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A rapid, seedling-based bioassay for identifying low cadmium-accumulating individuals of Durum wheat (*Triticum turgidum* L.)

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Summary

We have developed a seedling-based bioassay that is capable of identifying low Cd-accumulating phenotypes (homozygous and heterozygous) after 96–120 h of exposure. Our experiments were conducted using ^{109}Cd as a tracer at subtoxic concentrations to simulate conditions that might be experienced in the field. Supply of Cd (10^{-11} M) to 4-d old seedlings for 0–8 h resulted in no differences in root and leaf Cd content between the low (TL05) and high (TL04) Cd-accumulating isolines. Increasing time of exposure produced significant differences in leaf Cd accumulation between isolines, with differences becoming most pronounced after the bulk of applied cadmium (∼95%) was depleted from absorption solutions (≥ 72 h). Similar results were obtained with 8-d old seedlings, where differences between genotypes were more pronounced in young leaves (2nd leaf) or shoot bases. Individuals from five low and high Cd-accumulating near isogenic pairs (50 individuals/isoline) were screened using Cd concentration of shoot bases as the screening criterion. Mean scores within each isoline pair were significantly different, although overlap of individual scores was observed at intermediate foliar Cd concentrations. The 2nd leaf to root Cd content ratio, which reflects root to shoot translocation, provided a better parameter to distinguish low from high Cd-accumulating isolines. Plants used for this bioassay could also be rescued for subsequent experimental crosses, providing a rapid and cost-effective tool for early detection of the low Cd-accumulating phenotype.

Abbreviations: Cd –, Cadmium; EDDHA, Ethylenediamine – Di(o-hydroxyphenylacetic acid)

Cadmium (Cd) is rapidly emerging as one of the most important environmental contaminants of our time. Environmental contamination arises from several activities, including extraction and production of Cd from ore, use in industrial processing, and fossil fuel combustion. In addition, Cd has become a widespread pollutant in agricultural soils through the use of sewage sludge amendments (Chaney, 1980) and phosphate fertilizers (Ryan et al., 1982). Cadmium is readily taken up by plant roots and translocated to above-ground tissues, where it becomes available for human consumption.

Scientific and political attention has focused on risks associated with elevated levels of Cd in the environment. The World Health Organization (Codex Alimentarius) is currently considering a new guideline for Cd content of marketed grain (0.1–0.2 mg Cd kg^{-1}). If adopted, this guideline would put pressure on Canadian producers to reduce Cd levels in Durum wheat grain. To address this problem, Agri-

culture and Agri-Food Canada recently developed five pairs of near-isogenic Durum wheat genotypes. These isoline pairs, each of which includes a low and a high Cd-accumulating genotype, provide a resource for breeders who are working to develop means of reducing grain-Cd levels and subsequent human exposure to Cd. In field trials, Cd levels in grain from low Cd-accumulating lines averaged 60% lower than levels in high Cd-accumulating lines (Clarke et al., 1997b). Incorporation of the low Cd-accumulating trait into locally adapted wheats is hampered by the lack of short-term, seedling-based bioassay for identifying single, low Cd-accumulating phenotypes in segregating populations. Segregating populations have to be grown to maturity to identify low accumulating phenotypes, an effort that places a significant burden on breeding programs. Penner et al. (1995) successfully identified a RAPD marker which maps within 4.6 cM of the Cd accumulation loci. Unfortunately, this genetic marker indicates the presence of the recessive, high Cd-accumulating allele. Thus, it cannot distinguish homozygous, high Cd-accumulating genotypes from heterozygous, low Cd-accumulating genotypes.

The objective of this study was to develop a rapid, seedling-based bioassay for identifying single, low Cd-accumulating phenotypes (homozygous and heterozygous).

