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Research paper

Identification of quantitative trait loci and candidate genes for cadmium tolerance in *Populus*

Brahma Reddy Induri[1], Danielle R. Ellis[1], Gancho T. Slavov[1,2], Tongming Yin[3], Xinye Zhang[4], Wellington Muchero[5], Gerald A. Tuskan[5] and Stephen P. DiFazio[1,6]

[1]Department of Biology, West Virginia University, 53 Campus Drive, Morgantown, WV 26506-6057, USA; [2]Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth SY23 3EB, UK; [3]The Key Lab of Forest Genetics and Gene Engineering, Nanjing Forestry University, Nanjing 210037, China; [4]Hubei Forestry Academy, Wuhan 430079 China; [5]Biosciences Division, Oak Ridge National Laboratory, PO Box 2008, Oak Ridge, TN 37831-6422, USA; [6]Corresponding author (spdifazio@mail.wvu.edu)

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Understanding genetic variation for the response of *Populus* to heavy metals like cadmium (Cd) is an important step in elucidating the underlying mechanisms of tolerance. In this study, a pseudo-backcross pedigree of *Populus trichocarpa* Torr. & Gray and *Populus deltoides* Bart. was characterized for growth and performance traits after Cd exposure. A total of 16 quantitative trait loci (QTL) at logarithm of odds (LOD) ratio ≥ 2.5 were detected for total dry weight, its components and root volume. Major QTL for Cd responses were mapped to two different linkage groups and the relative allelic effects were in opposing directions on the two chromosomes, suggesting differential mechanisms at these two loci. The phenotypic variance explained by Cd QTL ranged from 5.9 to 11.6% and averaged 8.2% across all QTL. A whole-genome microarray study led to the identification of nine Cd-responsive genes from these QTL. Promising candidates for Cd tolerance include an NHL repeat membrane-spanning protein, a metal transporter and a putative transcription factor. Additional candidates in the QTL intervals include a putative homolog of a glutamate cysteine ligase, and a glutathione-S-transferase. Functional characterization of these candidate genes should enhance our understanding of Cd metabolism and transport and phytoremediation capabilities of *Populus*.

Keywords: cadmium, metal transporter, microarray, phytoremediation, *Populus*, quantitative trait loci.

Introduction

One of the challenges posed by phytoremediation of cadmium (Cd) contamination is the development of plants that are tolerant of the deleterious effects of this toxin, which has no known nutritional value in plants. Symptoms of Cd toxicity include leaf roll, chlorosis and reductions in root and shoot growth, even at minute concentrations (Milone et al. 2003, Almeida et al. 2007). Cadmium compromises the structure and function of many proteins by acting on sulphydryl groups (Vanassche and Clijsters 1990) and affects the permeability of membranes by altering lipid composition (Ouariti et al. 1997).

In addition to disrupting the ion and water balance of plants, Cd affects various physiological processes such as photosynthesis, respiration, nitrate assimilation, metabolite accumulation and enzyme activity (Smeets et al. 2005, Solti et al. 2008). Cadmium decreases the photosynthetic activity of plants by changing chloroplast ultrastructure (Sandalio et al. 2001) and altering stomatal closure. Cadmium also interferes with the uptake and distribution of other nutrients (Shao et al. 2008, Rodriguez-Serrano et al. 2009), most notably iron (Fe) (Siedlecka and Krupa 1999, Fodor et al. 2005, Solti et al. 2008). Cadmium has also been shown to affect the electron transport system, interact with antioxidative defense systems and induce lipid peroxidation (Smeets et al. 2005, Rodriguez-Serrano et al. 2009).

