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Isolation and partial characterization of a lead-accumulating *Brassica juncea* mutant

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Abstract A new screening method for non-destructive, high-sensitivity, high-throughput isolation of plant mutants capable of accumulating large amounts of heavy metals has been developed. This method is based on incubating seedlings in a solution containing radioisotopes of the metals of interest and visualizing the tissue accumulation of these metals with a phosphorimager. We used this technique to isolate mutants of *Brassica juncea* (L.) Czern with increased accumulation of Cd and Pb for use in phytoremediation, an emerging technology using plants to remediate polluted soil and water. Approximately 50,000 M2 seedlings were screened and 21 mutants were recovered that retained increased accumulation through the third generation. Mutant 7/15–1 is characterized by enhanced Pb accumulation per unit of root fresh weight, stunted root growth, and decreased root cell size. Data indicate that roots of 7/15–1 contain more cell-wall material on a fresh-weight basis than roots of the wild-type, which may at least partially explain its ability to accumulate more Pb.

Key words *Brassica juncea* · Screening · Mutants · Cadmium · Lead

Introduction

Phytoremediation, or the use of plants to remove toxic metals and other pollutants from soil, water and air, has received substantial attention in recent years (Salt et al. 1995; Cunningham and Ow 1996; Chaney et al. 1997; Raskin et al. 1997; Salt et al. 1998). Rhizofiltration, a subset of phytoremediation, refers to the remediation of polluted water in which hydroponically cultivated plant roots are used to absorb, concentrate, and precipitate toxic metals (Raskin et al. 1994; Salt et al. 1995). Dushenkov et al. (1995) first demonstrated the ability of roots of hydroponically cultivated *Brassica juncea* to absorb toxic metals from aqueous solutions effectively. Roots of this plant concentrated Cd and Pb 134- and 563-fold, respectively, above initial solution concentrations. In addition to being suitable for rhizofiltration, *B. juncea* can translocate Pb and other heavy metals from aqueous medium (Kumar et al. 1995) or soils (Blaylock et al. 1997) to shoots, making it the plant of choice for the remediation of contaminated soil (Salt et al. 1998).

While developing rhizofiltration, it was observed that etiolated seedlings of *B. juncea* grown in aerated water were even more effective at removing toxic metals from water than roots of older plants (Salt et al. 1997). The seedlings concentrated Cd and Pb 500- and 1250-fold over solution concentrations, respectively. This phenomenon is based on the increased ion absorption and adsorption properties of seedlings due to a large surface-to-volume ratio and an exceedingly high surface ion-exchange capacity (Raskin et al. 1997). In the case of Cd, biological uptake also plays an important role in metal removal from the solution. Cd taken up by the root cells is bound to phytochelatins and possibly organic acids (Salt et al. 1997).

Little is known about the cellular mechanisms of Pb accumulation in plants or seedlings. In older plants, surface adsorption and precipitation may be the dominant mechanisms of Pb uptake. Desorption studies in barley roots exposed to Pb showed that the metal binds reversibly to anionic binding sites (Broyer et al. 1972). These sites include the carboxyl groups of polygalacturonic acid (Salt, unpublished data) and negatively charged pectin sites normally occupied by calcium ions (Lane et al. 1978).

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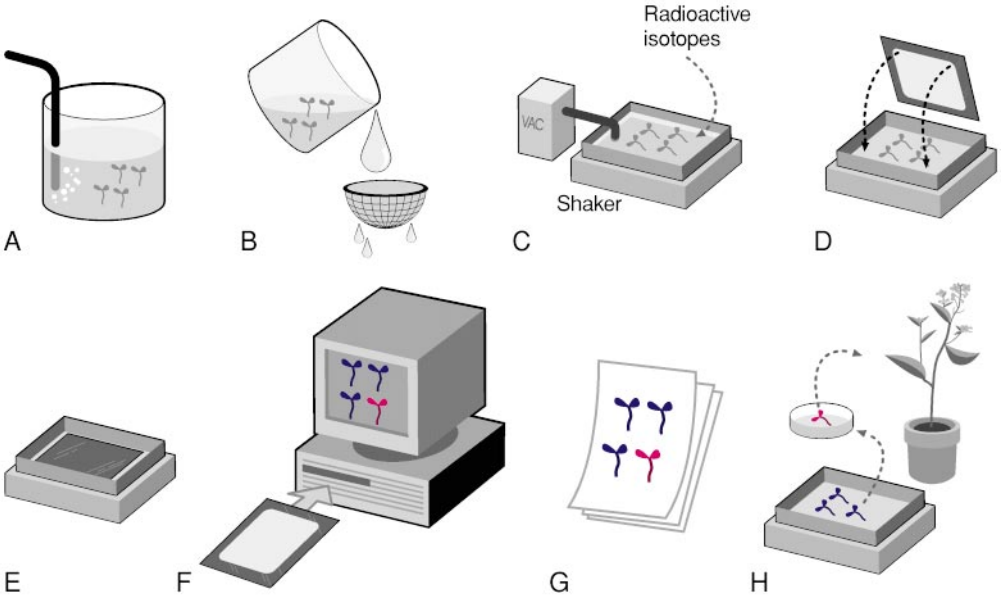