Materials and methods

Plant material

The majority of experiments reported here made use of a pair of near-isogenic lines of *Triticum turgidum* that differ in Cd accumulation in mature grain (Clarke et al. 1997b). Isoline TL04 accumulates roughly 2-fold more Cd in grain than isoline TL05. Seeds of each isoline were surface sterilized in 1.2% sodium hypochlorite for 20 min and imbibed overnight in a solution of 0.005 g/L Vitavax to limit fungal growth. Germinated seeds were placed on nylon mesh suspended over 15 L of aerated nutrient solution(pH 6.0), containing 0.4 mM KNO_3, 1.0 mM $Ca(NO_3)_2$, 0.3 mM NH_4NO_3, 0.3 mM $Mg(NO_3)$, 0.16 mM K_2HPO_4, 0.1 mM K_2SO4, 2.0 μM $MnCl_2$, 6.0 μM H_3BO_3, 0.5 μM $ZnSO_4$, 0.15 μM $CuSO_4$, and 0.10 μM Na_2MoO_4. Iron was supplied as Fe(III)EDDHA at a concentration of 11 μM with a 3 μM excess of $FeCl_3$ (EDDHA has a low affinity for Cd). In all experiments, plants were grown in a controlled environment with a 16 h/20 °C day and 8 h/15 °C night. Light was provided from a mixture of fluorescent tubes and incandescent bulbs. Photosynthetically active radiation was 500 μmol photons $m^{-2}s^{-1}$ at the top of mature plants and 350 μmol photons $m^{-2}s^{-1}$ at the level of the lowest leaf.

Dose response of isolines TL04 and TL05 to Cd in nutrient solution

Ten-d old seedlings of isolines TL04 and TL05 were transferred to plastic buckets (8 plants from a single isoline in each bucket) containing 15 L of aerated nutrient solution (pH 6.0) supplemented with 0–100 μM Cd as $CdCl_2$. At the end of the experiment (16 days), roots were rinsed three times with distilled water and plants were divided into shoots and roots, dried in an oven at 65 °C, and weighed.

Short- and long-term ^{109}Cd labeling of seedlings

Four-d old seedlings of isolines TL04 and TL05 were transferred to 20 mL plastic scintillation vials (1 seedling per vial) containing 10 mL of nutrient solution (pH 6.0) and 4.0×10^{-11}M $^{109}CdCl_2$(9.5 Bq mL^{-1}). Three replicate vials were harvested after 0, 1, 2, 3, 6 and 8 h (short-term) or 6, 12, 24, 48, 72, and 96 h (long-term). Roots were rinsed in 10 mL of deionized, distilled water to remove surface ^{109}Cd. Roots and shoots were harvested and dried for determination of total ^{109}Cd. Spent exposure solutions and water rinses were collected to determine the time-course of ^{109}Cd depletion from solution and verify complete recovery of added ^{109}Cd.

Determination of ^{109}Cd activity in plant materials and solutions

All ^{109}Cd radioactivity determinations were performed on dried plant material or solutions evaporated to dryness using either a Beckman 4000 or 8000 γ-counter. Data are reported as a percentage of total Cd activity supplied in exposure solutions per unit mass of plant material or as mass of Cd per unit mass of plant material.

Spatial variation in accumulation of ^{109}Cd in leaves and shoot bases

Eight-d old seedlings of isolines TL04 and TL05 were exposed to ^{109}Cd for 120 h using the techniques described above. Above-ground plant material was harvested and separated into 5 segments as follows: 1)

