Differential accumulation of cadmium in near-isogenic lines of durum wheat: no role for phytochelatins

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Abstract Certain cultivars of some crops, including durum wheat (*Triticum durum* Desf.), have a propensity to accumulate cadmium in the grain. In the 1980s, a Canadian wheat breeding program generated five pairs of near-isogenic lines of durum wheat that vary in cadmium-accumulation. Within each pair, one member accumulates twofold to threefold higher concentrations of cadmium in the shoot and grain. However, the physiological explanation for the high-low phenotype is unknown. We studied correlations between concentrations of cadmium and non-protein thiols, including phytochelatins, in these five pairs of near-isogenic lines to test the hypothesis that differential retention of cadmium-binding complexes in the root would explain the phenotype. The expected high-low pattern of cadmium accumulation was found in three of the pairs. In one pair, cadmium was positively correlated with cysteine and glutathione in the roots and with phytochelatins 2 and 4 in the shoots but in another pair cadmium was strongly negatively correlated with phytochelatins 2 and 4 in the shoots and unrelated to cysteine or glutathione. No correlations between concentrations of cadmium and the non-protein thiols were found in the third pair or in the remaining two pairs. The production of phytochelatins is a

well-described response to cadmium but the lack of consistent correlation between cadmium and non-protein thiols in these five near-isogenic lines indicates that complexation with non-protein thiols does not explain differential translocation of cadmium in durum wheat.

Keywords Cadmium · Durum wheat · Metal translocation · Phytochelatins · *Triticum durum*

Introduction

Cadmium is regulated in most countries. The acceptable limit for cadmium in urban soils worldwide ranges from 0.08 to 83,000 mg kg⁻¹ but 95 % of the values are less than 100 mg kg⁻¹ and the median limit is 7 mg kg⁻¹ (Jennings, 2013). Atmospheric deposition is a primary source of cadmium and the use of commercial fertilizers derived from rock phosphate (Roberts 2014) and sewage sludge (reviewed in Bingham 1979) can be an additional source to agricultural soils. As awareness of contaminants in fertilizers has increased, so too have restrictions on the concentrations of cadmium in such fertilizers (e.g., CCME 2010; EC 1986, 2003; US EPA 2016, among others). To protect consumers, limits for cadmium in arable soil are lower than the limits for other types of land use; for example, arable soil in Canada can contain no more than 1.4 mg Cd kg⁻¹ soil (CCME 1999).

Among cereal crops, durum wheat (*Triticum durum* Desf.) is particularly susceptible to the accumulation of cadmium (reviewed in Grant et al. 2008). The current CODEX Alimentarius Commission limit for wheat grain is 0.2 mg kg⁻¹ (CAC 2000), which is adequate to protect consumers. In the 1980s, however, concentrations of cadmium in the grain of some lines of Canadian durum wheat

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were at or above the limit for safe consumption (Clarke et al. 2002; Grant et al. 2008). This triggered a breeding program that resulted in five pairs of near-isogenic lines of durum wheat that differ only in the proportion of total cadmium that accumulates in the grain (Clarke et al. 1997b). Within each pair, the lines are characterized as being either 'low' or 'high' cadmium accumulators, although the total amount of cadmium taken up is the same (Greger and Lofstedt 2004; Harris and Taylor 2004; Hart et al. 2006). The low or high designation describes the relative concentrations in the grain; low cadmium lines retain more cadmium in their roots (Harris and Taylor 2013; Hart et al. 2006; Perrier et al. 2016; Tavares et al. 2015). By sowing only the low cadmium accumulators, the risk to consumers has been minimized. However, questions about the genetic and physiological factors that explain differential accumulation of cadmium in the grain of near-isogenic lines of durum wheat remain unanswered.

