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Production of Organic Acids and Adsorption of Cd on Roots of Durum Wheat (Triticum turgidum L. var. durum)

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Production of organic acids and adsorption of Cd on roots of durum wheat

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

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Abstract

A number of isolines of durum wheat (*Triticum turgidum* var *durum*) differ in their translocation of Cd. In the field, the high isolines accumulate twice the Cd in leaves and grain when compared to the low isolines. The hypothesis that differential accumulation of Cd is associated with differential production of organic acids was tested by measuring Cd content in tissues, Cd partitioning within the root, and organic acids in tissues. In solution culture, the high and low isolines of W9261-BG did not differ in any of the variables measured. Within W9260-BC, the low isoline had half the Cd in its shoot, 30% more tightly-bound Cd in the root and higher concentrations of fumaric, malic, and succinic acids in the root compared to the high isoline. Differential Cd accumulation may be linked to differential adsorption and retention of Cd in the roots of the low Cd-accumulating isolines, possibly via chelation with organic acids.

Keywords: Cadmium; LMWOAs; *Triticum turgidum*; Isolines; Adsorption.

Introduction

Durum wheat (*Triticum turgidum* L. var *durum*) is one of the main staple foods consumed worldwide, and is one of several crops that tend to accumulate high concentrations of cadmium (Cd) when grown in Cd-contaminated soil (Hart et al. 2006). Health concerns associated with Cd-contaminated crops have caused the international food standard organization, the Codex Alimentarius Commission, to propose a 0.2 mg Cd kg^{-1} limit for wheat and rice grains meant for the international market (CAC 2005). This proposed limit, when adopted, may have dire consequences for major durum wheat exporters, such as Canada, where the natural accumulation of Cd in the grains varies from 0.1 – 0.5 mg Cd kg^{-1} depending on the cultivar (Garret et al. 1998). To limit the Cd concentration in wheat grains, five pairs of isolines of durum wheat were derived through classical breeding (Clarke et al. 1997a). Each pair of isolines can be categorized as high and low Cd-accumulating isolines, with the high member of each pair having twice the Cd content in their leaves and seeds when compared to the low Cd-accumulating isoline (Clarke et al. 1997a; Harris and Taylor 2001).

Many factors including rates of Cd uptake, xylem translocation from root to shoot and production of metal-binding chelators have been studied to date but the observed differential partitioning of Cd in these isolines has yet to be clearly explained. Berkelaar and Hale (2000) associated the differences in Cd accumulation between durum wheat cultivars Kyle (high Cd in grain) and Arcola (low Cd in grain) with differences in their root morphology; roots of Arcola accumulated 30% more Cd and also had more root tips and greater root surface area. Further, Hart et al. (1998) observed that Cd binds to the

root cell wall in durum wheat and the concentration dependence of the binding was linear, suggesting that apoplastic binding might be responsible for the observed decrease in root to shoot Cd translocation in the low Cd-accumulating cultivars. However, the relative involvement of the apoplast in differential Cd accumulation in the isolines of durum wheat remains unverified. Harris and Taylor (2001) determined that the movement of Cd^{2+} into the grains of durum wheat treated with $^{109}Cd^{2+}$ was partly correlated with remobilization of Cd from the flag leaf.

The roles of chelators such as phytochelatins (PCs) and low molecular weight organic acids (LMWOAs) in differential Cd accumulation in durum wheat have also been studied. Stolt et al. (2003) determined that neither PC chain length nor PC isoform varied with differential Cd accumulation in spring bread wheat (*Triticum aestivum* L.) and durum wheat. They hypothesized that differential accumulation of Cd in wheat grain might be due to differential Cd partitioning in the root. Thus, a closer look at the mechanisms of adsorption, absorption and storage of Cd within roots might aid in elucidating the mechanism behind differential Cd concentrations in the isolines of durum wheat. Cieśliński et al. (1998) investigated the effects of LMWOAs exuded in the rhizosphere of durum wheat cultivars Arcola (low Cd in grain) and Kyle (high Cd in grain). They found that Kyle exuded more LMWOAs into the rhizosphere when compared to Arcola, and suggested that this might be responsible for increased mobilization and uptake of Cd in Kyle. It is imperative to know the relative contribution of endogenous organic acids in either conferring metal-tolerance to plants or in facilitating uptake of metals by roots as we seek characteristics that define low Cd-accumulating cultivars.

The objective of the current study was to investigate the relationship between LMWOAs and Cd in the shoot and root tissues to test the hypothesis that differential accumulation of Cd is associated with differential production of LMWOAs. In addition, we also investigated the partitioning of Cd in roots by measuring the relative amounts of Cd adsorbed to the roots (an estimate of the apoplast) and in the roots (an estimate of the symplast). The experimental results were modeled using visual MINTEQ 2.53 (Gustafsson 2006), a chemical speciation program, to better understand the proportion of Cd that could be bound to various LMWOAs produced in the plant tissues.

Materials and Methods

Plant material, Germination and Growth Conditions

Two pairs of isolines of durum wheat (*Triticum turgidum* L. var *durum*) originally derived by Clarke et al. (1997a) were used. The two pairs are designated as W9260-BC-L and W9260-BC-H, and W9261-BG-L and W9261-BG-H (L= low Cd-accumulating isoline, H = high Cd-accumulating isoline). The pairs were selected from $F_{4:6}$ families that segregated for Cd concentration in the grain; the W9260-BC pair was derived from the cross DT617/DT471 and the W9261-BG pair from the cross DT630/DT471 (Clarke et al. 1997a). DT617 and DT630 are high Cd-accumulating and DT471 is low Cd-accumulating (Clarke et al. 2002). The isoline pairs and parental lines were developed under field conditions in Saskatchewan Canada and North Dakota USA (reviewed in Grant et al. 2008). Phenotypic analysis of the F_2 generations of five different crosses of durum wheat indicated that Cd accumulation in the grain was controlled by a single gene,

dominant for low Cd-accumulation (Clarke et al. 1997b). Recently, Knox et al. (2009) used quantitative trait locus (QTL) analysis of double haploid F_1 plants derived from low and high Cd-accumulating parental lines of durum wheat to map the locus of the gene for Cd-accumulation (*Cdu1*) on chromosome 5B.