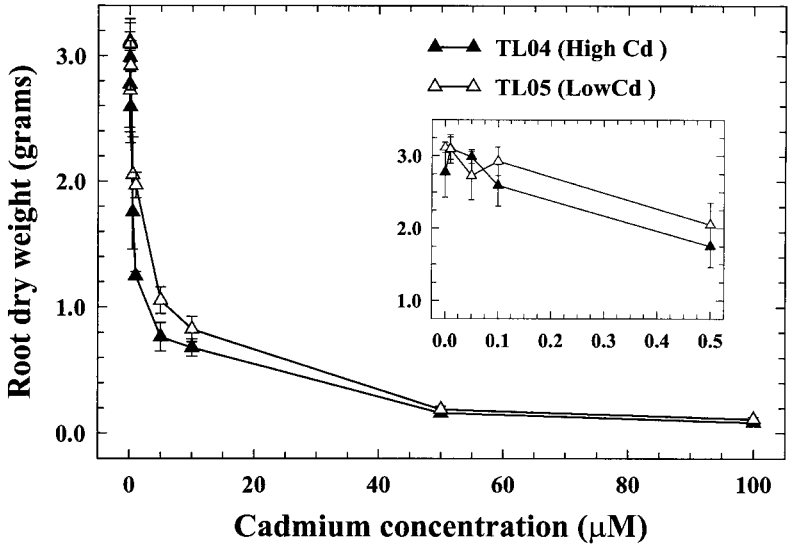

Figure 1. Dose response of isolines TL04 and TL05 of Durum wheat to $CdCl_2$ in nutrient solution. Ten-d old plants were grown for 16 days in nutrient solutions containing 0–100 μM Cd with no further Cd additions throughout the experimental period. Data from low Cd treatments ($\leq$ 0.5 μM Cd) are presented as an inset. Values are means $\pm$ S.E. (n = 3).

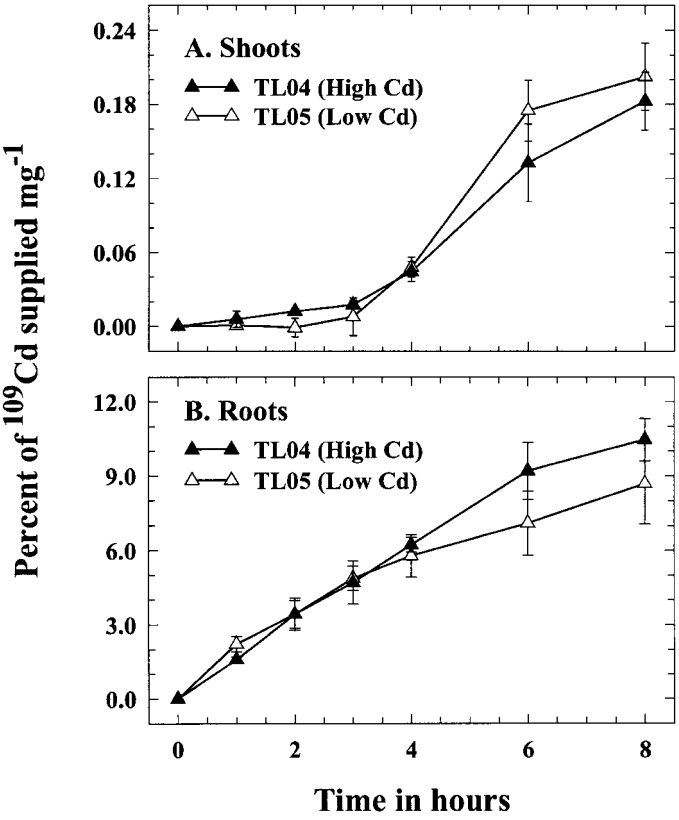

Figure 2. Cadmium distribution in shoots (A) and roots (B) of high and low Cd-accumulating isolines of Durum wheat over an 8 h period. ^{109}Cd activity is expressed as a percentage of the total radioactivity supplied to the 4-d old seedlings per mg DW of tissue. Values are means $\pm$ S.E. (n = 3).

the tip (3 cm) of the first leaf, 2) the base (2–3 cm) of the first leaf, 3) and 4) corresponding segments of the second leaf and, 5) the remaining shoot base (2–3 cm, including the stem and remaining leaf bases). Samples were prepared and analyzed for ^{109}Cd.

Testing the bioassay

Experiments were conducted to test the efficacy of using Cd accumulation in shoot bases or a leaf (2^{nd}) to root ratio for discriminating between high and low Cd-accumulating individuals. Eight-d old seedlings of each of 5 isoline pairs (high/low Cd), TL04/TL05, SF08/SF07, BG01/BG02, BG05/BG04, and A4/A2 (Clarke et al., 1997a) were exposed to 4.0×10^{-11}M $^{109}CdCl_2$ (5.5 Bq mL^{-1}; pH 6.0) for 120 h using the techniques described above. An additional 5 mL of Cd-free nutrient solution was added to each vial after 72 h of exposure to provide additional nutrients and ensure roots were completely submerged. The 2^{nd} leaf or shoot bases (including the stem and all leaf bases) and roots were harvested following exposure and analyzed for ^{109}Cd. Data were expressed as percent of total ^{109}Cd supplied per unit mass of plant material and as frequency of occurrences for tissue concentration classes or leaf/root ratios to visualize the separation achieved between high and low Cd-accumulating lines.