Fig. 1A–H Schematic representation of the metal accumulation screen. **A** Three hundred 3-day old seedlings were grown in the dark at 23°C completely submerged in aerated nutrient solution [0.7 mM $Ca(NO_3)_2$, 0.5 mM $Mg(NO_3)_2$, 1.5 mM KNO_3]. **B, C** At 5-days old, seedlings were transferred from the growth container to a 26.5×38×8 cm polyethylene tray with 1 l of fresh nutrient solution containing 0.16 µM of Cd traced with ^{109}Cd (1.2 µCi/l), and 0.12 µM of Pb traced with ^{210}Pb (0.34 µCi/l). The trays are placed on an orbital shaker at 25 rpm in the dark at room temperature. **D** After 5 h of incubation, the solution was removed from each tray using vacuum aspiration. Seedlings are re-arranged in the trays to prevent overlap. They are sprayed with tap water and covered with a film of plastic wrap to keep them hydrated and to prevent contamination of the phosphorimager screen. **E** A pre-erased screen was placed face-down on the seedlings in each tray and exposed in the dark for 30 min. **F** The screen was analyzed in a phosphorimager using IPLabGel software. **G** A life-sized color hard copy of the image was generated and used to pick out the seedlings with high accumulation. **H** High accumulators were rescued and grown for seed

Plants with genetically enhanced heavy metal uptake would be very useful as tools for understanding the biological mechanisms of metal uptake and as superior cultivars for phytoremediation applications. With this in mind, we developed a system for rapid and accurate screening of *B. juncea* mutants with an enhanced accumulation of heavy metals. Previously reported screening systems for isolating mutants with altered responses to heavy metals were based on the tolerance or sensitivity of plants to particular metals (Howden and Cobbett 1992; Murphy and Taiz 1995). The high-throughput method we have developed allows:(1) accurate quantification of metal accumulation, (2) determination of the spatial and temporal distribution of metal in a plant and in plant parts, and (3) screening for the accumulation of multiple metals simultaneously. We have used it to isolate several *B. juncea* mutants with increased Pb accumulation at the seedling stage. We have also partially characterized one of the isolated mutants.

Materials and methods

Plant material

Seeds of a true cultivar of Indian mustard, (*Brassica juncea* (L.) Czern, cv 426308) were obtained from the USDA/ARS Plant Introduction Station of Iowa State University. Seeds were mutagenized by presoaking in 0.1 M phosphate buffer for 3 h, followed by 6 h of soaking in 266 µM of ethylmethane sulfonate in 0.1 M phosphate buffer (0.1 g of seed per ml of solution) mixed continuously on a shaker, rinsing for 2 h in tap water and dried overnight. Plants were allowed to self, and the M2 generation was used to screen for mutants. The wild-type seeds used in the experiments were taken from the same seed stock employed for mutagenesis.

Growth Conditions

For all experiments, 1 g of seeds was germinated and grown in the dark in 6-l plastic containers at 23° C completely submerged 1 L of aerated nutrient solution [0.7 mM $Ca(NO_3)_2$, 0.5 mM $Mg(NO_3)_2$, 1.5 mM KNO_3]. Aeration was provided by a glass pipette connected to an aquarium pump. For screening, seeds were surface-sterilized by soaking in 1.3% sodium hypochlorite solution for 15 min, washed, and allowed to imbibe nutrient solution at 4° C (24–48 h) to promote uniform germination.