The concentrations of Cd in the leaves of the isolines are highly correlated ($r > 0.87$, $p<0.01$) with the concentrations of Cd in the grain (Clarke et al. (1997b); therefore, the phenotype (differential Cd-accumulation) can be assessed without having to wait for the grain to fill. The W9260-BC and W9261-BG isoline pairs were selected based on a preliminary study in which differential Cd accumulation (i.e. high Cd-accumulating isoline contained twice the Cd in the leaves compared to the low Cd-accumulating isoline) was reproduced in hydroponic culture (Bahrami, McGarvey and Macfie, unpublished data). Seedlings were grown following a modified method based on Archambault et al. (2001). Briefly, seeds were surface-sterilized with 1% sodium hypochlorite for 20 min, rinsed three times with distilled water, and thereafter imbibed overnight in aerated solution containing 0.005 g L^{-1} Vitavax (a systemic fungicide; Uniroyal Chemical Ltd, Calgary, AB, Canada). Aquaria were each filled with 10 L of nutrient solution containing 1.0 mM $Ca(NO_3)_2$, 1.0 mM K_2HPO_4, 0.4 mM KNO_3, 0.3 mM NH_4NO_3, 0.1 mM K_2SO_4, 0.01 mM $FeCl_3$, 0.01 mM Na_2EDTA, 6.0 µM H_3BO_3, 2.0 µM $MnCl_2$, 0.5 µM $ZnSO_4$, 0.15 µM $CuSO_4$, and 0.1 µM Na_2MoO_4, adjusted to pH 6, and the treated seeds were placed on mesh suspended over the aerated nutrient solution. The aquaria were completely covered for 24 h with black plastic to prevent seeds from drying out. Thereafter, the aquaria were uncovered and placed in a controlled environment room

set to a day/night temperature regime of 20/18°C with a 16 h light period and a 8 h dark period; the fluorescent light intensity was 200 ± 15 µmol $m^{-2}\cdot s^{-2}$. Seedlings of uniform size were selected for hydroponic culture experiments.

Experimental Treatment

On day 6, the seedlings were transferred into 1.4 L culture vessels (four plants per vessel and four replicate vessels per treatment unless stated otherwise) filled with the nutrient solution described above (pH 6), and aerated. Seedlings were placed between thin pieces of upholstery foam and suspended in slits cut into the lids of the culture vessels. The culture vessels were covered with black cloth to limit algal growth, and placed in the controlled environment room. The nutrient solutions were replenished every other day to safeguard against nutrient deficiencies. The solutions in culture vessels were brought up to 1.4 L daily with dH_2O to account for water loss due to evapotranspiration. Eight-day-old plants were treated with $CdCl_2\cdot 4H_2O$ (0 and 0.1 µM) added to fresh nutrient solutions. This low concentration of Cd was chosen to mimic the Canadian prairie soil with natural Cd deposition, given that the mean soil Cd concentration is 0.28 mg Cd kg^{-1} (Chan and Hale 2004) and Cd concentrations greater than 0.1 µM have been shown to induce stress in durum wheat (Archambault et al. 2001). The aerated nutrient solutions with or without Cd^{2+} supplementation were replenished every other day. Plant tissues were harvested on day 8 after treatment with or without $CdCl_2$, and fresh weight was recorded.

Measurement of Cd

To estimate the amount of Cd adsorbed to the root surface, half of the roots in each culture vessel (n=3) were rinsed in dH_2O for 30 s followed by a 30 min wash in 1 mM $CaSO_4$ and another 30 s wash in dH_2O (Gadapati and Macfie 2005); Cd adsorbed to the root surface will be removed by cation exchange between Cd^{2+} and Ca^{2+}. To measure the total amount of Cd associated with the roots, the other half of the roots in each vessel were washed in dH_2O for 30 s to rinse the nutrient solution from the root surface. With these treatments, the amount of adsorbed Cd can be obtained by subtracting the amount of Cd in the $CaSO_4$-washed roots from the amount of Cd in the unwashed roots. All plant tissues were blotted dry with Kimwipe® tissues, separated into roots and shoots, oven-dried at 60°C to constant weight and analyzed for Cd.

Dried plant tissues were cut into 2 – 3 mm pieces and approximately 0.1 – 0.2 g were weighed into individual test tubes. To the individual samples, 1.5 mL nitric acid (Omni-Trace®) was added. Each test tube was capped with a marble to keep the sample in the tube and allow for pressure release. Racks of test tubes were allowed to sit at room temperature overnight, then placed on a sand-filled tray and heated to 90 – 100°C until the tissues were fully digested. Tomato leaves (NIST standard reference material #1573a) and reagent blanks were included in the digestion process to allow for easy determination of percentage recovery of Cd in the tissue sample and possible contamination. When cooled to room temperature, the samples were filtered using 9 cm VWR brand filter paper (qualitative 413) and the volume brought up to 25 mL with

dH_2O. The samples were analyzed for total Cd by inductively coupled plasma- atomic emission spectroscopy (ICP-AES) using a Perkin-Elmer Optima 3300 dual view ICP-AES, with a RF generator power of 1300 Watts, gas flow rate of 15 L min^{-1}, auxiliary flow rate of 0.5 L min^{-1}, nebulizer flow rate of 0.8 L min^{-1}, pump (for sample) flow rate of 1.0 L min^{-1}, and an analyte line of Cd 226.507 nm, with a detection limit of 8 – 10 ppb for Cd.

