with non-polymorphic populations for a given marker. This can be seen particularly with CAD 5B in much of the material grown in 2013. In 20 out of 24 trials however, at least one of these markers correctly identified grain Cd phenotypes at least 80% of the time. In 15 out of 24 trials, all three markers correctly identified at least 80% of grain Cd phenotypes. Ex_c2001 was not included due to the high frequency of linkage breaks with the low grain Cd QTL on chromosome 5B, which resulted in generally low prediction values. Markers on chromosome 2B were also excluded since they did not explain enough variation to significantly differentiate between high and low grain Cd lines

Table 21. Percent of breeding lines each marker correctly identified Cd phenotype†.

Year	Trial	Marker Name		
		Cad 5B‡	1343§	1775¶
2013	Langdon EDA1	63%	61%	61%
	Langdon PYT10	13%††	79%	77%
	Langdon PYT11	‡‡	83%	83%
	Langdon PYT12	‡‡	79%	77%
	Langdon PYT13	51%††	75%	69%††
	Williston PYT11	‡‡	81%	81%
	Williston PYT14	80%	81%	68%*
2014	Langdon URDN	97%	88%	84%
	Langdon PYT4	89%	86%	85%
	Langdon PYT5	91%	89%	90%
	Langdon PYT6	86%	80%	81%
	Minot AYT7	91%	94%	90%
	Williston URDN	94%	84%	88%
	Williston AYT5	‡‡	94%	90%
	Williston AYT7	88%	90%	86%
	Williston PYT3	93%	86%	86%
	Williston PYT4	91%	87%	84%
	Williston PYT5	87%	85%	86%
	Williston PYT6	83%	82%	83%
	Williston PYT7	84%	85%	87%
	Williston PYT8	96%	87%	86%
	Williston PYT9	90%	93%	94%
	Williston PYT10	88%	87%	88%
	Williston PYT11	93%	87%	56%††

† Lines with the low Cd marker and Cd concentrations within 1 $LSD_{0.01}$ of Strongfield were considered correct, as were lines with the high Cd marker and Cd concentrations above the sum of the $LSD_{0.01}$ and Strongfield.

‡ Located on chromosome 5B.

§ Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

¶ Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

†† Trial contained populations that were not polymorphic for the marker.

‡‡Except for Strongfield, the marker was not polymorphic.

Mixed Model for Cd Across Years, Locations, and Trials

Separate GLM models created using SAS 9.3 for CAD 5B, Ex_c1343, Ex-c1775, and Ex_c2001 revealed that greater than 98% of the variation was accounted for when year, location, and genotype were included in the model. Using Type III sum of squares, Ex_c1343 and

Ex_c2001 had the highest and lowest mean square and F-values, respectively. Location X marker interactions were highly significant for markers CAD 5B, Ex_c1343, and Ex_c1775, with all mean squares having F-values greater than 40. This suggests that soil and weather conditions in each location highly influence grain Cd. Grain Cd estimates of lines with allelic forms of CAD 5B, Ex_c1343, Ex-c1775, and Ex_c2001 grown in Langdon, Minot, and Williston were obtained using MIXED models from SAS 9.3 (Table 22; Figures 7-10 in Appendix). Estimates of grain Cd in lines solely based off the first three markers were not more than one µg/kg different from 59 µg/kg for the three low Cd alleles; however, estimates for lines with the high Cd marker alleles ranged from 122-142 µg/kg. Lines with the same marker allele are estimated to have nearly twice the grain Cd concentration when grown in Minot and Williston than those grown in Langdon. Marker Ex_c2001 predicted grain Cd far less effectively overall due to the non-polymorphic state of the marker in most of the experimental lines grown in Langdon and Williston.

Table 22. Mixed model estimates of grain Cd based on marker data and location.

Location	Allele (L=low, H=high)	Marker Estimate (µg/kg)			
		Cad 5B[1]	Ex_c1343[2]	Ex_c1775[3]	Ex_c2001[4]
*	L	59.9	59.0	58.9	76.0
	H	122.5	142.2	137.7	93.5
Langdon	L	39.9	33.4	33.1	48.8
	H	65.3	82.7	80.0	47.7
Minot	L	76.0	79.1	79.0	98.7
	H	175.7	194.6	192.9	141.5
Williston	L	63.8	64.6	64.5	80.4
	H	126.5	149.1	140.2	91.3

[1] Located on chromosome 5B.

[2] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[3] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[4] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

* Overall estimate without specifying location.

Heritability of Grain Cd in Trials Across Locations

Broad sense heritability values for all trials grown in two locations ranged from 0.75 to 0.90, indicating grain Cd concentration was not heavily influenced by genotype X environment interactions (Table 23). Average heritability of trials grown in Langdon and Williston was 0.82 and the trial grown in Williston and Minot was 0.90. Standard errors of heritability ranged from 0.14 to 0.16. These results agree with previous findings that grain Cd uptake in durum is highly heritable (Clarke et al 1997; Abuhammad 2013). Previous studies have found that high heritability of a trait can result in higher prediction accuracy of markers as well (Sallam et al 2015). The slightly higher heritability estimate of AYT7 may be due to the Minot location, which had the highest grain Cd values and the largest differences between low and high Cd phenotypes.

Table 23. Broad sense heritability of grain cadmium in five yield trials across two locations in North Dakota

Trial	Year	Locations	Broad sense heritability	Standard error
PYT11	2013	Langdon Williston	0.75	±0.15
AYT7	2014	Minot Williston	0.90	±0.16
PYT4	2014	Langdon Williston	0.90	±0.14
PYT5	2014	Langdon Williston	0.75	±0.14
PYT6	2014	Langdon Williston	0.88	±0.14

DISCUSSION

Previous validation studies in wheat have identified markers capable of predicting large proportions of phenotypic variance for various traits such as disease resistance and grain quality (Spielmeyer et al., 2003; Pumphrey et al., 2007; Smith et al., 2011; Bernardo et al., 2013; Neelam et al., 2013). The validation study conducted by Abuhammad (2013) on markers associated with grain Cd was limited in scope and only tested two populations with marker Ex_c1343, whereas our study included most populations from our 2013 and 2014 yield trials and six previously identified markers in linkage disequilibrium with Cd related QTL on chromosomes 2B and 5B.