Results and discussion

Dose response experiments showed that isolines TL04 and TL05 exhibited similar dose-dependent reductions in growth when challenged with solutions of Cd ranging from 0 to 1×10^{-4} M (Figure 1). Root growth of both isolines was inhibited by more than 30% at 5×10^{-7}M Cd (Figure 1, inset). Complete inhibition of growth was observed at 5×10^{-5} M Cd and higher. Since our objective was to detect genotypic differences at sub-toxic levels, these results dictated a maximal Cd concentration of 1×10^{-7}M for further experiments.

The first step in developing a short-term bioassay was to determine the time course of accumulation in roots and shoots of seedlings. Short-term accumulation of ^{109}Cd into roots of isolines TL04 and TL05 was initially linear with time, but rates of accumulation began to decline after 4–6 h of exposure (Figure 2B). Cadmium accumulation in shoots began after 3 h, after which time the rate of accumulation increased to a maximum between 4 and 6 h of exposure (Figure 2A). Cadmium levels in roots were approximately

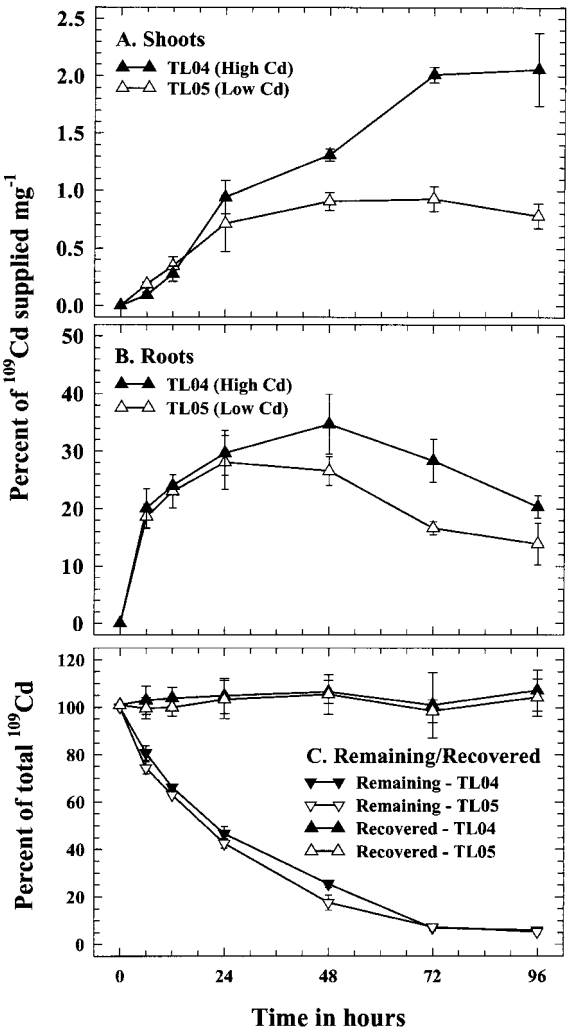

Figure 3. Cadmium concentrations in shoots (A) and roots (B) of high (TL04) and low (TL05) Cd-accumulating isolines of Durum wheat over a 96 h period. ^{109}Cd activity is expressed as a percentage of the total radioactivity supplied to the 8-d old seedlings per mg DW of tissue. Depletion of Cd in labeling solutions and total recovery of added Cd over a 96-h period are reported as percentage of total ^{109}Cd radioactivity supplied (C). Values are means ± S.E. (n = 4).

40 times greater than in shoots at the end of the 8h exposure period. No differences between isolines were observed.