The genetic explanation for the high-low cadmium phenotype in durum wheat is almost complete. The dominant (Clarke et al. 1997a) *Cdu1* allele (Zimmerl et al. 2014), which is on chromosome 5B (Knox et al. 2009), explains about 90 % of the low cadmium phenotype of the grain (Zimmerl et al. 2014). We do not know what this gene encodes, although many have suggested that it could be a tonoplast transporter in root cells (Adeniji et al. 2010; Harris and Taylor 2004; Perrier et al. 2016; Tavares et al. 2015). Such a transporter alone, however, cannot explain retention of cadmium in the roots. The plant would also require the formation of stable cadmium complexes in the cytosol and vacuole.

The physiological component(s) of the phenotype are more elusive. The consensus is that differential root to shoot translocation of cadmium explains the difference between low and high lines (Adeniji et al. 2010; Greger and Lofstedt 2004; Harris and Taylor 2004, 2013; Hart et al. 2006; Perrier et al. 2016; Stolt et al. 2003). Hart et al. (1998) and Perrier et al. (2016), however, believe that the phenotype is explained by transport of cadmium from the leaves to the grain.

Theories to explain retention of cadmium in the roots of the low lines include binding to cell walls and accumulation in vacuoles (Hart et al. 2006). Perrier et al. (2016) showed that root cation exchange capacity was not correlated with cadmium in the grain of 10 cultivars of durum wheat, suggesting that adsorption to the cell walls is unlikely to explain retention of cadmium in the roots of low lines. It is more likely that cadmium is sequestered in the root vacuoles. While cytosolic chelators play important roles in metal homeostasis, two types of chelators act together to store cadmium in vacuoles: low molecular weight organic acids and phytochelatins (Sanita di Toppi and Gabbriellini 1999).

Three vacuolar organic acids (citric, malic and oxalic acids) form stable cadmium complexes and probably play a role in cadmium tolerance (Krotz et al. 1989). Adeniji et al. (2010) studied two pairs of near-isogenic lines of durum wheat (W9260-BC and W9261-BG) and reported higher concentrations of malate in the roots of the low cadmium lines and higher concentrations of citrate in the roots of the high lines; they speculated that cadmium-malate complexes were stored in root vacuoles and cadmium-citrate complexes were carried up the plant in the xylem.

Phytochelatins are synthesized from glutathione in the cytosol by the enzyme phytochelatin synthase, which is activated by the cadmium ion (Cd^{2+} ; Grill et al. 1998). Phytochelatins 2, 3 and 4 are found in durum wheat (Paradiso et al. 2008; Stolt et al. 2003). The interplay between phytochelatins, organic acids and cadmium is reviewed in Sanita di Toppi and Gabbriellini (1999) and summarized here. Chelation in the cytosol results in low molecular weight cadmium-phytochelatin complexes that are transported into the vacuole, where addition of a sulphur ion creates a high molecular weight complex. The phytochelatins likely return to the cytosol and the cadmium ions form stable complexes with vacuolar organic acids. Stolt et al. (2003) found no relationship between phytochelatins in the roots and cadmium in the leaves of two cultivars of durum wheat, but these cultivars also did not vary in aboveground cadmium concentrations. In one pair of near-isogenic lines of wheat (W9262-339A), soluble cadmium was associated with phytochelatins in the roots of both the low and high line, suggesting that vacuolar influx of cadmium did not differ between the two lines (Hart et al. 2006). However, phytochelatins and other non-protein thiols can also bind cadmium in the cytosol (Sanita di Toppi and Gabbriellini 1999), travel in the xylem to the shoots (Saathoff et al. 2011) and move within the plant in the phloem (Medoza-Cozatl et al. 2008).

We studied five pairs of near-isogenic line of durum wheat (*T. durum* Desf.) to test the hypothesis that retention of cadmium in the roots of the low cadmium accumulating lines of durum wheat is correlated with higher concentrations of phytochelatins and three other non-protein thiols: cysteine, γ -glutamyl cysteine and glutathione.