The RAPD marker discovered by Penner et al. (1995) explained a similar level of grain Cd variation as the markers Cad 5B, Ex_c1343, and Ex_c1775 in this study. It is also likely these three markers are strongly linked to the same grain Cd gene that Clarke et al. (1997) observed segregating in ratios expected from a single qualitative gene. Knox et al. (2009) concluded that this QTL discovered by Penner et al. (1995) resided on chromosome 5B, which is in agreement with results from the bi-parental and association mapping studies conducted by Abuhammad (2013). Results from this study indicate that most of the grain Cd variation in populations with both a high and low Cd parent can be explained by at least one of the above markers residing on chromosome 5B.

Abuhammad (2013) found no significant correlations between grain Cd and yield, test weight, and plant height, suggesting no major pleiotropic effects or unfavorable linkages exist with these important agronomic traits. However, the list of traits correlated with grain Cd in that study was not exhaustive and was conducted on only a single bi-parental mapping population; therefore in the future unfavorable linkages may need to be broken. The vernalization locus *Vrn-*

B1 mapped approximately 3 cM distal to the marker 'ScOpc20' (converted to Cad 5B), which is strongly linked to *Cdu1* on chromosome 5B (Penner et al. 1995; Iwaki et al., 2002; Weibe et al., 2010). Since no vernalization problems arose while creating low Cd populations in our germplasm, it can be reasonably assumed that the favorable allele was already in coupling in low Cd parental lines. *Tsn1*, a well characterized qualitative gene responsible for insensitivity to the toxin Ptr ToxA released by some tan spot (*Pyrenophora tritici-repentis*) races, was also located on the long arm of chromosome 5B in the deletion bin 0.75-0.76 (Faris et al., 1996; Faris et al., 2010; Lu et al., 2006). Using a marker tightly linked to *Tsn1* found by Lu et al. (2006), Knox et al. (2009) determined that the marker linked to *Tsn1* mapped approximately 12 cM proximal from *Cdu1*, which resides in deletion bin 0.76-0.79. According to Singh et al. (2006), several elite durum wheat cultivars, many of which are commonly grown in North Dakota and used as parents in our breeding program, exhibit necrosis due to Ptr ToxA. If the favorable allele for Tsn1 was known to be present in a low Cd line, it may be possible to select for both traits simultaneously using Cad 5B or Ex_c1343. The region of 5BL containing *Cdu1* and *Tsn1* has been characterized as a gene-rich region, suggesting there may be several genes with currently unknown allelic variation suitable for use in future breeding efforts (Lu et al., 2006; Weibe et al., 2010).

Based on the results, markers Cad 5B and Ex_c1343 will be used to screen for low Cd genotypes. These markers, especially Ex_c1343, consistently co-segregate with an allele controlling grain Cd in the diverse genetic backgrounds of our germplasm. Though Cad 5B was not polymorphic in some of the material, it appears to have a closer linkage distance than Ex_c1343, and will likely be more reliable in populations segregating for both markers. In addition, all lines with the low Cd allele for these two markers had grain Cd levels below the

proposed limits of 200 µg/kg and all 64 grain samples above this limit had the high Cd allele. These markers will be used for marker assisted selection to select low Cd $F_{4:5}$ lines before planting them in expensive replicated trials.

Though in many trials markers Ex_c1775and Ex_c2001 on chromosome 5B both explained significant levels of grain Cd variation, they will not be utilized due to lower polymorphic nature or higher linkage distance compared to the two other markers on chromosome 5B. Variance from these markers would also be captured by Ex_c1343, so their use would be unnecessary. The associations between the markers Ex_c1996 and Ra_rep_c1067 on chromosome 2B and Cd levels were generally poor and currently would be of little practical value in marker assisted selection for low grain Cd. If further reductions of grain Cd are desired once the low Cd allele on chromosome 5B is introgressed, marker Ex_c1996 would be a better candidate marker for identifying lines with the minor effect QTL on chromosome 2B (Abuhammad 2013). This marker was polymorphic in much of the 2013 and 2014 germplasm and predicted some grain Cd variation, though population structure was clearly responsible for the abnormally high r^2 values in certain trials.

Since Cad 5B and Ex_c1343 are diagnostic for alleles that are responsible for a large proportion of variation of a trait which is highly heritable, creating low Cd cultivars will be straight forward with selection of the marker alleles associated with low Cd. Late generation yield trials will still be phenotyped for grain Cd to confirm marker results; however, the high prediction accuracy of these markers will eliminate the need for expensive phenotyping of preliminary yield trials. Using marker assisted selection prior to preliminary yield trials will reduce resources spent on field plots for lines that would eventually be discarded in advanced yield trials due to high grain Cd. Also, by removing high Cd lines earlier in the breeding

process, more low Cd lines can be tested, increasing the probability of identifying desirable lines for release. This research may prove particularly important for creating durum cultivars for growers with soils similar to those at the Minot and Williston experiment stations, where grain samples from many of the lines with the high Cd allele contained more Cd than the proposed limit (Codex, 2011).

REFERENCES

AbuHammad, W. 2013. Identification of Quantitative Trait Loci Associated With A Low Cadmium Uptake Gene In Durum Wheat (*Triticum Turgidum* L. var. *durum*). Ph.D. Diss. North Dakota State Univ., Fargo.