In long-term experiments (96 h), the rate of Cd accumulation in roots was initially rapid (24 h) before declining to near zero thereafter. Thus, concentrations of Cd in roots showed a net decline after 48 h (Figure 3B), reflecting depletion of Cd from exposure solutions, continued root growth causing dilution of

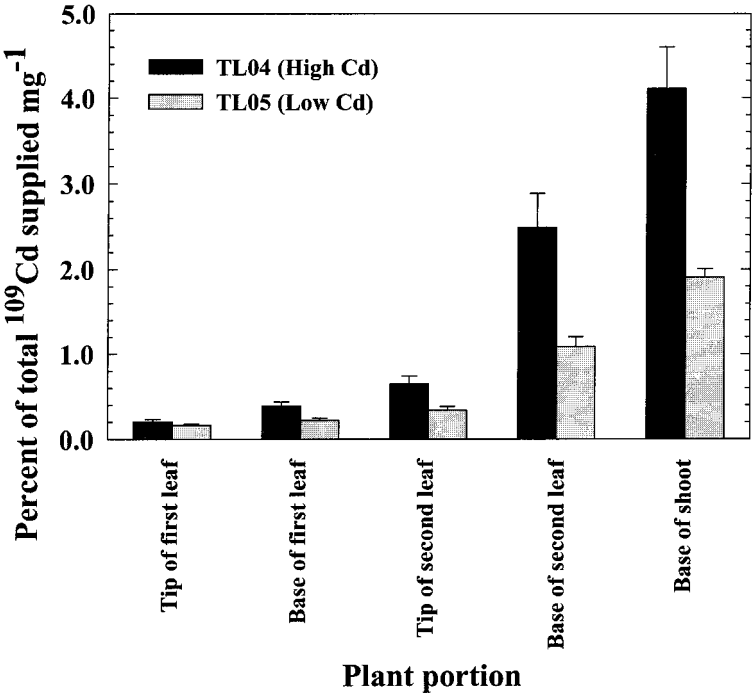

Figure 4. Cadmium concentrations in 1st leaf tips, 1st leaf bases, 2nd leaf tips 2nd leaf bases, and remaining shoot bases of 8-d old seedlings of high (TL04) and the low (TL05) Cd-accumulating isolines of Durum wheat. Concentrations are expressed as a percentage of ^{109}Cd supplied per mg DW. Values are means ± S.E. (n = 4).

Cd, and translocation of Cd to the shoot. Differences between isolines in Cd accumulation in shoots began to appear after 24 h of exposure and intensified over the remainder of the experimental period (Figure 3A). Differences were greater when data were expressed on a tissue mass basis (% total Cd supplied mg^{-1} dry weight) than on the basis of total activity (% total Cd supplied, data not shown), possibly due to the fact that isoline TL05 exhibited a greater growth rate than isoline TL04. The time-course of Cd depletion from exposure solutions was characterized by a rapid rate of depletion over the first 12–24 h of exposure, with the rate diminishing over the remainder of the exposure period (Figure 3C). Less than 6% of total ^{109}Cd supplied remained in solution by the end of the experiment (96 h). These results suggested that the depletion of Cd from solutions might be an important issue affecting our ability to separate isolines. Complete recovery of added Cd was achieved in all our experiments (see for example, Figure 3C). Often more than 95% of total Cd supply was recovered from plant tissues when plants were exposed for 96h or more, suggesting that most of the Cd supplied was available to the plants.

To determine whether spatial variation in Cd accumulation might allow for improved separation of low and high Cd-accumulating isolines, different leaf portions were harvested separately after 120 h of exposure. Greatest accumulation of ^{109}Cd occurred in the shoot base compared to leaf tips and leaf bases. In fact, accumulation in the shoot base was up to 40 times greater than in leaf tips (Figure 4). Differences between isolines in Cd accumulation in the tip of the second leaf, the base of the second leaf, and shoot base were similar, with concentrations in TL04 1.3 to 2.3 fold higher than in TL05.

In all our experiments, depletion of ^{109}Cd from solution occurred rapidly, although the time course of depletion varied with the initial size of plants. In experiments with 8-d old plants, more that 80% of total Cd was typically removed from solution within 24h (data from smaller, 4-d old plants are provided in Figure 3C). Thus, we examined the possibility of providing additional Cd to plants to reduce the extent of depletion and perhaps increase separation between isolines. When Cd concentrations were increased from 4×10^{-11}M to 4×10^{-7} M using unlabeled Cd, no improvement in separation between isolines was