Materials and methods

Plant material and growth conditions

Durum wheat (*T. durum* Desf.) seeds were obtained from Dr. John Clarke (Agriculture and Agri-Food Canada, Swift Current, Canada). Five pairs of near-isogenic lines (8982SF-L and 8982SF-H; 8982TL-L and 8982TL-H; W9262-BC-L and W9260-BC-H; W9261-BG-L and

W9261-BG-H; W9262-339A-L and W9262-339A-H) were studied. The last letter of each line's name indicates whether it is a high cadmium accumulator (H) or a low cadmium accumulator (L) according to Clarke et al. (1997b).

Durum wheat was cultivated following the methods of Archambault et al. (2001) with the following modifications. Seeds were surface-sterilized in 20 % sodium hypochlorite for 20 min, then soaked for 20 h in an aerated solution of 0.005 g L⁻¹ Vitavax (a systemic fungicide; Uniroyal Chemical Ltd., Calgary, Canada). Treated seeds were placed on nylon mesh suspended in aquaria such that the seeds rested at the surface of 5 L aerated nutrient solution (Table 1) adjusted to pH 6.0. The sides of the aquaria were covered in foil to inhibit algal growth. Aquaria were placed in a growth chamber set to 20 °C, 60 % relative humidity and a 16:8 h light:dark cycle (light intensity $168.3 \pm 0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$). For the first 24 h, the aquaria were completely covered with opaque plastic to prevent desiccation of seeds. After 4 days in aquaria, seedlings were transferred to 120 mL jars that were wrapped in foil and filled with 110 mL aerated nutrient solution (pH 6.0) containing the cadmium treatments (0–0.5 μM CdCl₂ depending on the experiment, see below). Five randomly selected seedlings were placed into folded foam (0.5 × 1 × 5 cm) strips and suspended in a slot cut into the plastic lid of each jar such that the root/shoot interface was at the surface of the solution. Each experimental treatment had 3 replicate jars. Plants were provided with fresh solution every 2nd day and harvested on the 8th day of treatment (12 days old). Eight days of treatment are sufficient because differences in aboveground (shoot) cadmium between the high and low lines are established by day 2 (Hart et al. 2006).

Biomass and photochemical efficiency of photosystem II

In this experiment, concentrations of cadmium in the growth solution were 0, 0.1, 0.25 or 0.5 μM . Dry biomass and maximum photochemical efficiency of photosystem (PS) II were used to assess cadmium-induced stress. A few

hours prior to harvest, 3 leaves (from 3 different seedlings) in each jar were dark-adapted for 30 min using light-exclusion clips. The maximum photochemical efficiency of PSII was calculated as the ratio between variable and maximum fluorescence, F_v/F_m (Maxwell and Johnson 2000), which was measured using a chlorophyll fluorometer (Opti-Sciences, OS-30, USA). The mean F_v/F_m for each replicate (i.e., each jar) was recorded. At harvest, roots were separated from the shoots and the tissues were dried to constant weight at 60 °C prior to recording biomass per jar.

Concentrations of cadmium and non-protein thiols

Based on the results of the first experiment, the concentrations of cadmium in the growth solution were chosen to be 0 or 0.1 μM . Higher concentrations of cadmium resulted in 5–40 % reductions in biomass and we wanted to assess the physiology of non-protein thiols in response to mild cadmium stress, not to cadmium toxicity. At harvest, cadmium ions adsorbed to the surface of the plant roots were removed using the method described by Adeniji et al. (2010), which involved soaking the roots for 30 min in 1 mM CaSO₄ followed by a quick rinse in reverse osmosis (RO) water. After blotting dry, approximately half of the root and shoot mass from each jar was dried to constant weight at 60 °C for cadmium analysis and the other half was used immediately for non-protein thiol analysis.