Adams, M. L., F. J. Zhao, S. P. McGrath, F. A. Nicholson, and B. J. Chambers. 2004. Predicting Cadmium Concentrations in Wheat and Barley Grain Using Soil Properties. Journal of Environmental Quality 33: 532-541. doi:10.2134/jeq2004.5320

Akesson, A., B. Julin, and A. Wolk. 2008. Long-term Dietary Cadmium Intake and Postmenopausal Endometrial Cancer Incidence: A Population-Based Prospective Cohort Study. Cancer Res 68: 6435-6441.

Akhunov, E.D., A.R. Akhunova, and J. Dvorak. 2005. BAC libraries of Triticum urartu, Aegilops speltoides and Ae. Taushcii, the diploid ancestors of polyploid wheat. Theor Appl Genet 111: 1617-1622.

Aoshima, K., J. Fan, Y. Cai, T. Katoh, H. Teranishi, and M. Kasuya. 2003. Assessment of bone metabolism in cadmium-induced renal tubular dysfunction by measurements of biochemical markers. Toxicology Letters 136: 183-192.

Archambault, D.J., E. Marentes, W. Buckley, J. Clarke, and G.J. Taylor. 2001. A rapid, seedling-based bioassay for identifying low cadmium-accumulating individuals of Durum wheat (*Triticum turgidum* L.). Euphytica. 117: 175-182.

Berglund, M., A. Akesson, P. Bjellerup, and M. Vahter. 2000. Metal-bone interactions. Toxicology Letters 112-113: 219-225.

Bernardo, A.N., R.L. Bowden, M.N. Rouse, M.S. Newcomb, D.S. Marshall, and G. Bai. 2013. Validation of Molecular Markers for New Stem Rust Resistance Genes in U.S. Hard Winter Wheat. Crop Sci. 53: 755-764.

Bingham F.T., G. Sposito, and J.E. Strong. 1984. The Effect of Chloride on the Availability of Cadmium. J. Environ. Qual. 13(1): 71-74.

Brzoska, M.M. and J. Moniuszko-Jakoniuk. 2001. Interactions between cadmium and zinc in the organism. Food and Chemical Toxicology 39(10): 967-980.

Cakmak I. and H. Marschner. 1988. Increase in Membrane Permeability and Exudation in Roots of Zinc Deficient Plants. Journal of Plant Physiology. 132(3): 356-361.

Chan, D. Y. and B. A. Hale. 2004. Differential Accumulation of Cd in Durum Wheat Cultivars, Uptake and Retranslocation as Sources of Variation. Journal of Experimental Botany. 55: 2571-2679. doi:10.1093/jxb/erh255

Chen, X., G. Zhu, S. Gu, T. Jin, and C. Shao. 2009. Effects of cadmium on osteoblasts and osteoclasts in vitro. Environmental Toxicology and Pharmacology 28: 232-236.

Clarke, J.M., D. Leisle, and G. L. Kopytko. 1997. Inheritance of Cadmium Concentration in Five Durum Wheat Crosses. Crop Science 37: 1722-1726.

Clarke, J.M., T.N. McCaig, R.M. DePauw, R.E. Knox, F.R. Clarke, M.R. Fernandez, and N.P. Ames. 2005. Strongfield durum wheat. Plant Science. 85: 651-654.

Codex Alimentarius Commission. 2011. Joint FAO/WHO Food Standards Programme Codex Committee on Contaminants in Foods. Rome, Italy. Available from ftp://ftp.fao.org/codex/meetings/CCCF/cccf5/cf05_INF.pdf

De Filippis, L.F. and H Ziegler. 1993. Effect of Sublethal Concentrations of Zinc, Cadmium and Mercury on the Photosynthetic Carbon Reduction Cycle of Euglena. Journal of Plant Physiology 142(2): 167-172.

Dvorak, J., P. Di Terlizzi, H.B. Zhang, and P. Resta. 1993. The evolution of polyploid wheats: identification of the A genome donor species. Genome 36: 21-31.

Elias, E.M., D.K. Steiger, and R.G. Cantrell. 1996. Evaluation of Lines Derived from Wild Emmer Chromosome Substitutions: II. Agronomic Traits. Crop Science 36(2): 228-233.

Elias, E.M. and F.A. Manthey. 2007a. Registration of 'Alkabo' Durum Wheat. Journal of Plant Registrations 1: 10-11.

Elias , E.M., and F.A. Manthey. 2007b. Registration of 'Divide' durum wheat. Journal of Crop Registrations 1:7-8.

Elias, E.M. and F.A. Manthey. 2007c. Registration of 'Grenora' Durum Wheat. Journal of Plant Registrations 1: 8-9.

Elias, E.M. and F.A. Manthey. 2016. Registration of 'Joppa' Durum Wheat. Journal of Plant Registrations doi:10.3198/jpr2015.11.0071crc

Elias, E.M., F.A. Manthey, and W.A. AbuHammad. 2014. Registration of 'Carpio' Durum Wheat. Journal of Plant Registrations 9: 78-82.

Faris, J.D., J.A. Anderson, L.J. Francl, and J.G. Jordahl. 1996. Chromosomal Location of a Gene Conditioning Insensitivity in Wheat to a Necrosis-Inducing Culture Filtrate from *Pyrenophora tritici-repentis*. Phytopathology 86(5): 1996.

Faris, J.D., J.P. Fellers, S.A. Brooks, B.S. Gill. 2003. A Barcterial Artificial Chromosome Contig Spanning the Major Domestication Locus Q in Wheat and Identification of a Candidate Gene. Genetics 164(1): 311-321.

Faris, J.D., Z. Zhang, H. Lu, S. Lu, L. Reddy, S. Cloutier, J.P. Fellers, S.W. Meinhardt, J.B. Rasmussen, S.S. Xu, R.P. Oliver, K.J. Simons, and T.L. Friesen. 2010. A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. PNAS 107(30): 13544-13549.