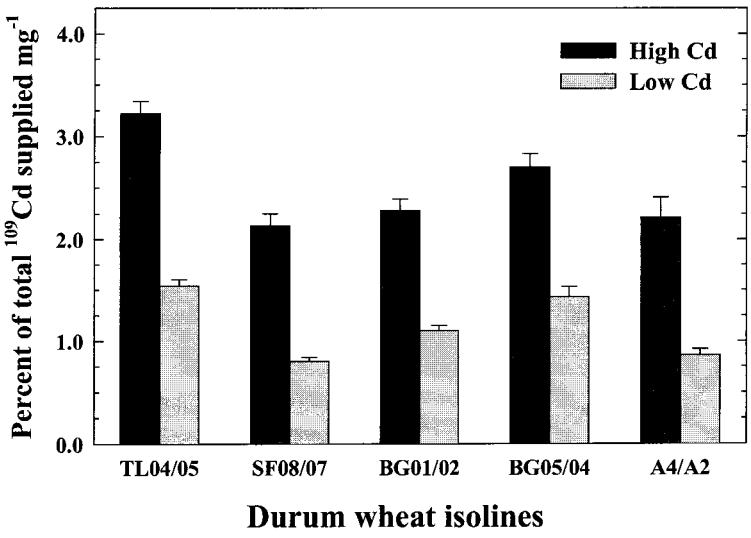

Figure 5. ^{109}Cd activity in shoot bases from five pairs of near-isogenic isolines of Durum wheat. Concentrations are expressed as a percentage of ^{109}Cd supplied per mg DW Values are means ± S.E. (n = 50).

observed (data not shown). At 4×10^{-5} M Cd, depletion was reduced to less than 20% after 96 h, but differences between isolines were abolished (data not shown). Under these conditions, symptoms of severe Cd toxicity were observed and plants ceased growth. Thus, increasing Cd concentration in exposure solutions was not effective in further separating isolines.

Our experiments suggest that the optimal, short-term protocol for separating high and low Cd-accumulating lines should involve exposing seedlings to low Cd concentrations (4×10^{-11} M) for 96–120 h, after which the second leaf or shoot base is harvested for analysis of ^{109}Cd content. We used these protocols to screen 50 individuals of each of 5 isoline pairs (high/low Cd-accumulating). In this experiment, high Cd-accumulating isolines accumulated 2 to 2.5-fold more Cd in shoot bases than their low Cd-accumulating counterparts (Figure 5). However, examination of Cd accumulation within individuals from each isoline pair showed some overlap between low and high Cd individuals (Figure 6A, 6B). While improved separation of individuals within populations would be of value, this protocol provides an effective bioassay for identifying extreme phenotypes.

Analysis of ^{109}Cd content in roots and shoots of high and low Cd-accumulating lines suggested that translocation of Cd from roots to shoots is an important factor affecting accumulation in aerial tissues. Thus, we explored the use of a leaf/root Cd content ratio for separating high and low phenotypes. Use of 2nd leaf/root ratios (Figure 6C, 6D) reduced the extent of overlap between high and low Cd-accumulating individuals within each isoline pair, providing for improved separation with isoline pairs. However, use of the ratio also introduced a new source of variation that was specific to each isoline pair. Thus, while separation between isolines within a pair improved, increased overlap was observed when comparing isolines across pairs. In fact, means for BC04 (low Cd; ratio = 0.110 ± 0.004) and TL04 (high Cd; ratio = 0.108 ± 0.004) were virtually identical (Figure 6C and D).

Incorporating the low Cd-accumulating trait into locally adapted wheats using a backcross approach is hampered by the lack of a short-term, seedling-based bioassay for identifying single, low Cd-accumulating phenotypes. Currently, segregating populations would have to be grown to maturity to identify low accumulating phenotypes, an effort which would place a significant burden on any breeding program. A RAPD marker that maps within 4.6 cM of the Cd accumulation loci has successfully been identified at Agriculture and Agri-Food Canada, Winnipeg (Penner et al., 1995). Unfortunately, this genetic marker indicates the presence of the recessive, high Cd-accumulating allele. Thus, it cannot distinguish homozygous, high Cd-accumulating genotypes from heterozygous, low Cd-accumulating genotypes (the desired genetic product of each backcross).