The concentrations of cadmium in root and shoot tissues were determined following the US Environmental Protection Agency test method SW-846 (US EPA 2005) as modified by Adeniji et al. (2010). Dried plant tissues were digested in heated nitric acid (OmniTrace®, EM Science, USA) prior to analysis using an inductively-coupled plasma atomic emission spectrometer (ICP-AES). A standard reference material (NIST #1573a tomato leaves) was used to calculate the efficiency of the digestion procedure ($98.2 \pm 1.5 \%$) and reagent blanks were used to verify the absence of sample contamination (lower detection limit = 0.02 mg L⁻¹). Certified cadmium solutions were used to generate a standard curve and one of the cadmium

Table 1 Composition of the nutrient solution

Macronutrients	Concentration (mM)	Micronutrients	Concentration (μM)
Ca(NO ₃) ₂ ·4H ₂ O	1.0	H ₃ BO ₃	6.0
K ₂ HPO ₄	1.0	MnCl ₂ ·4H ₂ O	2.0
KNO ₃	0.4	ZnSO ₄ ·7H ₂ O	0.5
Mg(NO ₃) ₂ ·6H ₂ O	0.3	CuSO ₄ ·5H ₂ O	0.15
NH ₄ NO ₃	0.3	Na ₂ MoO ₄	0.1
K ₂ SO ₄	0.1		
FeCl ₃ ·6H ₂ O	0.01		
Na ₂ EDTA	0.01

Fig. 1 Separation and identification of non-protein thiols. A mixture of pure standards was derivatized with mBrB then separated using HPLC-fluorescence and the identities of individual components were confirmed using mass spectrometry. The expected mass to charge (m/z) ratios and the mass spectra are indicated beside the corresponding

peaks. The analytical details are in the methods section. *cys* cysteine, *gamma-glu-cys* gamma-glutamyl cysteine, *GSH* glutathione, *NAC* N-acetyl-L-cysteine, *mBrB* monobromobimane, *PC2*, *PC3*, *PC4* phytochelatin 2, 3, and 4, respectively

standards was run after each batch of 20 samples to ensure instrument accuracy.

Extraction of non-protein thiols followed the method of Ebbs et al. (2002) with the following modifications. Fresh tissue was immersed in heated methanesulfonic acid (MSA) to denature enzymes, then homogenized using a Polytron® tissue grinder. Samples were processed in batches of six. After centrifugation at 13,000 rpm and 4 °C for 15 min, the supernatant was passed through a 0.2- μ m syringe filter, collected in a 2-mL Eppendorf tube and kept on ice until derivatized. In a preliminary experiment, the peak areas for replicate samples of individual standards varied ($\pm 10\%$) from day to day; therefore, an internal standard, N-acetyl-L-cysteine (NAC), was used for both analytical standards and plant samples. Derivatization of thiol groups with monobromobimane (mBrB) was performed using the method of Morelli and Scarano (2001), except that a mixture of 100 μ L plant extract and 20 μ L of 50 μ M NAC was derivatized. Samples were stored in the dark at 4 °C for 1–4 days before analysis; preliminary experiments revealed that samples would remain stable for up to 4 weeks if kept in the dark at 4 °C. Reagent blanks were similarly derivatized to identify reagent peaks during analysis.

Separation of non-protein thiols was achieved using high-performance liquid chromatography (HPLC) following the methods of Lavoie et al. (2009) with slight modifications. Analytes were separated with a Nucleosil reversed-phase C-18 column (250 mm $\times$ 4.6 mm, 5 μ m particle size) on a Waters™ 600E HPLC. A 60-min elution protocol used a linear gradient from 0.1 % trifluoroacetic

acid (TFA) in water to 0.1 % TFA in acetonitrile, and a flow rate of 1 mL min⁻¹. The injection volume was 100 μ L. The column was cleaned with 100 % acetonitrile for 5 min and then 0.1 % TFA for 5 min after each sample. Retention times for cysteine (Cys) glutathione (GSH), gamma-glutamyl cysteine (γ -glu-cys) and phytochelatin (*PC*)_{2–4} were verified using pure standards (Sigma-Aldrich, Oakville ON, Canada or Anaspec, Fremont CA, USA). The non-protein thiols were quantified by fluorescence detection (Shimadzu RF-551; excitation at 380 nm and emission at 470 nm) of their mBrB derivatives. The peak area for each non-protein thiol was normalized to the peak area of the NAC internal standard prior to data analysis and the number of thiol groups per molecule was taken into consideration when determining the concentrations of PCs.