Greger, M. and M. Lofstedt. 2004. Comparison of Uptake and Distribution of Cadmium in Different Cultivars of Bread and Durum Wheat. Crop Science. 44(2): 501-507.

Guo, J.H., X.J. Liu, Y. Zhang, J.L. Shen, W.X. Han, W.F. Zhang, P. Christie, K.W.T. Goulding, P.M. Vitousek, F.S. Zhang. 2010. Significant Acidification in Major Chinese Croplands. Science 327(5968): 1008-1010.

Hart, J. J., R. M. Welch, W.A. Norvell, L.A. Sullivan, and L.V. Kochian. 1998 Characterization of Cadmium Binding, Uptake, and Translocation in Intact Seedlings of Bread and Durum Wheat Cultivars. Plant Physiology 116(4): 1413-1420.

Hart, J.J., R.M. Welch, W.A. Norvell, and L.V. Kochian. 2002. Transport interactions between cadmium and zinc in roots of bread and durum wheat seedlings. Physiologia Plantarum 116: 73-78.

Hart, J. J., R. M. Welch, W. A. Norvell, and L. V. Kochian. 2006. Characterization of cadmium uptake, translocation and storage in near-isogenic lines of durum wheat that differ in grain cadmium concentration. New Phytologist, 172: 261–271. doi:10.1111/j.14698137.2006.01832.x

Helentjaris, T., M. Slocum, S. Wright, A. Schaefer, and J. Nienhuis. 1986. Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor Appl Genet. 72: 761-769 .

Huang, S., A. Sirikhachornkit, X. Su, J. Faris, B. Gill, R. Haselkorn, and P. Gornicki. 2002. Genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase of the Triticum/Aegilops complex and the evolutionary history of polyploid wheat. PNAS 99(12): 8133-8138.

International Agency for Research on Cancer. 1993. Cadmium and cadmium compounds. IARC Monogr Eval Carcinog Risks Hum 58: 119-237.

Iretskaya, S.N., S.H. Chien. 1999. Comparison of cadmium uptake by five different food grain crops grown on three soils of varying pH. Communications in Soil Science and Plant Analysis. 30(3&4): 441-448.

Iwaki, K., J. Nishida, T. Yanagisawa, H. Yoshida, and K. Kato. 2002. Genetic analysis of Vrn-B1 for vernalization requirement by using linked dCAPS markers in bread wheat (Triticum aestivum L.). Theor Appl Genet 104: 571-576.

Jantasuriyarat, C., M.I. Vales, C.J.W. Watson, and O. Riera-Lizarazu. 2004. Identification and mapping of genetic loci affecting the free-threshing habit and spike compactness in wheat (Triticum aestivum L.). Theor Appl Genet 108: 261-273.

Jiao, Y., C.A. Grant, and L.D. Bailey. 2004. Effects of phosphorus and zinc fertilizer on cadmium uptake and distribution in flax and durum wheat. Journal of the Science of Food and Agriculture. 84: 777-785.

Joppa, L.R. and N.D. Williams. 1988. Genetics and breeding of durum wheat in the United States. In G. Fabriana and C. Lintas (eds.) Durum Chemistry and Technology. St. Paul, MN: Am. Assoc. of Cereal Chem. p. 47-68.

Kazantzis, G.. 2004. Cadmium, Osteoporosis and Calcium Metabolism. BioMetals 17: 493-498.

Knapp, S. J., W.W. Stroup, and W.M. Ross. 1985. Exact Confidence Intervals for Heritability on a Progeny Mean Basis. Crop Science. 25(1): 192-194.

Knox, R. E., C. J. Pozniak, F. R. Clarke, J. M. Clarke, S. Houshmand, and A. K. Singh. 2009. Chromosomal location of the cadmium uptake gene (Cdu 1) in durum wheat. Genome 52: 741-747.

Leisle, D., F.G. Kosmolak, and M. Kovacs. 1981. Association of glume color with gluten strength and gliadin proteins in durum wheat. Canadian Journal of Plant Science 61(1): 149-151.

Li, Y. M., R. L. Chaney, A. A. Schneiter, J. F. Miller, E. M. Elias, and J. J. Hammond. 1997. Screening for Low Grain Cadmium Phenotypes in Sunflower, Durum Wheat and Flax. Euphytica 94: 23-30.

Lu, H.-J., J.P. Fellers, T.L. Friesen, S.W. Meinhardt, and J.D. Faris. 2006. Genomic analysis and marker development for the *Tsn1* locus in wheat using bin-mapped ESTs and flanking BAC contigs. Theor Appl Genet 112: 1132-1142.

Maccaferri, M., A. Ricci, S. Salvi, S.G. Milner, E. Noli, P.L. Martelli, R. Casadio, E. Akhunov, S. Scalabrin, V. Vendramin, K. Ammar, A. Blanco, F. Desiderio, A. Distelfeld, J. Dubcovsky, T. Fahima, J. Faris, A. Korol, A. Massi, A.M. Mastrangelo, M. Morgante, C. Pozniak, A. N'Diaye, S. Xu, R. Tuberosa. 2015. A high-density, SNP-based consensus map of tetraploid wheat as a bridge to integrate durum and bread wheat genomics and breeding. Plant Biotechnology Journal 13: 648-663.

McKenna, I.M., R.L. Chaney, and F.M. Williams. 1993. The Effects of Cadmium and Zinc Interactions on the Accumulation and Tissue Distribution of Zinc and Cadmium in Lettuce and Spinach. Environmental Pollution 79: 113-120.

Mench, M., D. Baize, and B. Mocquot. 1997. Cadmium availability to wheat in five soil series from the Yonne district, Burgundy, France. Environmental Pollution 94(1): 93-103.