Screening for Mutants with Enhanced Cd and Pb Accumulation

Primary Screen

For the primary screen, batches of approximately 300 3-day old seedlings were transferred from the growth container to 26.5×38×8 cm polyethylene trays (Fisher, catalog No. 15–236–1A) using a #10 standard sieve to remove seed coats and nutrient solution. Each tray was filled with 1 l fresh nutrient solution (described above) containing 0.16 µM of Cd with ^{109}Cd as a tracer (1.2 µCi/l), and 0.12 µM of Pb with ^{210}Pb as a tracer (0.34 µCi/l). The trays were placed on an orbital shaker at 25 rpm in the dark at room temperature. After 5 h of incubation, the solution was removed by vacuum aspiration (Fig. 1A,B,C). Samples of the incubation solution taken after 5 h showed that Cd was not depleted whereas Pb was depleted by approximately 20%. Seedlings were re-arranged in the trays to ensure that they did not overlap, lightly sprayed with tap water, and covered with a film of plastic wrap to keep

them hydrated and to prevent contamination (Fig. 1D). A pre-erased screen (20×25 cm storage phosphor screen, Molecular Dynamics, Sunnyvale, Calif.) was placed face-down on the seedlings in each container and exposed in the dark for 30 min (Fig. 1E). The screen was then analyzed in a phosphorimager (Molecular Dynamics, Model SI, Sunnyvale, Calif.) using IPLabGel software (Signal Analytics Corporation, Version 1.5, Vienna, Va.) to produce an image on the computer. Controls were adjusted to colorize the image such that increasing radioactivity in the seedling was represented by a color continuum where yellow > pink > blue. The concentration of radioactive metals used in the incubation solution was chosen so that seedlings with wild-type metal accumulation would be blue, while those accumulating two times or more that amount would be pink (Fig. 1F). For each image, the colors were adjusted until an internal radioactivity standard became a dark pink, and the color range was standardized to a normalization minimum and maximum of 0 and 1200, respectively. The internal radioactivity standard was prepared by placing 3450 cpm of ^{109}Cd in 5 ml of solution on a piece of Whatman #1 filter paper and laminating it. The radioactivity standard was placed in the upper right-hand corner of each incubation tray. A life-sized color hard copy of the image was generated and used to identify seedlings with greater than average radionuclide accumulation (Fig. 1G). Any seedling identified as an enhanced accumulator was immediately re-exposed a second time to verify that the signal on the phosphorimager was not due to the seedling being pressed closer to the screen than other seedlings. High accumulators were rescued and grown for seed (Fig. 1H).

Discrimination between Pb and Cd accumulating abilities

Since the incubation solution used in the primary screen contained both Cd and Pb, it was necessary to determine which metal the rescued mutants were accumulating above wild-type levels. M3 progeny testing positive for the hyperaccumulating phenotype in the secondary screen were separately re-screened in ^{109}Cd and ^{210}Pb.

Growth of rescued M2 seedlings

Putative mutants were rescued on 1.2% agar containing 55 mM *myo*-inositol, 12 mM thiamin, 20 mM pyridoxine, 2 mM KNO_3, 5 mM $Ca(NO_3)\cdot 2H_2O$, 2 mM $MgSO_4$, 2 mM KH_2PO_4, 0.89 mM Fe-tartrate, 46.26 µM H_3BO_3, 9.095 µM $MnCl_2\cdot 4H_2O$, 0.765 µM $ZnSO_4\cdot 7H_2O$, 0.312 µM $CuSO_4\cdot 5H_2O$ and 0.106 µM MoO_3 (adapted from Smith et al. 1994) in order to provide a supportive environment for growth. Plants were kept in a growth chamber (10-h photoperiod, fluorescent lighting; 22°C; 50% RH) inside vertically placed 9-cm Petri dishes until two true leaves emerged, at which time they were transferred to PROMIX-brand soil and grown to seed in a greenhouse equipped with supplementary lighting (15-h photoperiod; 20–23°C). Plants were allowed to self. Because many plants of different lines were grown in close proximity, pollination bags were placed on inflorescences.

Secondary screen

Progeny of putative mutants (M3) were re-screened as in the primary screen. Each putative mutant line and the wild-type line was incubated in its own tray. Solutions were mixed as a batch and then divided between the trays. Putative mutant seedlings were assayed when they were 4-days old instead of 3-days old because many had slow root growth and were too small to screen at 3 days. Wild-type seedlings were used at 3 days and were approximately the same size as the putative mutants. Putative mutant and wild-type seedlings were analyzed and compared on one phosphorimager screen. The seedlings from each mutant line with a radionuclide "hyperaccumulating" phenotype were rescued and grown to establish a seed stock.

Quantification of Cd and Pb in seedlings and roots

To quantify uptake, mutant and wild-type controls were incubated side by side in a solution mixed as a batch and divided into separate trays. The solution composition was the same as in the screening, except that the specific activity of ^{210}Pb was increased to 0.5 µCi/l to ensure detection by scintillation counting. After 5 h of incubation, the metal concentration in individual seedlings or roots was determined. Roots were excised, blotted, weighed and their radioactivity quantified. To quantify ^{109}Cd, whole seedlings or roots were placed in plastic tubes, crushed with a glass rod and analyzed in a γ-counter (Packard Instrument Company, Model C5002, Meriden, Conn.) with the window set to 15–35 keV and a counting time of 3 min.