Organic Acid Analyses

Plant tissues were carefully monitored during growth and development to ensure adequate nutrient supply and to raise the confidence in organic acid data because LMWOAs may be produced by nutrient-deficient plants as in the case of lupin, *Lupinus albus* L. cultivars Minori and Nelly, where exudation of citric acid increased significantly under phosphorus deficiency (Egle et al. 2003).

Organic acids in the plant tissues

Following harvest, plant tissues (roots and shoots) were prepared using the modified method of Sanita di Toppi et al. (2007). The roots were severed from the shoot and fresh tissues (approximately 1.0 g) were ground to powder in a chilled mortar using liquid nitrogen, collected in 50 mL centrifuge tubes and frozen (-80°C) until analysis. For analysis, 5 mL of dH_2O was added to the tissue samples and the mixture was heated for 20 min in a water bath at 80°C to denature the degradative enzymes. The mixture was

then centrifuged at 2500 x g for 10 min at 25°C and 300 µL of the supernatant was injected directly on a Dionex Ion Chromatograph (IC) for the determination of LMWOAs (see next section). Where appropriate, the supernatant was diluted with dH_2O to reduce overloaded peaks and obtain more resolved and identifiable peaks.

Identification of organic acids

The method used for the separation and identification of LMWOAs using IC followed that of Liu et al. (2007) with slight modifications. Briefly, the LMWOAs were separated with a Dionex ICE-ASI Ion exclusion column (4 x 250 mm) with the following column conditions: 1.5 mM heptafluorobutyric acid (HFBA) as eluent, eluent flow of 80 on the dial, nitrogen pressure of 7.5 psi, run time was 11.90 min, and 40 µL injection volume. The suppressor settings for the Dionex ion chromatograph were as follows: regenerant was 9.7 mM tetrabutyl ammonium hydroxide (TBAOH), regenerant flow was 3.5 ml min^{-1} and nitrogen pressure was 15 psi. Spiking experiments involving the addition of known concentrations of organic acid standards to the supernatants were carried out in order to validate the identities of the observed peaks.

Data Analyses

All statistical analyses were performed using SigmaStat Version 2.03. Two-way analysis of variance (ANOVA) was used to determine the effects due to isolines and Cd treatment on biomass and Cd content and Tukey's test was used to determine significant

differences between means ($P < 0.05$). The t-test was used to determine the differences in proportion of Cd adsorbed to roots of low and high Cd-accumulating isolines. Two-way ANOVA was used to determine the differences in mean values of total LMWOAs in low and high Cd-accumulating isolines. Due to significant interactions between isolines and individual LMWOAs, one-way ANOVAs were used to analyze the isolines, and Tukey's test was used to determine significant differences between treatments.

Visual MINTEQ (version 2.53), a chemical speciation program, was used to estimate the proportion of measured Cd that was bound to each of the measured LMWOAs in the tissues. Fumaric acid did not exist in the visual MINTEQ database, so we approximated Cd-fumaric acid interactions by replacing fumaric acid with tartaric acid, the most similar LMWOA in the database (fumaric acid pka1=3.02, pka2=4.44; tartaric acid pka1=2.98, pka2=4.34). The average amounts of LMWOAs and Cd in the Cd-treated roots and shoots of each low and high Cd-accumulating isoline were modeled; these amounts were calculated by multiplying mean concentration by mean mass for each tissue. Modelling was done at pH values 4.5 – 7.5 to span the range of pH in the cytoplasm, vacuole and cell wall. In a review by Kurkdjian and Guern (1989) the pH of the cytoplasm ranged from 6.8 to 7.5 in roots and was 7.5 in leaves, and the pH of the vacuole ranged from 4.6 to 6.0 in roots and was 5.3 in leaves. Cell wall free space pH ranges from 4.5 to 6.5 (Jacobs and Ray 1976). We recognize that other metal-binding sites (such as the cell wall matrix) exist within plant tissue, the model simply gives an estimate of the potential for Cd to bind to the various LMWOAs.

Results

Plant Biomass and Cd Distribution

During the course of the experiments, all plants appeared healthy and there were no visible signs of Cd-induced stress or nutrient deficiencies. Neither the fresh nor dry weights of W9260-BC shoots varied between treatments (Fig. 1a and 1b, respectively). The fresh and dry weights of W9260-BC roots were more variable, with the root fresh weight of the low Cd-accumulating isoline being higher in treated plants (Fig 1a) and the dry weight for the high Cd-accumulating isoline being lower in control plants (Fig. 1b). Based on the ratio of dry weight to fresh weight, shoots of W9260-BC contained 85 – 95% water, and roots contained 91 – 96% water. The root and shoot fresh (Fig 1d) and dry (Fig 1e) weights of W9261-BG did not differ between treatments or isoline. Based on the ratio of dry weight to fresh weight, W9261-BG shoots contained 95 – 96% water, and roots contained 97 – 98% water.

The Cd concentration in the shoots of the high Cd-accumulating isoline of W9260-BC was two-fold greater than that of the low Cd-accumulating isoline when treated with 0.1 µM Cd (Fig. 1c). No differences were found in concentrations of Cd in Cd-treated roots of the low and high Cd-accumulating isolines of W9260-BC. In W9261-BG (Fig. 1f), the Cd concentrations in the root and shoot tissues did not vary with treatment or isoline. However, Cd concentrations in Cd-treated W9261-BG were 50 – 75% lower in shoots than those of Cd-treated W9260-BC, and at least 90% lower in roots.