Mitchell, L.G., C.A. Grant, and G.J. Racz. 2000. Effect of nitrogen application on concentration of cadmium and nutrient ions in soil solution and in durum wheat. Canadian Journal of Soil Science. 80(1): 107-115.

Muramatsu, M. 1986. The vulgare surper gene, Q: its universality in durum wheat and its phenotypic effects in tetraploid and hexaploid wheats. Canadian Journal of Genetics and Cytology 28(1): 30-41.

Nalam, V.J., M.I. Vales, C.J.W. Watson, S.F. Kianian, and O. Riera-Lizarazu. 2006. Map-based analysis of genes affecting the brittle rachis character in tetraploid wheat (*Triticum turgidum* L.). Theor Appl Genet 112:373-381.

National Agricultural Statistics Service, Agricultural Statistics Board, U.S. Department of Agriculture (2015). Acreage. Available from <www.usda.gov/nass/>

Nawrot, T., M. Plusquin, J. Hogervorst, H.A. Roels, H. Celis, L. Thijs, J. Vangronsveld, E.V. Hecke, and J.A. Staessen. 2006. Environmental exposure to cadmium and risk of cancer: a prospective population-based study. Lancet Oncol 7: 119-126.

Neelam, K., G. Brown-Guedira, and L. Huang. 2013. Development and validation of a breeder-friendly KASPar marker for wheat leaf rust resistance locus Lr21. Mol Breeding 31: 233-237.

Norvell, W. A., J. Wu, D. G. Hopkins, and R. M. Welch. 2000. Association of Cadmium in Durum in Durum Wheat Grain with Soil Chloride and Chelate-Extractable Soil Cadmium. Soil Science Society of America 64: 2162-2168. doi:10.2136/sssaj2000.6462162x

Nriagu, J.O. and J.M. Pacyna. 1988. Quantitative assessment of worldwide contamination of air, water and soils by trace metals. Nature 333: 134-139.

Oliver, D. P., R. Hannam, K. G. Tiller, N. S. Wilhelm, R. H. Merry, and G. D. Cozens. 1994. Heavy Metals in the Environment: The Effects of Zinc Fertilization on Cadmium Concentration in Wheat Grain. Journal of Environmental Quality 23: 705-711. doi:10.2134/jeq1994.00472425002300040013x

Peng, J., Y. Ronin, T. Fahima, M.S. Röder, Y. Li, E. Nevo, and A. Korol. 2003. Domestication quantitative trait loci in Triticum dicoccoides, the progenitor of wheat. PNAS 100(5): 2489-2494.

Penner, G.A., J. Clarke, L.J. Bezte, and D. Leisle. 1995. Identification of RAPD Markers Linked to a Gene Governing Cadmium Uptake in Durum Wheat. Genome. 38.

Pozniak, C.J., S.L. Fox, and D.R. Knott. 2009. CDC Verona durum wheat. Can. J. Plant Sci. 89:321-324.

Pumphrey, M.O., R. Bernardo, and James A. Anderson. 2007. Validating the *Fhb1* QTL for Fusarium Head Blight Resistance in Near-Isogenic Wheat Lines Developed from Breeding Populations. Crop Sci. 27: 200-206.

Rafalski, J.A. and S.V. Tingey. 1993. Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. Trends in Genetics 9(8): 275-280.

Sallam, A.H., J.B. Endelman, J.-L. Jannink, and K.P. Smith. 2015. Assessing Genomic Selection Prediction Accuracy in a Dynamic Barley Breeding Population. The Plant Genome. 8(1): 1-15.

Schön, C.C., A.E. Melchinger, J. Boppenmaier, E. Brunklaus-Jung, R.G. Herrmann, and J.F. Seitzer. 1994. RFLP Mapping in Maize: Quantitative Trait Loci Affecting Testcross Performance of Elite European Flint Lines. Crop Sci. 34: 378-389.

Schroder, J.L., H. Zhang, K. Girma, W.R. Raun, C.J. Penn, and M.E. Payton. 2010. Soil Acidification from Long-Term Use of Nitrogen Fertilizers on Winter Wheat. Soil Science Society of America Journal 75(3):957-964.

Semagn, K., R. Babu, S. Hearne, and M. Olsen. 2014. Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. Mol Breeding, 33: 1-14.

Singh, A.K., J.M. Clarke, R.E. Knox, R.M. DePauw, T.N. McCaig, M.R. Fernandez, and F.R. Clarke. 2012. Transcend Durum wheat. Can. J. Plant Sci. 92: 809-813.

Singh, P.K., M. Mergoum, S. Ali, T.B. Adhikari, E.M. Elias, J.A. Anderson, K.D. Glover, and W.A. Berzonsky. 2006. Evaluation of elite wheat germ plasm for resistance to tan spot. Plant Dis. 90: 1320-1325.

Smith, N., M. Guttieri, E. Souza, J. Shoots, M. Sorrells, and C. Sneller. 2011. Identification and Validation of QTL for Grain Quality Traits in a Cross of Soft Wheat Cultivars Pioneer Brand 25R26 and Foster. Crop Sci. 51: 1424-1436.

Sorahan, T. and N.A. Esmen. 2004. Lung cancer mortality in UK nickel-cadmium battery workers, 1947-2000. Occup Envrion Med 61: 108-116.

Spielmeyer, W., P.J. Sharp, and E.S. Lagudah. 2003. Identification and Validation of Markers Linked to Broad-Spectrum Stem Rust Resistance Gene Sr2 in Wheat (*Triticum aestivum* L.). Crop Sci. 43: 333-336.

Steemers, F.J., C. Weihua, G. Lee, D.L. Barker, R. Shen, and K.L. Gunderson. 2006. Whole-genome genotyping with the single-base extension assay. Nature Methods 3(1): 31-33.