Most phytoremediation research so far has been carried out on herbaceous plants that accumulate high levels of metals in

their cells naturally. However, their slow growth rates, annual habit, small stature, low biomass and/or narrow geographic adaptability make them unsuitable for phytoremediation on an operational scale (Eapen and D'Souza 2005). *Populus*, with its rapid growth, makes an ideal plant species for phytoremediation of soils polluted by heavy metals (Cunningham and Ow 1996). Furthermore, *Populus* is readily propagated from vegetative cuttings, and has a wide range of adaptability, high bulk root volume, high transpiration rate and large stature, all of which should enhance the efficacy of phytoremediation (Rockwood et al. 2004). Previous studies have assessed the potential of using *Populus* to remediate soils polluted by atrazine (Burken and Schnoor 1997), trichloroethylene (Newman et al. 1997), Cd (Robinson et al. 2000), selenium (Pilon-Smits et al. 1998) and zinc (Di Baccio et al. 2009). In addition, some hybrid *Populus* clones have shown elevated tolerance of Cd in field plantings relative to other tree species (Migeon et al. 2009), and there is considerable natural variation within the genus that could be exploited to enhance Cd tolerance further using biotechnological approaches.

To further characterize Cd tolerance in *Populus*, we (i) quantified phenotypic and genetic variation for Cd tolerance and Cd accumulation in a pseudo-backcross multiple generation *Populus* pedigree that was grown under greenhouse conditions; (ii) detected quantitative trait loci (QTL) for traits related to Cd tolerance including dry weight components and root volume; and (3) identified candidate genes in the QTL intervals based on transcriptional data from a microarray study.

Material and methods

Plant materials

An interspecific mapping population was developed at the University of Minnesota Natural Resources Research Institute by crossing *Populus trichocarpa* Torr. & Gray clone 93-968 from western Washington to *Populus deltoides* Bart. clone ILL-101 from southern Illinois. The resulting F_1 hybrid, 52-225, was in turn backcrossed to *P. deltoides* clone D-124 from Minnesota. Two-hundred and fifty-two full-sib progeny from the 'pseudobackcross' pedigree (Family 52-124) were used for the current study. Dormant cuttings were collected from a stoolbed in early spring prior to bud break and were stored at 4 °C for 5 weeks to meet chilling requirements. This facilitated uniform rooting of the cuttings, and enabled selection of plants with approximately uniform sizes at the initiation of our experiment. Two ramets per clone were used for both control and Cd treatments. A total of 1008 vegetative cuttings (252 genotypes × 2 treatments × 2 cuttings per genotype) were examined in this experiment.

Cuttings with approximate lengths of 15 cm and diameters of 1 cm were dipped in 1 : 10 dilution of a commercial liquid rooting hormone solution (Dip N Grow 1% indole-3-butyric acid, 0.5% naphthalene acetic acid) for 15 s, surrounded by foam stoppers and plugged into 3 cm holes at 8 cm spacing in the lids of polyethylene containers. Approximately 5 cm of the base of the cuttings was submerged beneath a dilute, continuously aerated nutrient solution, 0.1 × Johnson's solution (Siddique et al. 1990). The nutrient solution was composed of 400 μM NH_4NO_3, 400 μM KNO_3, 200 μM $Ca(NO_3)_2{\cdot}4H_2O$, 100 μM $MgSO_4{\cdot}7H_2O$, 50 μM K_2HPO_4, 20 μM KCl, 25 μM H_3BO_3, 0.5 μM $Na_2MoO_4{\cdot}2H_2O$, 2 μM $MnSO_4{\cdot}4H_2O$, 0.5 μM $CuSO_4{\cdot}5H_2O$, 2 μM $ZnSO_4{\cdot}7H_2O$, 0.5 μM $CoCl_2$ and 20 μM Fe-Na EDTA. The pH was ~5.4. One primary shoot was maintained per cutting.

Once the cuttings developed roots and shoots, they were transplanted into 126 polyethylene containers (24 cm × 12 cm × 38 cm) following a partially balanced incomplete block design, with two treatments (Cd and control), 126 containers as incomplete blocks and two replicates of each genotype in each treatment. Each container had one ramet from each of eight different genotypes at 9 cm spacing and contained 7 l of the 0.1× Johnson's solution described above. The solutions were changed every 3 days throughout the experiment (from 19 May 2007 to 10 July 2007) to maintain nutrient and oxygen levels and to avoid algal growth.