The proportions of Cd that were adsorbed to Cd-treated roots of the two pairs of isolines were investigated to further decipher the mechanisms behind the differential Cd accumulation in the aboveground tissues. In the low Cd-accumulating isoline of W9260-BC, only 8.2±5.1% of the Cd was adsorbed to the root, which was significantly lower (P=0.01) than the proportion of Cd adsorbed to the root of the high Cd-accumulating isoline (43.9±5.7%). In W9261-BG, the percentage of Cd adsorbed to the root did not differ (P = 0.88) between isolines; low = 31.8±3.0%; high = 33.8±12.0%.

Organic Acid Content in Durum Wheat Tissues

In the low Cd-accumulating isoline of W9260-BC, total LMWOA concentrations were higher in roots and shoots treated with Cd relative to their controls (Fig. 2a). In contrast, total LMWOAs in the high Cd-accumulating isoline treated with Cd declined in the root and did not vary in the shoots compared to their respective controls (Fig. 2a).

Each of citric, fumaric, malic, oxalic and succinic acids were found in both isolines of W9260-BC (Fig. 2b-f). Concentrations of oxalic acid (Fig. 2e) were 4 – 20 times higher in shoots and 5 – 10 times higher in roots as compared to the other LMWOAs. In the low Cd-accumulating isoline, concentrations of fumaric, malic, oxalic and succinic acids in the root, and of fumaric and succinic acids in the shoot were greater in the Cd-treated plants. Contrasting results were found in the high Cd-accumulating isoline where concentrations of citric acid in the root and shoot, and of succinic acid in the root, decreased in Cd-treated plants.

No differences in total LMWOA concentration were found in roots and shoots of low and high Cd-accumulating isolines of W9261-BG treated with Cd relative to their controls; although, shoots of the high Cd-accumulating isoline had higher concentrations of LMWOAs than the low Cd-accumulating isoline regardless of Cd-exposure (Fig. 3a).

In W9261-BG, only four LMWOAs were found in the root and shoot tissues, and these were in the order oxalic > malic > fumaric = succinic (Fig. 3 b-e). With only two exceptions, treatment with Cd did not affect the concentrations of any LMWOA in roots or shoots within any pair of isolines. The two exceptions were for the low Cd-accumulating isoline grown in the presence of Cd; the concentrations of oxalic acid in the root (Fig. 3d) and succinic acid in the shoot (Fig. 3e) were higher than their respective controls.

Modeling using visual MINTEQ

Our experimental results were modeled to estimate the proportion of total Cd that was bound to the various LMWOAs. Estimated speciation of Cd at different pHs in the root and shoot tissues of the low and high Cd-accumulating isolines of W9260-BC and W9261-BG is presented in Table 1. In no instance was the formation of Cd-fumaric or Cd-succinic acid complexes predicted to account for more than 0.15% of the total Cd in any tissue (data not shown) so these two LMWOAs were not included in the table. Within the range of pH tested (4.5 – 7.5), greater than 88% of the total Cd in both tissues of all isolines was predicted to be bound to oxalic acid, the LMWOA in highest abundance. Up to 1.5% of the total Cd was predicted to be the Cd^{2+} free ion even though

the concentrations of LMWOAs were 1 – 3 orders of magnitude higher than the concentrations of Cd in the tissues. Although only approximately 2 – 10% of the total Cd in the tissue was predicted to be bound to citric or malic acids, some interesting patterns appeared.

In W9260-BC, in which the high Cd-accumulating isoline accumulated two-fold higher concentrations of Cd than did the low Cd-accumulating isoline, the speciation of Cd did not differ markedly in the shoot tissues (Table 1). However, the proportion of Cd estimated to be bound to citric acid was approximately 8 times higher in the roots of the high Cd-accumulating isoline than in the low Cd-accumulating isoline, across the range of pH modeled. In the roots of the high Cd-accumulating isoline, the proportion of Cd estimated to be bound to malic acid was about one third of that in the low isoline.

In W9261-BG, in which no differential translocation of Cd was detected, the distribution of Cd in the roots of the isolines did not differ but the proportion of Cd estimated to be bound to malic acid in the shoots was 10-fold higher in the high Cd-accumulating isoline (Table 1).

Discussion

During the course of the experiments, both pairs of isolines showed no symptoms of Cd-induced stress; hence, we are confident that the results reflect constitutive physiological differences between the isolines.

Under our experimental conditions, only one pair of isolines (W9260-BC) showed the expected two-fold higher concentrations of Cd in shoots of the high Cd-accumulating

isoline. Elevated concentrations of Cd in the shoots of the high Cd-accumulating isoline were not associated with decreased concentrations in the roots; however, the large variance in measurements for the low Cd-accumulating isoline in our study may have reduced the chances of finding a significant difference. Harris and Taylor (2004) and Hart et al. (2006) also found no differences in the root Cd uptake between the durum wheat isoline pairs of 8982-TL and W9262-339A.

The lack of differential Cd accumulation in the shoots of the W9261-BG isolines in our study may be attributable to the relatively low Cd content in their tissues. The reason for Cd-treated W9261-BG isolines accumulating 50 – 90% less Cd and having 50% dry mass as compared the W9260-BC isolines is not clear. However, it is unlikely to be related to a genetic difference between W9260-BC and W9261-BG. While the two pairs of isolines share the same low Cd-accumulating parent (DT471) and have different high Cd-accumulating parents (DT617 and DT630; Clarke et al. 1997a), the Cd-accumulating trait is specific to differential Cd-accumulation; the isoline pairs do not differ in differential translocation of Ca, Cu, Fe, K, Mn, Na or Zn, nor in agronomic traits including yield (Clarke et al. 2002). It is possible that hydroponic culture or conditions in the controlled environment room resulted in reduced transpiration of the W9261-BG isolines, which could explain reduced growth and Cd-uptake. However, in a preliminary study performed 1 year prior to this study, differential accumulation of Cd was observed in the W9261-BG pair of isolines.