Tanksley S.D., N.D. Young, A.H. Paterson, and M.W. Bonierbale. 1989. RFLP mapping in plant breeding: new tools for an old science. Nature Biotechnology 7: 257-264.

Tanno, K. and G. Willcox. 2006. How Fast Was Wild Wheat Domesticated? Science 311(5759): 1886.

Trzcinka-Ochocka, M., M. Jakubowski, G. Razniewska, T. Halatek, and A. Gazewski. 2004. The effects of environmental cadmium exposure on kidney function: the possible influence of age. Environmental Research 95: 143-150.

Waalkes, M.P. and S. Rehm. 1992. Carcinogenicity of Oral Cadmium in the Male Wistar (WF/NCr) Rat: Effect of Chronic Dietary Zinc Deficiency. Toxicol. Sci. 19(4): 512-520.

Waalkes, M.P. and S. Rehm. 1994. Chronic Toxic and Carcinogenic Effects of Cadmium Chloride in Male DBA/2NCr and NFS/NCr Mice: Strain-Dependent Association with Tumors of the Hematopoietic System, Injection Site, Liver, and Lung. Fundamental and Applied Toxicology 23(1): 21-31.

Waalkes, M.P., S. Rehm, and M.G. Cherian. 2000. Repeated Cadmium Exposures Enhance the Malignant Progression of Ensuing Tumors in Rats. Toxicol. Sci 54(1): 110-120.

Willcox, G.. 2005. The distribution, natural habitats and availability of wild cereals in relation to their domestication in the Near East: multiple events, multiple centres. Veget Hist Archaeobot 14: 534-541.

Wiebe, K., N.S. Harris, J.D. Faris, J.M. Clarke, R.E. Knox, G.J. Taylor, and C.J. Pozniak. 2010. Targeted mapping of Cdu1, a major locus regulating grain cadmium concentration in durum wheat (*Triticum turgidum* L. var *durum*). Theor Appl Genet. 121: 1047-1058.

Williams, C.H. and D.J. David. 1973. The effect of superphosphate on the cadmium content of soils and plants. Australian Journal of Soil Research. 11(1): 43-56.

Wu, L. C. Tan, L. Liu, P. Zhu, C. Peng, Y. Luo, and P. Christie. 2012. Cadmium bioavailability in surface soils receiving long-term applications of inorganic fertilizers and pig manure. Geoderma. 173-174: 224-230.

Zhang, L.P., A. Khan, D. Nino-Lui, M.R. Foolad. 2002. A molecular linkage map of tomato displaying chromosomal locations of resistance gene analogs based on a *Lycopersicon esculentum* x *Lycopersicon hirsutum* cross. Genome 45(1): 133-146.

Zhao, Z.Q., Y.G. Zhu, H.Y. Li, S.E. Smith, and F.A. Smith. 2003. Effects of forms and rates of potassium fertilizers on cadmium uptake by two cultivars of spring wheat (Triticum aesitivum, L.) Environmental International 29: 973-978.

Zhu, Guoying, H. Wang, Y. Shi, S. Weng, T. Jin, Q. Kong, and G. Nordberg. 2004. Environmental cadmium exposure and forearm bone density. BioMetals. 17: 499-503.

Zook, E. G., F. E. Greene, and E. R. Morris. 1970. Nutrient Composition of Selected Wheats and Wheat Products. 6. Distribution of Manganese, Copper, Nickel, Zinc, Magnesium, Lead, Tin, Cadmium, Chromium and Selenium as determined by atomic absorption spectroscopy and colorimetry. Cereal Chemistry. 47: 720-731.

APPENDIX

Table A1. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT6 grown in Williston, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13504	+[‡‡]	+	+	-	-	44.9
D13528	+	+	+	.	+	45.4
D13554	+	+	+	-	+	46.2
D13561	+	+	+	-	+	46.9
D13524	+	+	+	.	+	49.1
Highest Cd Lines						
D13527	+	+	+	-	-	173.2
CARPIO	-	-	-	-	-	161.1
TIOGA	-	-	-	-	-	161.1
D13485	+	+	+	-	+	149.6
ALKABO	-	-	-	-	+	146.9
Checks						
JOPPA	-	-	-	-	-	140.2
CARPIO	-	-	-	-	-	161.1
TIOGA	-	-	-	-	-	161.1
DIVIDE	-	.	-	-	.	140.2
ALKABO	-	-	-	-	+	146.9
STRONGFIELD	+	+	+	+	+	81.6
Statistics						
r^2	0.41*	0.38*	0.35*	0.00	0.01	
$LSD_{0.05}$						38.2
$LSD_{0.01}$						50.6
CV%						24.2%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Table A2. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT7 grown in Williston, North Dakota in 2014.

Genotype	**Marker Name**					**Cd Concentration (µg/kg)**
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13639	+[‡‡]	+	+	-	-	37.6
D13609	+	+	+	-	+	37.8
D13591	+	+	+	-	+	38.3
D13569	+	+	+	-	+	41.1
D13637	+	+	+	-	+	43.5
Highest Cd Lines						
DIVIDE	-	-	-	-	+	178.2
ALKABO	-	-	-	-	+	175.2
CARPIO	-	-	-	-	-	165.3
JOPPA	-	-	-	-	-	154.3
TIOGA	-	-	-	-	-	151.3
Checks						
JOPPA	-	-	-	-	-	154.3
CARPIO	-	-	-	-	-	165.3
TIOGA	-	-	-	-	-	151.3
DIVIDE	-	-	-	-	+	178.2
ALKABO	-	-	-	-	+	175.2
STRONGFIELD	+	+	+	+	+	76.0
Statistics						
r^2	0.51*	0.71*	0.70*	0.00	0.00	
$LSD_{0.05}$						29.9
$LSD_{0.01}$						39.6
CV%						19.8%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Table A3. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT8 grown in Williston, North Dakota in 2014.