The experiment was carried out with a target day temperature of 25 °C and a night temperature of 21 °C and a photoperiod of 18 h. Artificial lighting was provided by high-density mixed halide lights and photosynthetically active radiation (PAR) was recorded for every container used in the study, at plant height (average PAR = 224.05 μmol/m^2/s and standard deviation = 55.08). Temperature was maintained by an evaporative cooling system.

Cuttings were grown for 40 days and one-half of the containers were randomly selected to receive a 25 μM $CdCl_2$ treatment for an additional 2 weeks. The remaining half of the containers were maintained as described above for 2 weeks and used as controls. The Cd concentration was selected based on pilot experiments that revealed substantial phenotypic variation in this family without excessive mortality. Fresh $CdCl_2$ was added each time solutions were changed. Before and after 20 days of treatment, measurements were taken on shoot length, cutting diameter, shoot diameter, PAR, root collar diameter (the diameter recorded right above the origin point of the uppermost root on a cutting) and rooted length for each cutting (portion of the cutting occupied by roots). Furthermore, root volume was estimated using water displacement in graduated cylinders. After 2 weeks, roots and shoots were harvested, separated into paper bags and dried in an oven at 60 °C for 6 days. Roots were washed with deionized water and blotted on paper towels before being stored in the bags. Dry weights were recorded for leaves, roots and shoots separately, and

total dry weights were calculated by summing all components.

Statistical analysis of phenotypic data

Phenotypic data were tested for normality using the Shapiro–Wilk test (Shapiro and Wilk 1965) and logarithmic transformations were performed to mitigate heteroscedasticity of variance versus predicted values. Data were analyzed with mixed linear models using SAS JMP, version 8.0 (SAS Institute Inc., Cary, NC, USA). The general form of the statistical model was

$$Y_{ijk} = \mu + T_i + B_j + G_k + \varepsilon_{ijk}$$

where Y_{ijk} was the measurement of response variable Y (total dry weight, root dry weight, leaf dry weight, stem dry weight or root volume) for genotype k in container j under treatment i, μ was the population mean, T_i was the effect of treatment i, B_j was the effect of incomplete block (container) j, G_k was the effect of genotype k and ε_{ijk} was the experimental error. When significant ($P \leq 0.05$), the rooted length and dry weight of each cutting and PAR for each container were also included as covariates. Genotype and all interactions with genotype were treated as random effects in the model, whereas all remaining variables were treated as fixed effects.

Broad-sense heritabilities (H^2) were calculated as $H^2 = V_G/(V_G + V_\varepsilon)$ based on the variance components for genotype (V_G) and error (V_ε) estimated using restricted maximum likelihood (REML) in mixed linear models that were fitted separately for control and Cd treatments. This was done to assess differences in variances and heritabilities under these two conditions.

The effect of Cd on each genotype (hereafter referred to as 'Cd effects') was estimated by subtracting its best linear unbiased predictor (BLUP) under Cd conditions from that under control conditions (Coles et al. 2010) for all biomass traits described above. The genotypes with the largest BLUP differences were identified as Cd sensitive and those with the smallest BLUP differences were identified as Cd tolerant. Genetic correlations among traits were calculated using BLUPs and the REML method in SAS JMP, version 8.0.

Linkage map construction

To construct a genetic map for Family 52-124, 188 progeny were genotyped using 590 amplified fragment length polymorphism markers. In addition, 418 progeny were genotyped using 287 simple sequence repeat (SSR) markers (Yin et al. 2009) that were chosen based on their physical locations along chromosomes to achieve an even distribution of markers on each linkage group. Four aneuploid trees were identified by genotyping with fully informative SSR markers and these individuals were later removed from the mapping data set. Marker generation, genotyping and nomenclature were performed as described previously (Tuskan et al. 2004, Yin et al. 2004). Map construction was conducted using JoinMap 3.0 under the CP cross type (Van Ooijen 2001).