Concentrations of Cd were 4 – 10 times higher in roots than in shoots, which is a common result that has been attributed to apoplastic binding of Cd to the root cell walls. For example, Perriguey et al. (2008) found that maize had 28 times greater concentrations

of Cd in roots than in shoots, and suggested that binding of Cd in the root apoplast reduced Cd translocation to the shoot. Hart et al. (1998) and Chan and Hale (2004) also speculated that most of the root-Cd in durum wheat cultivars is restricted to the apoplast, and Cd was mostly associated with the cell wall in roots of *N. tabacum* and *T. caerulescens* (Boominathan et al. 2003). In our study < 40% of the Cd was adsorbed to the root. However, the method we used to desorb Cd from the root may not have removed 100% of the apoplastic Cd, especially past the first few layers of the root cortex.

Nevertheless, desorption of Cd in our study revealed that differential Cd accumulation in the W9260-BC isolines of durum wheat was associated with more Cd being sequestered in the putative root symplast of the low Cd-accumulating isoline. This is consistent with Harris and Taylor (2004) who reported that reduced Cd translocation in shoots of a low Cd-accumulating isoline of durum wheat was associated with a lower Cd concentration in the xylem sap; they hypothesized that the roots of the low Cd-accumulating isolines have an efficient mechanism of retaining Cd. It is possible that this retention may be attributable to sequestration and/or complexation with organic ligands in the root symplast, possibly LMWOAs in the vacuole.

In our study, roots of the isoline in which a greater proportion of Cd was retained in the root did contain greater concentrations of LMWOAs. Within the low Cd-accumulating isoline of W9260-BC, treatment with Cd resulted in increased concentrations of fumaric, malic, oxalic and succinic acids in the roots. In contrast, concentrations of malic, citric, fumaric and succinic acids decreased in the roots in response to Cd in the high Cd-accumulating isoline. Because the concentrations of oxalic acid were high relative to other LMWOAs in both high and low Cd-accumulating

isolines, chelation with oxalic is unlikely to explain differential retention of Cd in the roots. Even though the modeling results confirm that over 88% of the Cd in the root tissues was predicted to be complexed with oxalic acid, differences in concentrations of other LMWOAs might explain differential retention of the remaining Cd, especially when one combines absolute concentrations of Cd and LMWOAs with the results of the chemical speciation modelling. Specifically, the 2.5-fold higher estimated proportions of Cd-malic acid in roots of the low Cd-accumulating isoline relative to the high Cd-accumulating isoline suggests that some of the Cd retained in the root might be complexed with malic acid. Interestingly, the root of the high Cd-accumulating isoline had 8-fold higher estimated proportion of Cd-citric acid compared to the low Cd-accumulating isoline. Although not tested here, it is possible that Cd is translocated to the shoot as a citric acid complex. In the shoot, Cd-malic acid was the second-most prevalent Cd-complex in both low and high Cd-accumulating isolines.

The lack of differences in concentrations of LMWOAs in roots and shoots of W9261-BG grown in control solution as compared to those from plants treated with Cd may be related to the fact that these isolines did not differ in Cd content, and very little Cd was taken up by these isolines. However, the estimated higher proportion of Cd bound to malic acid in the shoot of the high Cd-accumulating isoline is consistent with the results of the W9260-BC isolines and suggests that Cd-malic complexes might be important to Cd sequestration.

Our results indicate that Cd-organic acid complexes sequestered in the root might explain the restricted Cd translocation from root to shoot in the low Cd-accumulating isolines of durum wheat. Also, though not tested in this study, there is a possibility that

the increased LMWOAs measured in the shoot of the low Cd-accumulating isoline might form complexes with Cd and thus restrict Cd loading into the grains. Similar results have been reported by Guo et al. (2007) who found an increased production of LMWOAs in maize (*Zea mays* L.) roots treated with Cd, suggesting that LMWOAs produced might be involved in Cd tolerance and detoxification. Similar to the studies on isolines of durum wheat, LMWOAs have been implicated in Zn hyperaccumulation and tolerance in *Arabidopsis halleri* by Zhao et al. (2000), who found no significant differences in concentrations of citric and malic acids in shoots but a positive increase in concentrations of both acids in roots treated with Zn. In contrast, Nakamura et al. (2008) detected citric and malic acids in the xylem exudate of oilseed rape (*Brasicca napus* L.), providing inconclusive evidence for citric acid and malic acid as Cd chelators in the xylem exudate.

The yet to be identified gene responsible for differential Cd-accumulation in durum wheat appears to code for a trait that affects retention of Cd in the root (Berkelaar and Hale 2000) and/or root to shoot translocation of Cd (Chan and Hale 2004, Harris and Taylor 2004) as opposed to uptake of Cd into the root. While we know the trait for low Cd-accumulation in the grain is dominant (Clarke et al. 1997b), and the gene is located on chromosome 5B (Knox et al. 2009), the function of this gene is not known. The results of our study indicate that the answer may lie in organic acid biochemistry. It is tempting to speculate that the gene for differential Cd accumulation affects a pathway that results in inter-conversion of citric and malic acids. The more malic acid produced the more Cd is bound in the root, the more citric acid produced the more Cd is translocated to the shoot. It is equally plausible that the gene directly affects sequestration of Cd in the root; for example, there might be a Cd-specific transporter that

moves Cd into the vacuole where it is subsequently chelated with malic acid or another LMWOA. As soon as the gene is identified, experiments can begin to elucidate its function.