Genotype	**Marker Name**					**Cd Concentration (µg/kg)**
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13749	+[‡‡]	+	+	.	.	37.6
D13743	+	+	+	-	-	40.9
D13713	+	+	+	-	+	41.0
D13695	+	+	+	-	-	43.6
D13662	+	+	+	-	-	44.7
Highest Cd Lines						
D13697	-	+/-	+/-	-	-	173.9
ALKABO	-	-	-	-	.	173.3
CARPIO	-	-	-	-	-	156.0
TIOGA	-	-	-	-	-	153.3
DIVIDE	-	-	-	-	+	141.8
Checks						
JOPPA	-	-	-	-	-	125.9
CARPIO	-	-	-	-	-	156.0
TIOGA	-	-	-	-	-	153.3
DIVIDE	-	-	-	-	+	141.8
ALKABO	-	-	-	-	.	173.3
STRONGFIELD	+	+	+	+	+	78.9
Statistics						
r^2	0.69*	0.46*	0.46*	0.00	0.01	
$LSD_{0.05}$						21.6
$LSD_{0.01}$						28.6
CV%						15.1%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Table A4. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT9 grown in Williston, North Dakota in 2014.

Genotype	**Marker Name**					**Cd Concentration (µg/kg)**
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13831	+[‡‡]	+	+	.	.	34.1
D13812	+	+	+	-	+	35.9
D13827	+	+	+	-	+	40.4
D13797	+	+	+	-	-	42.4
D13759	+	+	+	-	.	43.9
Highest Cd Lines						
TIOGA	-	-	-	-	-	168.3
CARPIO	-	-	-	-	-	168.3
JOPPA	-	-	-	.	.	153.6
DIVIDE	-	-	-	-	+	146.5
ALKABO	-	-	-	-	-	142.7
Checks						
JOPPA	-	-	-	.	.	153.6
CARPIO	-	-	-	-	-	168.3
TIOGA	-	-	-	-	-	168.3
DIVIDE	-	-	-	-	+	146.5
ALKABO	-	-	-	-	-	142.7
STRONGFIELD	+	+	+	+	+	75.5
Statistics						
r^2	0.64*	0.65*	0.66*	0.00	0.03	
$LSD_{0.05}$						22.6
$LSD_{0.01}$						30.0
CV%						15.3%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

Table A5. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT10 grown in Williston, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13874	+[‡‡]	+	+	-	-	34.8
D13865	+	+	+	.	+	41.2
D13854	+	+	+	-	-	42.1
D13850	+	+	+	.	-	42.4
D13889	+	+	+	-	+	42.6
Highest Cd Lines						
D13922	-	-	-	-	-	160.3
D13857	-	+	+	+	-	152.3
ALKABO	-	-	-	-	-	142.9
DIVIDE	-	.	-	.	.	135.6
D13918	-	-	-	-	-	134.7
Checks						
JOPPA	-	-	-	-	-	105.6
CARPIO	-	-	-	-	-	129.3
TIOGA	+	-	-	-	-	96.6
DIVIDE	-	.	-	.	.	135.6
ALKABO	-	-	-	-	-	142.9
STRONGFIELD	+	+	+	+	+	60.7
Statistics						
r^2	0.49*	0.43*	0.46*	0.04	0.07**	
$LSD_{0.05}$						25.3
$LSD_{0.01}$						33.5
CV%						18.4%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

** Indicates $p< 0.05$.

Table A6. Marker results and Cd concentrations of lowest and highest Cd accumulating lines and checks from the trial PYT11 grown in Williston, North Dakota in 2014.

Genotype	Marker Name					Cd Concentration (µg/kg)
	Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	
Lowest Cd Lines						
D13949	+[‡‡]	+	-	-	-	39.8
D13998	+	+	+	+	+	40.7
D13967	+	+	-	-	-	41.4
D13980	+	+	+	+	+	42.2
D13986	+	+	+	.	+	42.9
Highest Cd Lines						
ALKABO	-	-	-	-	.	166.6
CARPIO	-	-	-	-	.	159.4
D13994	-	-	-	.	.	154.0
TIOGA	-	-	-	-	.	152.8
D131032	+/-	+	+	+	-	144.2
Checks						
JOPPA	-	-	.	-	.	142.5
CARPIO	-	-	-	-	.	159.4
TIOGA	-	-	-	-	.	152.8
DIVIDE	-	-	-	-	+	142.9
ALKABO	-	-	-	-	.	166.6
STRONGFIELD	+	+	+	+	+	69.8
Statistics						
r^2	0.80*	0.58*	0.06†	0.02	0.00	
$LSD_{0.05}$						23.9
$LSD_{0.01}$						31.6
CV%						16.8%

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] The '+' and '-' indicate allele agreement and disagreement with the low Cd cultivar Strongfield, respectively. A '.' indicates marker data was missing.

* Indicates $p<0.01$.

† Indicates $p< 0.05$.