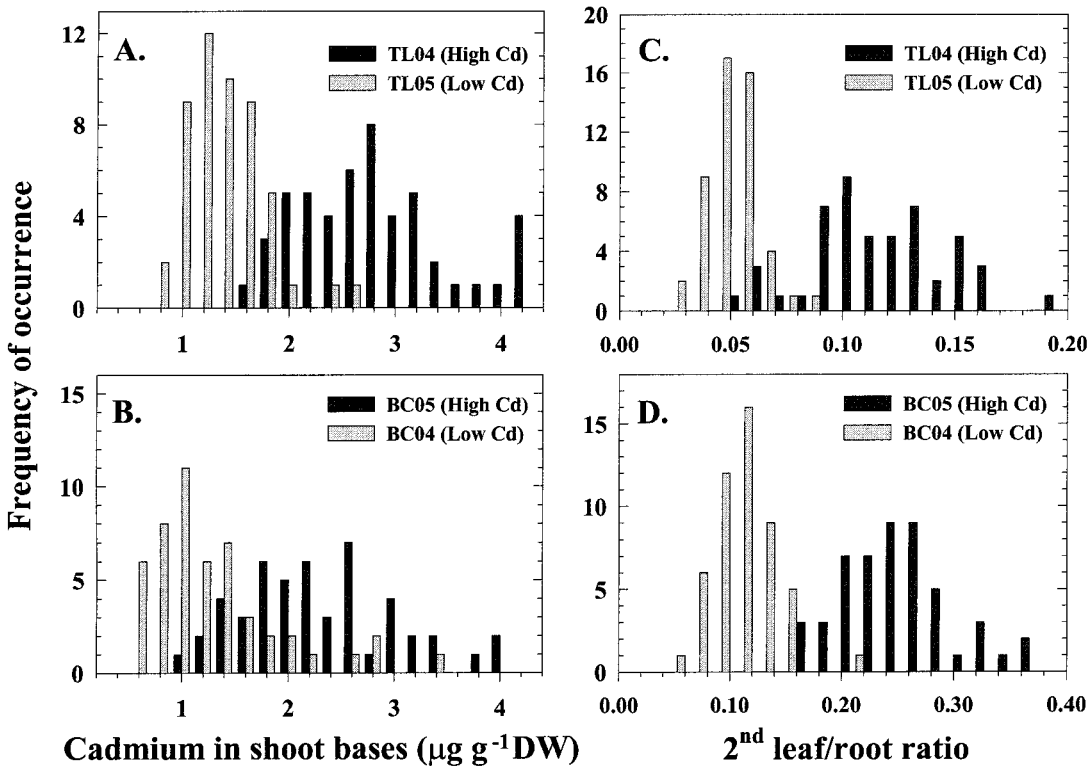

Figure 6. Frequency of occurrence of the shoot base Cd concentration (μg Cd g^{-1} DW) and the Cd accumulation index (2^{nd} leaf to root Cd content ratio) in two pairs of near-isogenic isolines of Durum wheat. Data are from single individuals from a group of 50 seedlings for each isoline. Note differences in the range of the x-axis scaling between panels for the 2^{nd} leaf to root Cd content ratio.

The seedling-based bioassay that we have developed provides a rapid, easy and cost-effective way of screening large numbers of seedlings for the low Cd-accumulating phenotype. The technique should be suitable for characterizing individuals within a single cross (2nd leaf/root ratio) or identifying extreme phenotypes in a broader range of genetic material (Cd content in leaf bases). The ^{109}Cd isotope allows use of short-term exposures and environmentally-relevant exposure conditions (low Cd concentrations). Hundreds of plants can be screened on a daily basis, for \$0.05 to \$0.50 per assay (costs vary with use of disposable items, but exclude personnel costs). Thus, the bioassay provides an important tool for physiological studies and can be used to accelerate breeding programs that require selection of desirable individuals or information about the behaviour of breeding populations (segregating vs. non-segregating). This would eliminate the need to grow large numbers of plants to maturity under field conditions and to subsequently determine the Cd content of grains. We are currently testing the bioassay on F3 populations produced from high/low Cd isoline pairs to verify the ability of the assay to predict accumulation of Cd in grains under field conditions.

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