Conclusions

Differential Cd accumulation in shoots of isolines of durum wheat may be linked to the differential Cd partitioning and retention in the root symplast of the low Cd-accumulating isolines. The increased retention of Cd in the root of low Cd-accumulating isolines may be associated with LMWOA-Cd complexes. The role of individual LMWOAs in forming complexes with Cd in the roots of the low Cd-accumulating isoline is worthy of further investigation as this might enhance our understanding of the roles of naturally-produced chelators as they relate to food safety.

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Table 1 Estimated percentage of total Cd apportioned to Cd-organic complexes or the free Cd^{2+} ion in shoots and roots of durum wheat grown for 8 days in nutrient solution supplemented with 0.1 μM $CdCl_2$. Total Cd in each tissue was calculated as concentration of Cd x tissue dry weight.

	pH	4.5	5	5.5	6	6.5	7	7.5		4.5	5	5.5	6	6.5	7	7.5
W9260-BC																
Shoot	Low	Total Cd = 2.1 μg							High	Total Cd = 3.7 μg						
	Cd-citric	0.03	0.1	0.2	0.3	0.4	0.4	0.4		0.1	0.3	0.6	1.1	1.5	1.7	1.7
	Cd-malic	3.6	5.3	6.5	7	7.2	7.3	7.3		4.6	6.6	8.0	8.7	8.9	8.9	8.9
	Cd-oxalic	95.1	93.5	92.2	91.5	91.2	91.1	91.1		94.4	92.3	90.5	89.4	88.9	88.6	88.5
	Cd^{2+}	0.9	0.8	0.8	0.7	0.7	0.7	0.7		0.9	0.8	0.7	0.7	0.7	0.7	0.7
Root	Low	Total Cd = 11.0 μg							High	Total Cd = 7.1 μg						
	Cd-citric	0.04	0.1	0.2	0.5	0.7	0.8	0.8		0.3	0.8	2.1	4.0	5.8	6.8	7.2
	Cd-malic	1.0	1.5	1.9	2.1	2.2	2.2	2.2		0.4	0.6	0.8	0.8	0.8	0.8	0.8
	Cd-oxalic	97.5	97	96.5	96.1	95.8	95.7	95.6		97.7	97.2	95.9	93.9	92.2	91.2	90.8
	Cd^{2+}	1.3	1.1	1.1	1.1	1.0	1.0	1.0		1.5	1.3	1.2	1.2	1.1	1.1	1.1
W9261-BG																
Shoot	Low	Total Cd = 0.5 μg							High	Total Cd = 0.3 μg						
	Cd-citric	0.05	0.1	0.3	0.6	0.9	1.0	1.1		0.03	0.1	0.2	0.3	0.4	0.4	0.5
	Cd-malic	0.4	0.6	0.7	0.8	0.8	0.8	0.8		4.0	5.8	7.1	7.6	7.8	7.9	7.9
	Cd-oxalic	98.2	98.1	97.8	97.5	97.2	97.0	96.9		95.0	93.2	91.9	91.1	90.8	90.7	90.7
	Cd^{2+}	1.3	1.1	1.1	1.1	1.0	1.0	1.0		0.9	0.8	0.8	0.8	0.8	0.8	0.7
Root	Low	Total Cd = 0.6 μg							High	Total Cd = 0.4 μg						
	Cd-citric	0.05	0.1	0.3	0.6	0.9	1.0	1.1		0.03	0.1	0.2	0.4	0.6	0.7	0.7
	Cd-malic	0.6	1.0	1.2	1.3	1.4	1.4	1.4		0.5	0.7	0.9	1.0	1.1	1.1	1.6
	Cd-oxalic	97.9	97.7	97.3	96.9	96.6	96.4	96.4		98.2	98.0	97.8	97.5	97.3	97.2	97.1
	Cd^{2+}	1.3	1.1	1.1	1.1	1.0	1.0	1.0		1.3	1.1	1.0	1.0	1.0	1.0	1.0

Figure Legends

Fig. 1 Effect of Cd on root and shoot fresh weights (a,d), dry weights (b,e) and Cd uptake (c,f) of low and high Cd-accumulating isolines of durum wheat. Different lower case letters denote significant differences ($P < 0.05$) within each tissue type. Error bars represent standard error of the mean (SEM) of three replicates

Fig. 2 Concentrations of low molecular weight organic acids in root and shoot tissues of W9260-BC; a total, b citric, c fumaric, d malic, e oxalic, f succinic. Different lower case letters within each tissue denote significant difference ($P < 0.05$) between treatments. Error bars represent SEM of four replicates

Fig. 3 Concentrations of low molecular weight organic acids in root and shoot tissues of W9261-BG; a total b fumaric, c malic, d oxalic, e succinic. Different lower case letters within each tissue denote significant difference ($P < 0.05$) between treatments. Error bars represent SEM of four replicates