The identities of the various non-protein thiols were also confirmed using liquid chromatography (Waters™ Alliance 2690) coupled to an orthogonal time-of-flight mass spectrometer (Waters™ Micromass LCT) following the methods of Taylor et al. (2008), except the gradient was extended to 50 min. Identities of the non-protein thiols were confirmed and peak resolution was sufficient for quantification (Fig. 1).

Data analysis

Two-way analyses of variance (ANOVA) were used to detect effects of cadmium and plant line on plant biomass, F_v/F_m , cadmium concentration, and concentrations of non-protein thiols. Post-hoc Tukey Tests were used to determine significant differences among means ($p < 0.05$) in cases

Fig. 2 Cadmium dose responses for durum wheat. Four-day-old seedlings of five pairs of near isogenic lines were transferred to nutrient solutions containing CdCl_2 and plants were harvested after 8 days of treatment. For clarity, the high and low members of each pair (*left and right panels*, respectively) are plotted in different panels for shoots (*top panels*) and roots (*bottom panels*) but all plant lines were included in each statistical analysis. Also for clarity, *letters* or

symbols denoting pair-wise significant differences have not been included. Overall, shoot dry mass varied among plant lines ($F = 38.5$, $p < 0.001$) and decreased with increasing concentration of cadmium in the growth medium ($F = 41.1$, $p < 0.01$). Root dry mass also varied among plant lines ($F = 12.4$, $p < 0.001$) and decreased with cadmium dose ($F = 43.9$, $p < 0.001$). No interactions between main effects were found for shoots ($p = 0.142$) or roots ($p = 0.108$)

where ANOVA detected significant main effects. Linear regression was used to assess the relationship between cadmium and concentrations of thiols in the tissues. Statistical analyses were done using SigmaPlot version 12.5.

Results and discussion

Cadmium dose responses

Plant mass declined with increasing cadmium in solution. When grown with $0.5 \mu\text{M}$ CdCl_2 , dry weight was 73–83 % of control for shoots (Fig. 2 top panels) and 60–65 % of control for roots (Fig. 2 bottom panels), indicating cadmium-induced toxicity. With the exception of roots of W9261-L and 8982TL-L, biomass of plants grown with $0.1 \mu\text{M}$ CdCl_2 (the concentration chosen for the non-protein thiol experiment) did not differ from those of control roots.

Across cadmium treatments, some lines had greater biomass than others. Specifically, shoots of lines W9261-BG and 8982SF weighed 10–25 % more than did shoots from the other lines in each cadmium treatment (Fig. 2 top panels). Differences among roots were less pronounced; roots of 8982SF-H weighed more than those of the other lines when grown in solutions with 0.25 or $0.5 \mu\text{M}$ CdCl_2 , and roots of W9261-BG-L and 8982SF-(H and L) weighed more under most cadmium treatments (Fig. 2 bottom panels). Shoots of the low lines (Fig. 2 right-side panels) had up to 15 % more biomass than the shoots of the corresponding high lines (Fig. 2 left-side panels). Such differences among lines are not unusual (Harris and Taylor 2013; Tavares et al. 2015) and do not result in different seed yields (Clarke et al. 2002).