Quantitative trait loci analysis

Quantitative trait loci were identified using MapQTL version 5.0 (Van Ooijen 2004). Interval mapping was performed initially to identify markers associated with putative QTL. Markers with significant effects in this analysis were then used as cofactors in restricted multiple QTL model mapping (Van Ooijen 2004). Logarithm of odds (LOD) scores were calculated at 1.0 cM intervals. Only QTL with LOD score > 2.5 are reported here.

Microarray study

A whole-genome microarray study was conducted using two genotypes that demonstrated differential Cd effects in the above QTL hydroponic study: a Cd-tolerant genotype (1–183) and a Cd-susceptible genotype (182). The experiment consisted of six polyethylene containers as described above but with only four plants per container. Each genotype was represented by two ramets per container with randomly assigned locations. Three containers were treated with 25 μM $CdCl_2$ and the remaining three containers were under control conditions. Three ramets from each genotype × treatment combination were harvested at 24 h, and three at 72 h after Cd treatment. Roots from each plant were thoroughly washed in deionized water and blotted dry and all material was quick frozen in liquid nitrogen and stored at –80 °C.

Plant tissue was ground in liquid nitrogen, RNA was extracted using the Qiagen RNA Mini kit (Qiagen, Germantown, MD, USA), and double-stranded DNA was synthesized using the Invitrogen SuperScript™ double-stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Labeling, hybridization and scanning were performed by NimbleGen (Roche NimbleGen, Madison, WI, USA). The raw data were normalized using the robust multichip averaging procedure, which performs a convolution background correction, quantile normalization and summarization based on a multi-array model fit robustly using the median polish algorithm (Bolstad et al. 2005). Differentially expressed genes (Cd treated versus control) were determined using rank product analysis (Breitling et al. 2004) using 1000 permutations and a percentage of false positives cutoff of 0.05.

Results

Cadmium effects

The effect of Cd was evident for all measured traits (Table 1). Cadmium symptoms included necrotic spots on leaves and browning of roots (Figure 1). The genotypes under Cd conditions showed poor growth compared with those under control conditions (Table 1). The effects of genotype, treatment and

Table 1. Summary statistics and heritability estimates for total dry weight and other measured traits along with their variance components.

	Mean (g)	SD[1]	Range	SE[2]	V_G[3]	V_p[4]	H^2_{BS}[5]
Control							
Total dry weight C	6.35	4.11	24.98	0.19	0.170	0.471	0.361
Leaf dry weight C	3.57	2.28	13.05	0.10	0.160	0.499	0.321
Root dry weight C	1.14	0.88	7.34	0.04	0.201	0.581	0.346
Stem dry weight C	1.63	1.16	7.39	0.05	0.233	0.597	0.390
Root volume C	1.62	0.94	6.00	0.04	0.045	0.235	0.191
Cadmium							
Total dry weight Cd	2.52	1.63	8.24	0.07	0.153	0.450	0.340
Leaf dry weight Cd	1.46	1.01	5.23	0.04	0.147	0.533	0.276
Root dry weight Cd	0.45	0.33	2.51	0.01	0.179	0.558	0.322
Stem dry weight Cd	0.60	0.43	2.70	0.02	0.153	0.529	0.288
Root volume Cd	0.97	0.64	9.00	0.02	0.024	0.218	0.110
Cd mg/g	0.34	0.27	1.51	0.01	0.108	0.859	0.126

[1]Standard deviation.
[2]Standard error.
[3]Genetic variance.
[4]Phenotypic variance ($V_G + V_\varepsilon$).
[5]Broad-sense heritability.

Figure 1. Morphological effects of Cd treatment in a hydroponic experiment: (a) control roots; (b) Cd-treated roots; (c) control leaf (d) and Cd-treated leaf.

their interaction were highly significant ($P < 0.001$) for total dry weight, root dry weight, leaf dry weight, stem dry weight and root volume. The covariate effects of cutting rooted length, cutting dry weight and PAR were also significant ($P < 0.001$) for all traits except for PAR for root volume (Table 2).