To quantify ^{210}Pb, whole seedlings or roots were digested and analyzed in a scintillation counter (Beckman Instruments, Model LS 5000TD, Fullerton, Calif.). For digestions, individual seedlings were placed in 200 µL concentrated nitric acid and heated to 150°C for 1 h or until the solution was transparent and colorless. Thereafter, 100 µl of 50% hydrogen peroxide was added to each tube. The mixture was returned to the heating block until effervescence ceased. Samples of approximately 300 µl were transferred to scintillation vials with 7.5 ml of a READY-SAFE liquid scintillation cocktail (Beckman, Fullerton, Calif.), vigorously mixed and counted. For samples of incubation solution, 500-µl aliquots were counted. The scintillation counter window was set for 0–1000 keV, with quench limits between 71 and 267 and a counting time of 3 min per sample. Sample errors were less than 5%.

Preparation of root cell walls

Root cell-wall content was determined using the method described by Lasat et al. (1996). Roots of 25 seedlings were excised and their fresh and dry weight determined as a batch. They were subsequently immersed in methanol-chloroform (2:1 v/v) solution for 3 days, washed in distilled water for 12 h, and dried at 60°C for 48 h. These roots were then used for Pb-accumulation experiments.

Desorption of roots in EDTA

After incubation of seedlings in $PbNO_3$ and ^{210}Pb, roots of half the seedlings were removed and the Pb content analyzed (non-desorbed) while the remaining half were transferred to an aerated ice-cold solution of 1.0 mM K_2EDTA and 5.0 mM MES, pH 6, for 15 min (desorbed) before Pb analysis. It was determined that by 15 min, desorption comes to equilibrium. The Pb content was obtained as described above.

Results

Isolation of Cd- and Pb-accumulating mutants

We developed a non-destructive, high-sensitivity, high-throughput method for isolating *B. juncea* (Indian mustard) mutants that accumulate large amounts of heavy metals such as Cd and Pb. This method is based on submerging an M2 population of 3-day old seedlings in nutrient solution containing Cd and Pb salts traced with radioactive isotopes of these metals. Metal accumulation in individual seedlings was visualized using a phosphorimager. In Fig. 2, the two seedling roots indicated with arrows are pink, specifying that they are superior accumulators and with radionuclide accumulation at least two-times higher than wild-type. Other seedlings have varying levels of accumulation. Dark-blue coloration

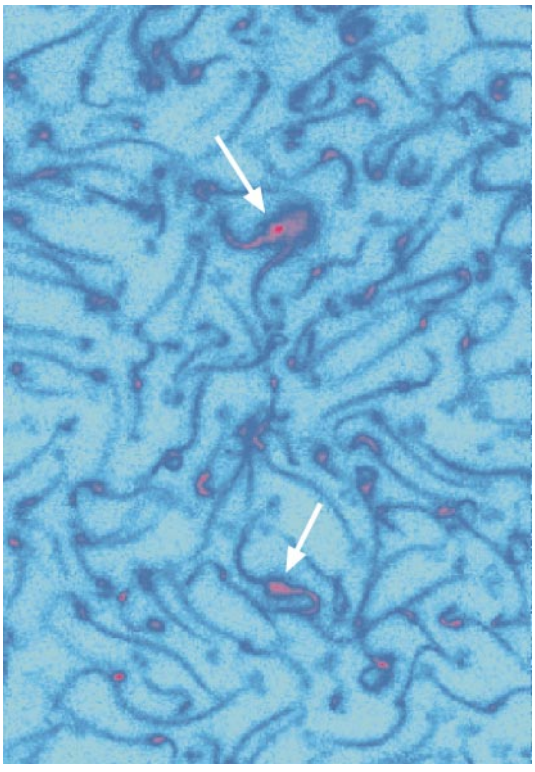

Fig. 2 Primary screening of *B. juncea* M2 seedlings. Seedlings have varying levels of accumulation. Two seedling roots indicated by *arrows* are superior accumulators