No evidence of cadmium-induced reduction in PS II photochemical efficiency was found; F_v/F_m ranged from 0.78 ± 0.04 to 0.82 ± 0.01 with no differences among

Fig. 3 Cadmium concentrations in durum wheat. Four-day-old seedlings of five pairs of plant lines were transferred to nutrient solutions containing 0 or 0.1 μM CdCl_2 and plants were harvested after 8 days of treatment. Plants grown in control solution, **a**, contained trace amounts of Cd. For Cd-treated plants, **b**, different lower case letters indicate differences among means within each tissue (2-way ANOVA followed by Tukey test, $p \leq 0.05$)

plant lines or between cadmium treatments ($F < 2.04$ and $p > 0.41$ in all cases). In healthy plants, F_v/F_m ranges from 0.75 to 0.85 (Maxwell and Johnson 2000). Chlorophyll fluorescence, however, may not be particularly sensitive to cadmium stress. For example, when Ouzounidou et al. (1997) grew bread wheat (*Triticum aestivum* L.) in 1000 μM CdCl_2 , F_v/F_m was 0.76 ± 0.06 —within the range expected of healthy plants—and yet the chloroplast structure had been damaged, as seen by wavy, rather than neatly stacked, thylakoid membranes.

Cadmium and non-protein thiol concentrations

Seedlings grown in control (no added cadmium) solutions had trace concentrations of cadmium on both shoots and roots (Fig. 3a). This likely includes residual cadmium from the seeds. The seeds of the high accumulators among these

five pairs of lines contain up to 0.35 μg cadmium per g seed, depending on the year of harvest (Clarke et al. 2002). Only three of the five pairs of lines had the expected high and low differential translocation of cadmium to the shoots: W9261-BG, 8982SF and 8982TL (Fig. 3b). Inconsistent low and high phenotypes of these lines have also been reported by Adeniji et al. (2010) and Greger and Lofstedt (2004), who also measured cadmium in leaves (rather than in grain) and grew the plants in hydroponic solutions. When the same five pairs of near-isogenic lines were grown in an agricultural field, the high line within each pair consistently had greater concentrations of cadmium in the grain, as compared to the low line, although the magnitude of the difference varied with field location and year (Clarke et al. 2002).

As phytochelatins are being synthesized in response to cadmium treatment, one might expect the concentrations of the precursors (especially glutathione, γ -glutamyl cysteine and cysteine) to decrease, unless there is upregulated synthesis of those molecules in response to the demand. In maize (*Zea mays* L.) grown in solution culture with 3 μM CdSO_4 , the concentration of glutathione decreased in direct proportion to simultaneous increases in phytochelatins 2–4 (Tukendorf and Rauser 1990). In contrast, in mung bean (*Vigna radiata*) concentrations of both glutathione and phytochelatins increased by 30 % and 600 %, respectively, in response to cadmium treatment (Nahar et al. 2016). The concentration of cysteine is less responsive to cadmium than is glutathione. For example, concentrations of cysteine in maize roots did not vary across 0–10 μM CdSO_4 treatments whereas concentrations of glutathione were 25 % less than control for plants grown in 0.1 μM cadmium and 75 % less than control for those grown in 10 μM cadmium (Tukendorf and Rauser 1990). In pea (*Pisum sativum* L.), cysteine did not respond to 20 μM cadmium treatment and glutathione concentrations decreased to about 30 % of control (Rüegsegger et al. 1990).

In our experiment, we saw no consistent response of the precursors of phytochelatins to cadmium although cysteine increased in response to cadmium in the shoots of three of the lines and γ -glutamyl cysteine decreased in response to cadmium in the roots of two of the lines (Fig. 4). There were some significant differences between the high and low lines within pairs of near-isogenic lines (as indicated by asterisks) but these were not necessarily for the pairs of lines that had the expected low versus high phenotype (i.e., lines W9261-BG, 8982SF and 8982TL), nor did they correspond to whether the plants were grown under control conditions (left-side panels) or were treated with cadmium (right-side panels). In addition, when a difference was found, it was sometimes the low line and sometimes the high line that had higher concentrations of these three non-protein thiols. Because there were no consistent patterns

Fig. 4 Concentrations of cysteine, γ -glutamyl cysteine and glutathione in durum wheat. Four-day-old seedlings of five pairs of plant lines were transferred to nutrient solutions containing 0 or 0.1 μM CdCl_2 and plants were harvested after 8 days of treatment. The names of three near isogenic lines have been abbreviated to fit on the *x*-axes