Table 2. Results of ANOVA for traits measured in the greenhouse hydroponic study that showed significant differences between Cd and control conditions.

	Total dry weight		Leaf dry weight		Root dry weight		Stem dry weight		Root volume	
Trait	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Genotype	1.97	<0.0001	1.86	<0.0001	1.76	<0.0001	1.97	<0.0001	1.61	<0.0001
Treatment	589.94	<0.0001	525.75	<0.0001	335.03	<0.0001	555.28	<0.0001	215.37	<0.0001
Genotype × treatment	1.49	0.0002	1.41	0.0009	1.28	0.0124	1.53	<0.0001	1.34	0.0044
Rooted length of cutting	10.08	0.0016	5.99	0.0147	16.76	<0.0001	7.71	0.0057	13.92	0.0002
Cutting dry weight	139.78	<0.0001	130.92	<0.0001	56.23	<0.0001	146.18	<0.0001	51.94	<0.0001
PAR	6.87	0.0091	4.85	0.0281	4.30	0.0388	9.18	0.0026	0.70	0.4027

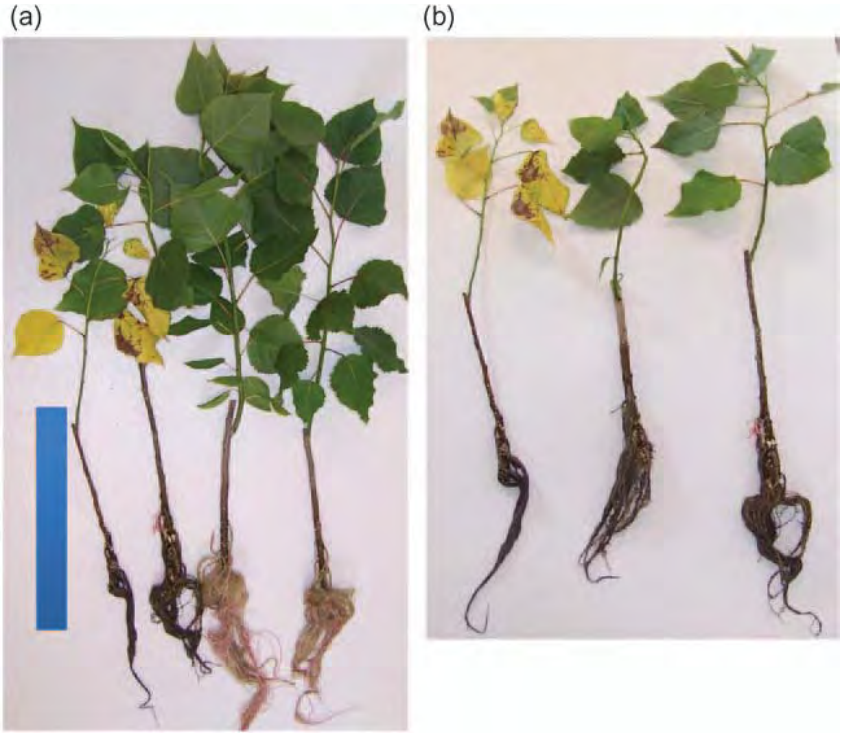

Figure 2. Genotypic variation in visible effects of Cd treatment. (a) Differences between control (right two plants) and Cd (left two plants) treatments. (b) Variation among genotypes under Cd conditions. Blue bar is 30.5 cm.

There was extensive variation in Cd responses among genotypes (Figures 2 and 3). Broad-sense heritabilities of total dry weight and its components were relatively low and comparable between control (range $H^2 = 0.19–0.39$) and Cd conditions (range $H^2 = 0.11–0.34$, Table 1). Root volume showed the lowest heritabilities under either control ($H^2 = 0.19$) or Cd ($H^2 = 0.11$) conditions. In contrast, total dry weight had heritabilities of 0.36 and 0.34 in control and Cd conditions, respectively (Table 1).