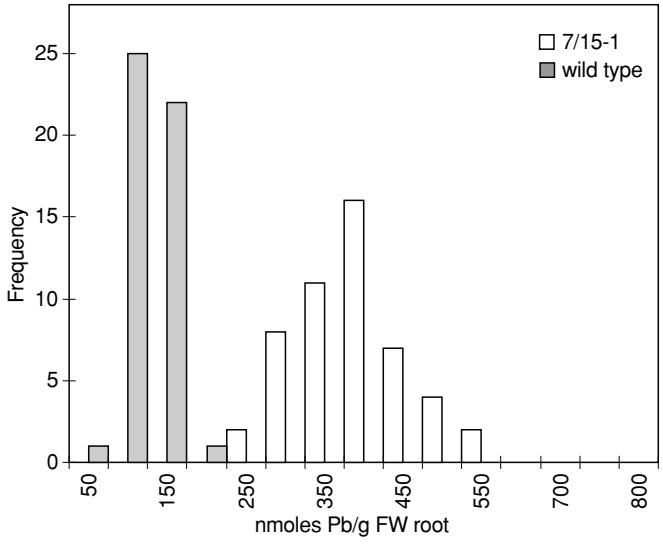

Fig. 3 Distribution of Pb accumulation in the roots of wild-type and 7/15–1 M4 populations. The mean accumulation was 102±25 nmol of Pb/g FW, (n=48) for wild-type and 364±68 nmol of Pb/g FW, (n=50) for 7/15–1

represents basal levels of Cd and Pb accumulation. Plants easily survive the analysis, can be re-assayed if desired, rescued and grown for seed. Four trays can be run simultaneously and processed by one person in 1 day, for a total throughput of 1200 seedlings/day.

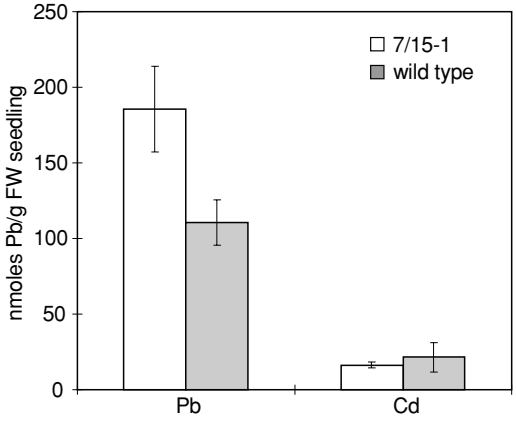

Fig. 4 Cd and Pb accumulation in seedlings of *B. juncea* mutant 7/15–1 and wild-type. The M3 progeny was re-screened in the presence of either ^{109}Cd, (1.2 µCi/l) or ^{210}Pb (0.5 µCi/l). Data points and error bars represent the means±SD of ten replicates

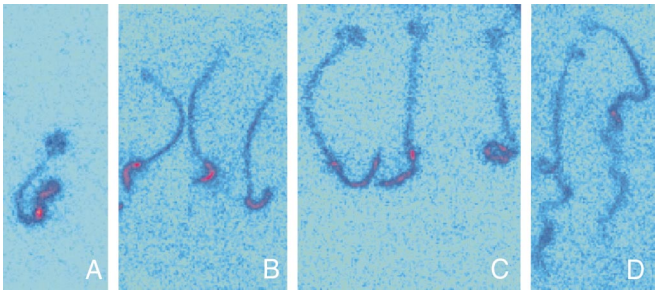

Fig. 5A–D Accumulation profile of Pb accumulation in *B. juncea* mutant 7/15–1. **A** Original M2 seedling 7/15–1 rescued in the primary screen. **B** M3 generation of 7/15–1 (100% segregation for the mutant phenotype). **C** M4 generation of 7/15–1. **D** Wild-type

Approximately 50,000 EMS-mutagenized M2 seedlings of *B. juncea* were screened using the phosphorimager screening system; 145 putative mutants were recovered and allowed to self. Of these, 45 died before producing seed and five were sterile. The M3 generation of the remaining 95 mutants was re-screened, with 21 mutants retaining a higher accumulation in 100% of the population.

Since the incubation solution used in the primary screen contained both Cd and Pb, all 21 mutant lines were re-tested in solutions in which only one metal was traced. Upon re-testing, all 21 lines showed increased accumulation:13 lines accumulated Pb only, one accumulated Cd only and seven accumulated both Cd and Pb.