Fig. 1

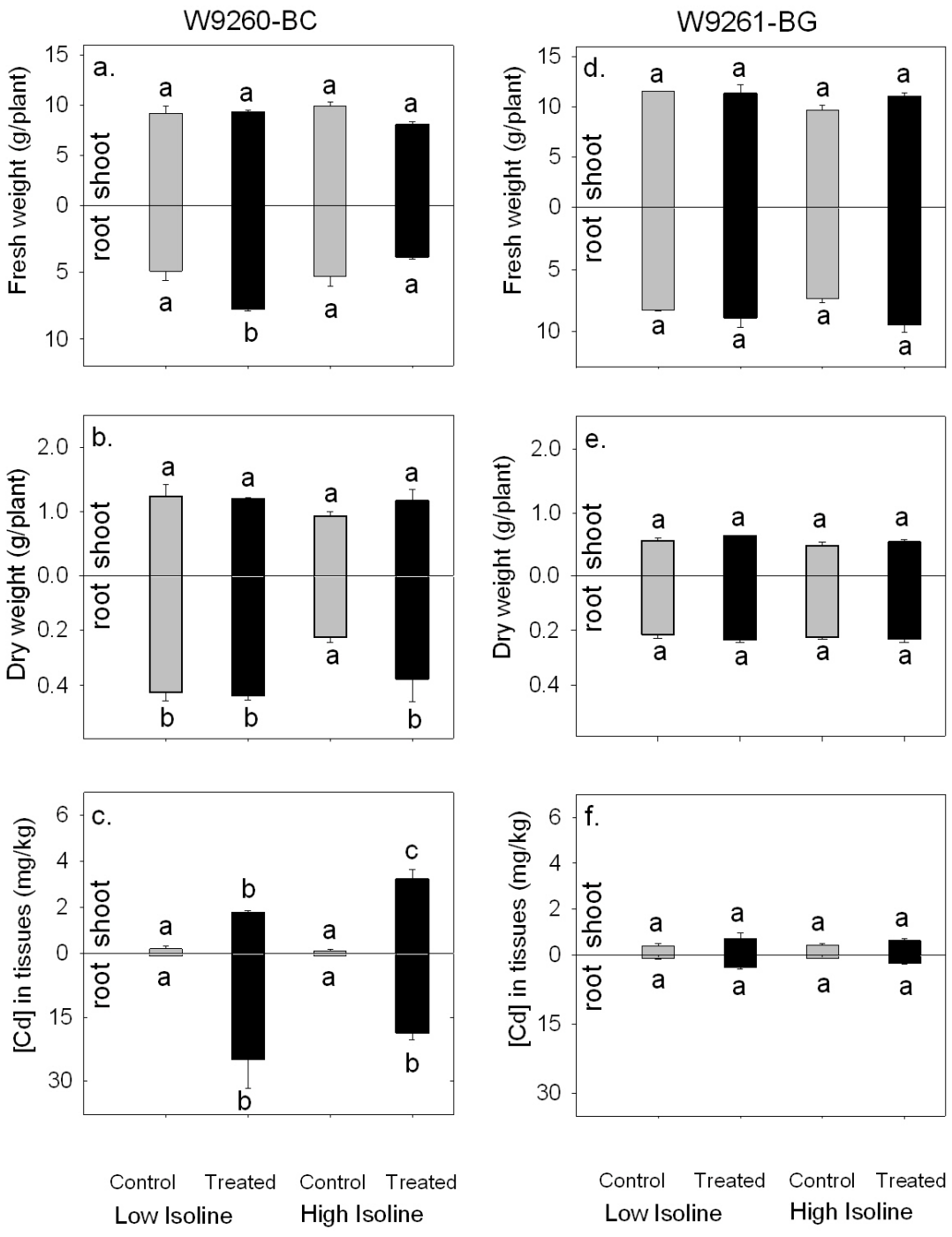

W9260-BC
W9261-BG
a.
b.
c.
d.
e.
f.
Fresh weight (g/plant)
Dry weight (g/plant)
[Cd] in tissues (mg/kg)
root
shoot
Control
Treated
Low Isoline
High Isoline