Quantitative trait loci identification

Quantitative trait loci were mapped for 16 phenotypes with a minimum LOD score of 2.5. Four of these QTL were identified

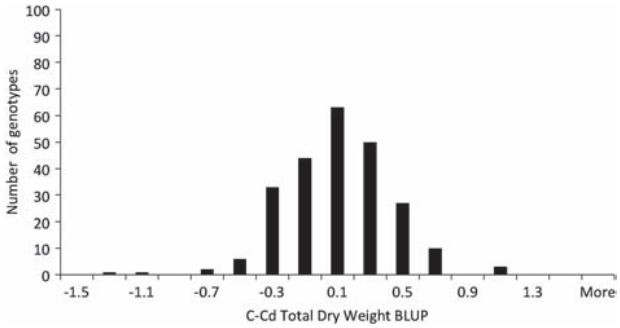

Figure 3. Distributions of the difference in BLUPs of total dry weight between control (C) and cadmium (Cd) treatments. Genotypes from the left portion of the curve are relatively tolerant of Cd treatment.

in control conditions and 12 under Cd conditions. Quantitative trait loci were located on LG III and XVI (Figure 4; Table 3). The phenotypic variation explained by QTL ranged from 5.9 to 11.6% and averaged 8.2% across all traits. Excluding co-localizing QTL (±1 LOD) and accounting for correlations among phenotypic traits (Table 4; see Figure S1 available as Supplementary Data at *Tree Physiology* Online), these 16 QTL correspond to four independent positions within the genome (Table 3), and ranged in size from ~15 to 30 cM (Figure 4).

Three QTL for dry weight and its components under control conditions were co-located on LG III along with three QTL for Cd effects on dry weight, collectively referred to as locus cd1 (Table 3). Two clusters of QTL were mapped to LG XVI at two discrete positions, the first of which (cd3) contained QTL for leaf dry weight under control conditions and Cd effects on total dry weight and its components (Table 3). For both cd1 and cd3, the allelic effects for dry weight measures under control conditions were in the same direction as those for QTL for Cd effects. However, the relative effect of the allele originating from *P. trichocarpa* was in opposite directions for these two loci, decreasing Cd tolerance at cd1 and enhancing Cd tolerance at cd3. The second cluster (cd2) on LG XVI contained QTL for total dry weight and its components under Cd conditions, as well as three QTL for Cd effects. In contrast to the other two loci, the allelic effects were opposite for dry weight measures and Cd effects. In this case, the allele from *P. trichocarpa* conferred reduced Cd tolerance on average. Finally, an apparently independent locus on LG XVI (cd4) explained 11% of the variation in leaf Cd concentration under Cd conditions (Table 3).

Candidate gene identification

The Cd response QTL intervals encompassed 1571 predicted genes from v2.0 of the *P. trichocarpa* genome annotation. In addition, across all genotypes and treatments there were 1748 different genes that showed up-regulation, and 672 genes that showed down-regulation in response to Cd treatment (see Figures S2 and S3 available as Supplementary Data at *Tree Physiology* Online). Nine of these differentially expressed genes were also present in the Cd response QTL intervals (Table 5).

Discussion

We observed substantial phenotypic variation in Cd effects on plant biomass and leaf Cd accumulation in Family 52-124, a pseudo-backcross pedigree derived from *P. trichocarpa* × *deltoides* hybrids. The effects of genotype, Cd treatment and their interactions were significant for all response variables measured in this study, including total dry weight, root dry weight, leaf dry weight, stem dry weight and root volume. Substantial variation in Cd tolerance is commonly observed within and among species. For example, Pietrini et al. (2010) used 50 μM of Cd (i.e., twice as high as in our experiment) to compare responses of multiple hybrid *Populus* clones involving six species. They observed significant variation among clones in the effects of Cd on biomass accumulation, photosynthetic responses, Cd content of plant parts and concentration of photosynthetic pigments. Interestingly, the variation in Cd responses observed among full siblings in our inter-specific cross is of magnitude similar to the variation observed among highly diverse clones that were exposed to a higher concentration of Cd. This illustrates the tremendous phenotypic variation captured by our crossing design and experimental conditions. The Cd-tolerant and Cd-susceptible genotypes identified in this study are a useful resource for future experiments targeted at physiological and molecular dissection of Cd tolerance.