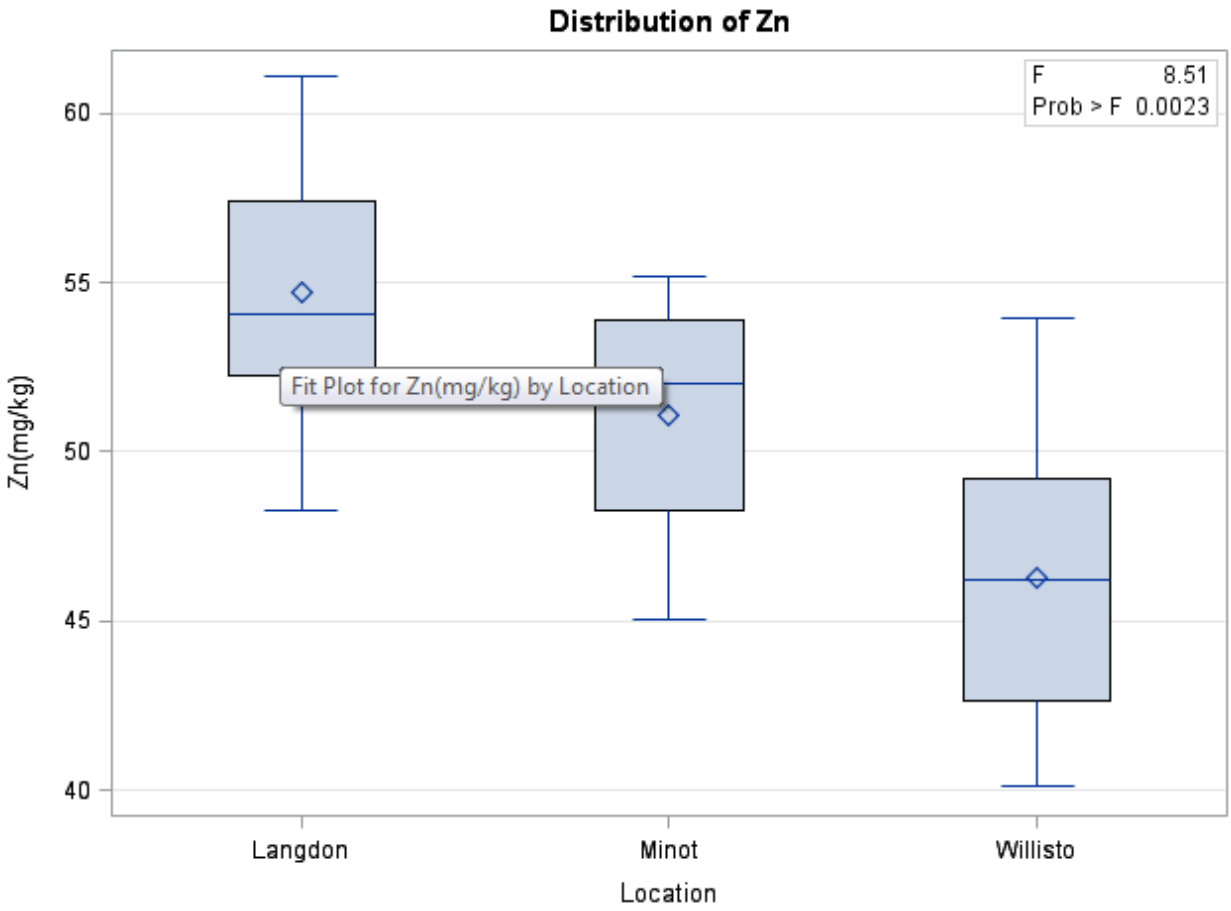

Figure A1. Soil Zn (mg/kg) levels in Langdon, Minot, and Williston with 10, 4, and 8 soil samples, respectively.

Table A7. Pearson correlation coefficients of each marker with grain Cd concentrations of lines in each trial.

Trial		Marker Name					
		Cad 5B[†]	1343[‡]	1775[§]	2001[¶]	1996[††]	1067[‡‡]
2013	Langdon EDA1	.75**	.72**	.76**	.45*	.01	§§
	Langdon PYT10	.09	.84**	.84**	§§	.15	§§
	Langdon PYT11	§§	.87**	.87**	§§	.27*	§§
	Langdon PYT12	§§	.82**	.83**	§§	.10	§§
	Langdon PYT13	.30*	.75**	.64**	§§	.04	.12
	Williston PYT11	§§	.84**	.86**	§§	.23*	§§
	Williston PYT14	.70**	.69**	.35*	§§	.01	§§
2014	Langdon URDN	.91**	.79**	.74**	.35	.22	§§
	Langdon PYT4	.91**	.90**	.90**	.10	.58**	§§
	Langdon PYT5	.83**	.83**	.83**	.22	.47**	§§
	Langdon PYT6	.78**	.64**	.62**	.04	.02	§§
	Minot AYT7	.84**	.84**	.83**	.45**	.18	§§
	Williston URDN	.92**	.83**	.79**	.44*	.28	§§
	Williston AYT5	§§	.92**	.92**	.10	.03	§§
	Williston AYT7	.88**	.88**	.86**	.37*	.08	§§
	Williston PYT3	.80**	.83**	.77**	.53**	.15	§§
	Williston PYT4	.89**	.86**	.87**	.09	.63**	§§
	Williston PYT5	.87**	.84**	.85**	.19	.46**	§§
	Williston PYT6	.64**	.62**	.59**	-.01	.10	§§
	Williston PYT7	.71**	.84**	.83**	.00	-.02	§§
	Williston PYT8	.83**	.68**	.68**	-.04	.09	§§
	Williston PYT9	.80**	.81**	.81**	-.03	.16	§§
	Williston PYT10	.67**	.66**	.68**	-.20	.27*	§§
	Williston PYT11	.89**	.76**	.24*	.13	.04	§§

[†] Located on chromosome 5B.

[‡] Abbreviation of marker Ex_c1343_2570756 located on chromosome 5B.

[§] Abbreviation of marker Ex_c17754_26503892 located on chromosome 5B.

[¶] Abbreviation of marker Ex_c20019_29052512 located on chromosome 5B.

[††] Abbreviation of marker Ex_c1996_3754394 located on chromosome 2B.

[‡‡] Abbreviation of marker Ra_rep_c106727_90434958 on chromosome 2B.

§§ Marker was not polymorphic in experimental lines.

** Indicates a p-value less than 0.01.

* Indicates a p-value less than 0.05.