(W9260-BC, W9261-BG and W9262-339A). Concentrations of non-protein thiols were determined by HPLC-fluorescence of derivatized samples. Asterisks indicate significant differences between the low and high members within a pair of near-isogenic lines (based on two-way ANOVA followed by Tukey test, $p \leq 0.05$)

between high and low lines in response to cadmium in our experiment, it seems clear that the high and low phenotype in durum wheat cannot be explained by differential concentrations of cysteine, γ -glutamyl cysteine or glutathione.

Concentrations of phytochelatins increase in plants in response to cadmium, with concentrations usually being higher in roots than in shoots. For example, in pea grown

with 20 μM cadmium, concentrations of total non-protein thiols doubled in shoots and increased tenfold in roots, relative to control plants (Rüeggsegger et al. 1990). In a single cultivar of durum wheat (cv Cresco) grown with 0 to 40 μM CdCl_2 , phytochelatins 2 and 3 in roots increased linearly with cadmium dose but were below detection limits in the shoots (Paradiso et al. 2008). Stolt et al. (2003)

Fig. 5 Concentrations of phytochelatins in durum wheat. Four-day-old seedlings of five pairs of plant lines were transferred to nutrient solutions containing 0 or 0.1 μM CdCl_2 and plants were harvested after 8 days of treatment. The names of three near isogenic lines have been abbreviated to fit on the x-axes (W9260-BC, W9261-BG and

W9262-339A). Concentrations of non-protein thiols were determined by HPLC-fluorescence of derivatized samples. Asterisks indicate significant differences between the low and high members within a pair of near-isogenic lines (based on two-way ANOVA followed by Tukey test, $p \leq 0.05$)

similarly reported increased phytochelatins in the roots of two cultivars of durum wheat (Topdur and Grandur) in response to increased cadmium, but no detectable phytochelatins in the shoots. Phytochelatins 2, 3 and 4 were found in control and cadmium-treated seedlings in our experiment, and were up to five times higher in roots and

shoots of cadmium-treated plants than in control plants (Fig. 5). This is in contrast with Stolt et al. (2003) for three reasons. In their experiment, two lines of spring wheat (*T. aestivum* L.) and two lines of durum wheat were grown in solution with 1–30 μM CdCl_2 . Concentrations of total PCs in their roots increased up to fourfold as cadmium

Fig. 6 Correlations between non-protein thiols and cadmium in shoots of durum wheat. Four-day-old seedlings of five pairs of plant lines were transferred to nutrient solutions containing 0 or 0.1 μM CdCl_2 and plants were harvested after 8 days of treatment. The names of three near isogenic lines have been abbreviated to fit on the graph (W9260-BC, W9261-BG and W9262-339A). Concentrations of non-protein thiols were determined by HPLC-fluorescence of derivatized samples. Lines indicate significant correlations between the two variables (linear regression, $p \leq 0.05$)

increased but (1) they did not detect phytochelatin in control roots, (2) phytochelatin 4 was not detected in roots grown with less than 30 μM , and (3) phytochelatin 3 was not detected in leaves of plants grown with less than 30 μM cadmium. These differences might be explained by the background cadmium present in our seed stock. Stolt et al. (2003) did not report whether cadmium was above detection limit in the control plants from their study, but if it was negligible then one would not expect to find phytochelatin in their control plants, and a higher dose of cadmium might have been required to bring concentrations of longer-chained phytochelatin above detection limits. Our results are also slightly different from those of Paradiso et al. (2008). They studied one cultivar of durum wheat (Creso)

for which control roots contained $43 \pm 6 \mu\text{g g}^{-1}$ cadmium (10 times higher than the plants in our study) and yet no phytochelatin was detected. There appear to be cultivar- and species-specific relationships between the amount of cadmium required to stimulate phytochelatin synthesis and accumulation. For another example, in maize roots grown in 0–10 μM CdSO_4 phytochelatin 3 was not detected in roots grown at concentrations lower than 0.05 μM cadmium, and phytochelatin 4 was found only in roots grown in at least 0.5 μM cadmium (Tukendorf and Rauser 1990).