Fig. 2

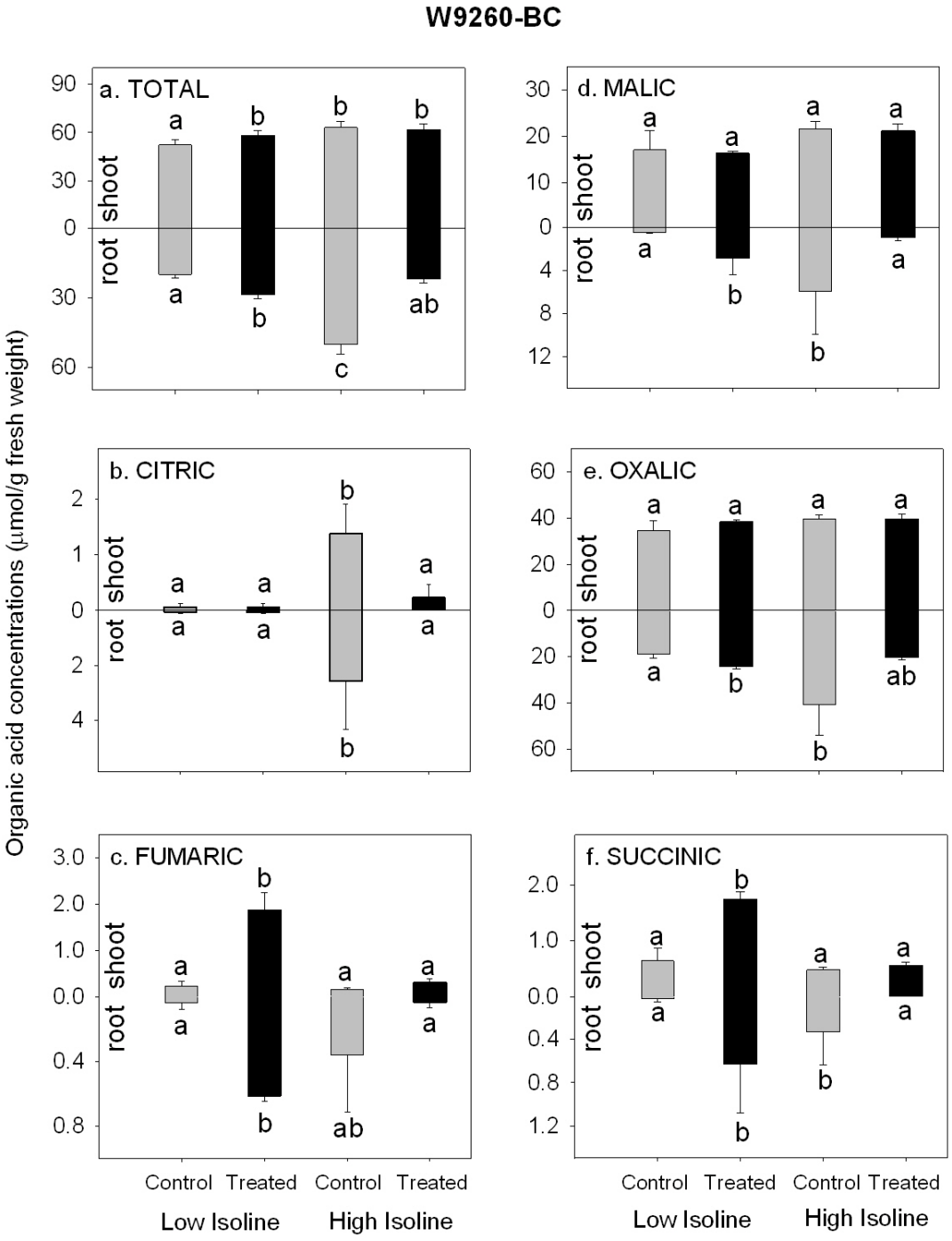
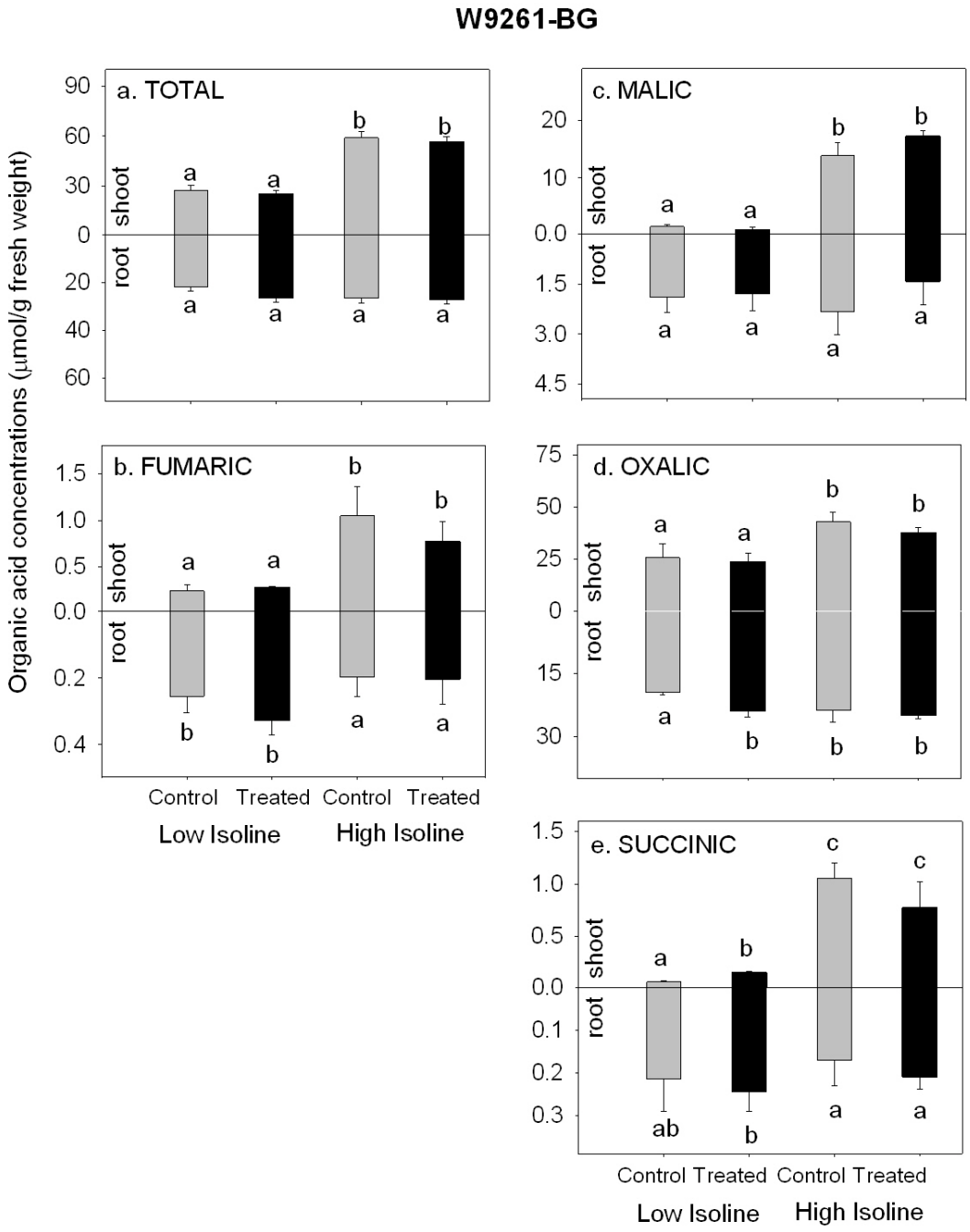

W9260-BC
Organic acid concentrations (µmol/g fresh weight)
a. TOTAL
b. CITRIC
c. FUMARIC
d. MALIC
e. OXALIC
f. SUCCINIC
root shoot
Control Treated Control Treated
Low Isoline High Isoline

Fig. 3


W9261-BG
a. TOTAL
b. FUMARIC
c. MALIC
d. OXALIC
e. SUCCINIC
Organic acid concentrations (µmol/g fresh weight)
shoot
root
Control
Treated
Low Isoline
High Isoline