Characterization of mutant 7/15–1

Mutant line 7/15–1 was chosen for further study, partly because it had one of the highest amounts of radioactivity accumulated per root surface area of all the mutants rescued. Increased root accumulation per unit surface area or per unit fresh weight (FW) is a particularly impor-

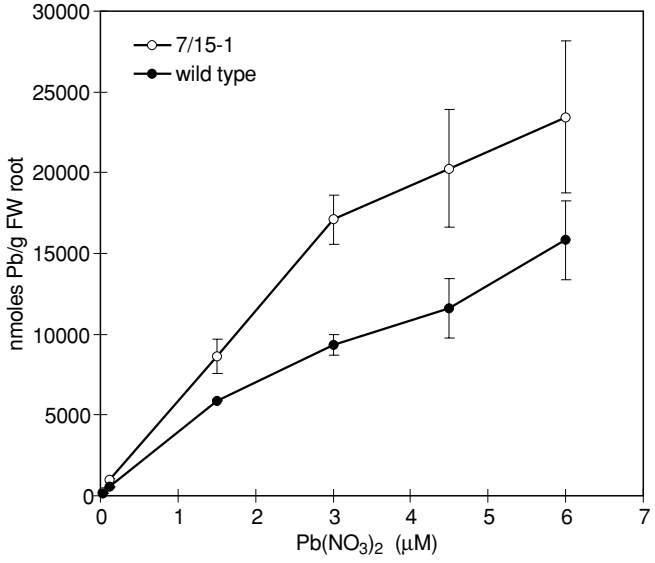

Fig. 6 Dose-response of *B. juncea* mutant 7/15–1 and wild-type roots. Data points and error bars represent the mean ± SD of ten replicates. The experiment was repeated with similar results

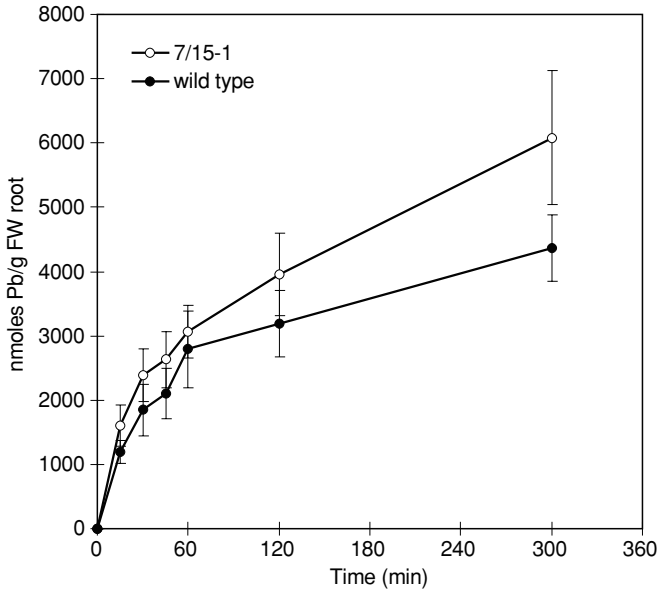

Fig. 7 Time course of accumulation of Pb in roots of *B. juncea* mutant 7/15–1 and wild-type. Roots were exposed to 1.5 μM $Pb(NO_3)_2$ (traced with 0.5 μCi ^{210}Pb) for different periods of time. Data points and error bars represent the mean±SD of ten replicates. The experiment was repeated with similar results

tant property for the rhizofiltration application. Roots of 7/15–1 seedlings were significantly shorter than the roots of wild-type seedlings of the same age. However, the roots of 3-week-old hydroponically cultivated 7/15–1 plants were equal in length and mass to the roots of wild-type plants (data not shown).

Mutant 7/15–1 exhibited greatly enhanced radionuclide accumulation in the roots of M4-generation seedlings (on a FW basis), as determined by phosphorimaging and scintillation counting. The average Pb accumulation in the roots of a population of 50 7/15–1 seedlings was 3.6-times higher than the wild-type (Fig. 3), with the distribution of root Pb uptake in the mutant population having almost no overlap between the mutant and wild-type populations. Whole 7/15–1 seedlings had enhanced accumulation of Pb only; Cd uptake in whole 7/15–1 seedlings was not significantly altered when compared to wild-type (Fig. 4). Figure 5 shows the inheritance of the enhanced Pb-accumulation phenotype through three generations. *B. juncea* is a self-pollinating species and therefore 25% of the M2 progeny will be homozygous for the mutation. The metal-accumulation phenotype was present in 100% of the M3 (11 individuals tested) and M4 generations (20 individuals tested) (Fig. 5B,C). Shoots of 7/15–1 also accumulated 1.5-times more Pb than the wild-type, as determined by tissue digestion and analysis (data not shown).