Although we detected increased phytochelatin upon exposure to cadmium, the responses among the high and low lines were inconsistent. Either no difference was found between the high and low lines within a pair, or the line

Fig. 7 Correlations between non-protein thiols and cadmium in roots of durum wheat. Four-day-old seedlings of five pairs of plant lines were transferred to nutrient solutions containing 0 or 0.1 μM CdCl_2 and plants were harvested after 8 days of treatment. The names of three near isogenic lines have been abbreviated to fit on the graph (W9260-BC, W9261-BG and W9262-339A). Concentrations of non-protein thiols were determined by HPLC-fluorescence of derivatized samples. Lines indicate significant correlations between the two variables (linear regression, $p \leq 0.05$)

with the higher concentration of phytochelatin was sometimes a low line and sometimes a high line. Hart et al. (2006) also reported increased phytochelatin in response to cadmium in durum wheat and suggested that, while cadmium-phytochelatin complexes appear to play a role in cadmium detoxification, they do not seem to play a role in differential translocation from root to shoot.

Because the concentrations of cadmium in shoots or roots of the five pairs of lines were not similar, despite being grown in the same concentration of cadmium in the nutrient solution, the relationship between concentrations of non-protein thiols and cadmium in shoots (Fig. 6) and roots (Fig. 7) was explored. If non-protein thiols accumulate in response to the absolute amount of cadmium taken up by a

plant, one would expect a positive correlation between these variables, especially in the three pairs of lines in which the low/high phenotype was seen (i.e., W9261-BG, 8982Sf and 8982TL). The patterns in shoots of line W9261-BG are closest to the predicted responses; concentrations of the precursors to phytochelatin declined with increased cadmium, and concentrations of phytochelatin 2 and 4 increased (Fig. 6). In each of 8982SF and 8982TL, however, concentrations of the precursor molecules increased in response to cadmium and either phytochelatin did not vary (8982SF) or declined (8982TL) as cadmium content increased. The lack of phytochelatin response in 8982SF might be a result of the narrow range of concentrations of cadmium found in the leaves of this line.

Only three correlations between non-protein thiol and cadmium concentrations were found in roots, and none of them were in pairs of lines that showed the low-high cadmium phenotype (Fig. 7). This may be because the ranges of cadmium concentrations in these lines were very narrow relative to the broad ranges in concentrations of phytochelatin.

Conclusions

Although a number of researchers have studied the same near-isogenic lines of durum wheat, the physiological explanation for differential aboveground accumulation of cadmium remains unclear. None of volume of water transpired (Quinn et al. 2011), influx of cadmium into roots (Harris and Taylor 2004), accumulation of low molecular weight organic acids (Adeniji et al. 2010) or phytochelatin (Hart et al. 2006; Stolt et al. 2003; this study) are solely responsible for the patterns of cadmium accumulation in near-isogenic lines of durum wheat, although all of the above variables certainly play roles in cadmium uptake and tolerance in all plants including durum wheat. It is possible that differences in sub-cellular (especially vacuolar) concentrations of organic acids and phytochelatin do differ between root cells of high and low lines of durum wheat, or that the cytosolic phytochelatin of the low lines are more efficient at shuttling cadmium to the root cell vacuoles. Analyses of bulk root tissues might not be capable of detecting such small scale differences. It is possible that the *Cdu1* gene encodes a tonoplast transporter, as has also been proposed by many (Adeniji et al. 2010; Tavares et al. 2015; Perrier et al. 2016; Harris and Taylor 2004), but the complexes with which cadmium is stored in the root cell vacuoles of the low lines remain unknown.

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