Concentration-dependence (Fig. 6) and time-dependence (Fig. 7) studies of Pb accumulation were performed with the 7/15–1 mutant to further characterize Pb uptake in its roots. In both mutant and wild-type, Pb accumulation increased linearly with increasing Pb concentration; however, at all tested concentrations the 7/15–1 mutant maintained a higher Pb accumulation per unit of root FW than the wild-type (Fig. 6). A time course of Pb accumulation was performed at a Pb concentration of 1.5 μM, a concentration well below the toxicity threshold of *B. juncea* for Pb. At all time points 7/15–1 accumulated more Pb on a root FW basis than the wild-type (Fig. 7).

In order to compare Pb binding to the root surface of 7/15–1 and the wild-type, we performed an EDTA desorption study. The optimum desorption period for this study was determined by washing seedlings previously incubated with Pb in 1 mM EDTA for up to 45 min. Very little ^{210}Pb was removed by EDTA after 15 min of incubation (data not shown), therefore 15 min was chosen as the optimum desorption time for subsequent experiments. As expected, roots of 7/15–1 seedlings accumulated 45% more Pb on a FW basis than the wild-type [541 (SD 141) and 373 (SD 97) nmol of Pb/g FW, respectively]. Accumulation in both 7/15–1 and the wild-type decreased after desorption with EDTA to 201 (SD 64) and 124 (SD 30) nmol of Pb/g FW, respectively; however, the percentage of Pb desorbed with EDTA was similar in the mutant and the wild-type (63 and 67%, respectively).

As mentioned above, roots of 7/15–1 are significantly shorter and thicker than wild-type roots (Fig. 8A,B). This phenotype is noticeable for at least 8 days after germination. The hypocotyls of 7/15–1 mutants are also stunted and thickened compared to wild-type. Light microscopy of mutant and wild-type roots showed that mutant roots were composed of cells which failed to elongate and were wider than the cells of the wild-type (Fig. 8C–F). In the mid-root region, cells of 7/15–1 were on average 36% the length of wild-type cells. These short cells were found either along the entire length of the

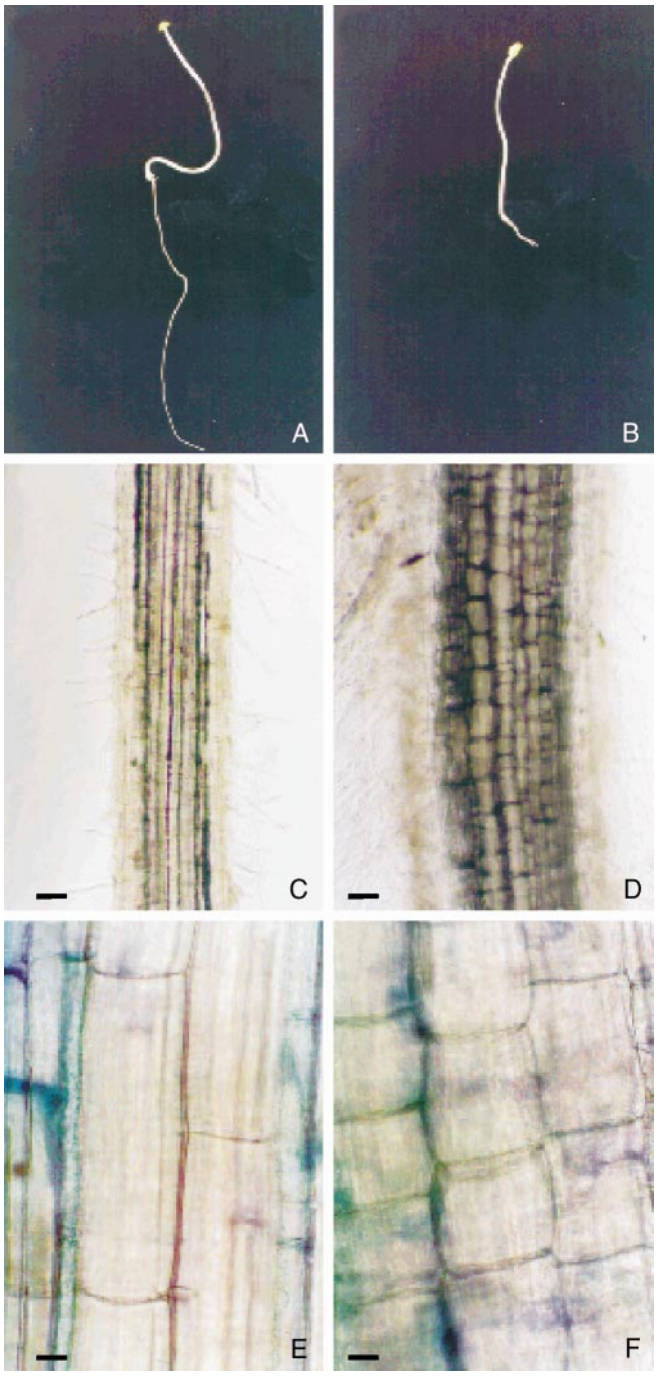

Fig. 8A–F Morphological comparison of *B. juncea* mutant 7/15–1 and wild-type seedlings. Five day old seedlings were grown submerged in aerated nutrient solution in the dark. **A** Wild-type. **B** 7/15–1. **C** Wild-type root. **D** 7/15–1 root. **C** and **D** were viewed with phase contrast on a Nikon Optiphot microscope. Bar=80 µm. **E, F** Comparison of the cell structure of *B. juncea* mutant 7/15–1 and wild-type. Five-day old whole roots were viewed with phase contrast on a Nikon Optiphot microscope. Photographed cells are immediately distal to the elongation zone. Tissue is stained with methylene blue. **E** Wild-type. **F** 7/15–1. Bar=20 µm

root or else in clusters separated by cells of normal length.

Since a large portion of Pb binds to, and precipitates in, cell walls, we compared the amounts of cell wall in FW equivalents of both mutant and wild-type roots. We found that 7/15–1 had 37% more cell wall per g of root FW than the wild-type; cell-wall preparations resulted in the recovery of 4.1×10^{-2} mg of cell walls from a g FW of 7/15–1 mutant roots versus 3.0×10^{-2} mg of cell walls from a g FW of wild-type roots.

Discussion

The screening system we have developed allows sensitive and rapid identification of plant mutants with enhanced uptake of ions from solution. This method can resolve differences in metal ion accumulation at organ or tissue levels, while utilizing very small amounts of radionuclides and relatively short exposure times.

We have used this screening method to isolate *B. juncea* mutants with enhanced accumulation of Cd and Pb, and to partially characterize one of the isolated mutants (7/15–1) through the M4 generation. Backcrosses were not performed on this mutant and, therefore, there may be additional mutations present affecting the phenotype of the 7/15–1 mutant. Further characterization is planned for the future. Mutant 7/15–1 had greatly enhanced Pb accumulation in the roots on a FW basis, and slightly enhanced accumulation in the shoots. This Pb "hyperaccumulating" phenotype was combined with a stunted root morphology. When the total Pb/root was calculated, accumulation by the mutant was comparable to that of the wild-type.

The microscopic structure of the mutant root explains the higher Pb accumulation on a FW basis. The volume of the mutant cells is much smaller than that of the wild-type cells, which translates into a greater cellular surface area, and thus a greater amount of cell wall on a per g FW basis, as directly confirmed by our data (see Results). Since cell wall binding and precipitation are the predominant mechanisms of Pb accumulation in plants (Salt et al. 1995), it is logical to conclude that the increased amount of cell wall per unit of root weight is the main reason for Pb hyperaccumulation in the root of this mutant. Cell-wall desorption experiments further confirmed this hypothesis. EDTA removed more Pb on an absolute basis from the cell walls contained in 1 g of mutant roots than from 1 g of wild-type roots (340 nmol/g FW for the mutant compared to 249 nmol/g FW for the wild-type). Biological uptake plays a primary role in Cd uptake in plants (Salt et al. 1997). We suggest that this is the reason why the greater cell-wall content in the 7/15–1 mutant did not translate into enhanced Cd accumulation.

Mutants with stunted roots have been isolated in *Arabidopsis thaliana* by several researchers. These include cob, lit, sab and shr (Benfey et al. 1993); rsw and reb (Baskin et al. 1992); and 5905, 1767, 7203, 4792 and 7133 (Holding et al. 1994). All of these mutants display

radial swelling in various root cell layers in addition to inhibition of elongation. 7/15–1 does not display radial swelling and does not resemble any of these mutants in gross root morphology. In addition, the stunted root phenotype in this mutant is only pronounced at the early stages of plant development. Rather, 7/15–1 roots resemble *Arabidopsis* roots exposed to exogenous ethylene in both gross and cellular morphology (Baskin and Williamson 1992; Baskin and Bivens 1995). Inhibition of cell elongation by ethylene is well-documented in the literature (Burg 1973; Lieberman 1979). However, the stunted phenotype was not reversed by seedling exposure to 2,5-norbornadiene (data not shown), a potent inhibitor of ethylene action at the receptor level (Sisler and Pian 1973). It is still possible that the 7/15–1 mutant has a lesion in the ethylene pathway downstream from hormone reception. Further studies are necessary to determine whether ethylene is involved in the stunted root phenotype of 7/15–1.

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