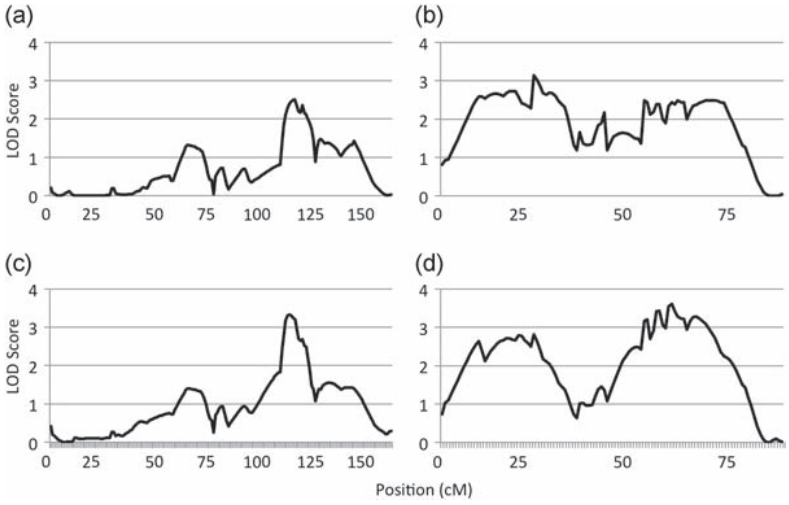

Figure 4. Logarithm of odds scores from the QTL analysis. (a) Total dry weight control: linkage group III. (b) Total dry weight Cd: linkage group XVI. (c) Total dry weight control–Cd: linkage group III. (d) Total dry weight control–Cd: linkage group XVI.

Table 3. Linkage group (LG), QTL positions, logarithm of odds ratio (LOD) scores, percent variation explained (PVE) and direction of effect of each QTL are given under the control (C) and cadmium-treated (Cd) conditions.

Locus	Trait	Linkage group	Position	LOD	PVE	Left marker	Right marker	T effects[1]	D effects[2]
cd1	Total dry weight C–Cd[3]	III	109.267	3.33	8.9	*CCCGA_8	*CACAC_218	−0.047	0.043
cd1	Root volume C–Cd	III	110.267	3.04	9	*CCCGA_8	*CACAC_218	−0.025	0.022
cd1	Leaf dry weight C–Cd	III	111.267	3.06	7.7	*CCCGA_8	*CACAC_218	−0.046	0.039
cd1	Total dry weight C	III	111.676	2.51	6.1	*CCCGA_8	*CACAC_218	−0.025	0.022
cd1	Root volume C	III	114.726	2.5	6.8	*CCCGA_1	*CCCAC_22	−0.014	0.013
cd1	Root dry weight C	III	110.267	2.66	6.6	*CCCGA_8	*CACAC_218	−0.021	0.022
cd2	Root dry weight C–Cd	XVI	22.784	2.99	7.4	G_3141	*CCCCT_202R	0.045	−0.030
cd2	Total dry weight C–Cd	XVI	26.609	2.81	6.8	*CCCCT_202R	*TCCGT_8R	0.047	−0.033
cd2	Leaf dry weight C–Cd	XVI	26.609	4.26	10	*CCCCT_202R	*TCCGT_8R	0.056	−0.042
cd2	Total dry weight Cd	XVI	26.609	3.14	7.8	*CCCCT_202R	*TCCGT_8R	−0.029	0.021
cd2	Root dry weight Cd	XVI	26.609	2.99	7.3	*CCCCT_202R	*TCCGT_8R	−0.027	0.017
cd2	Leaf dry weight Cd	XVI	26.609	4.6	11.6	*CCCCT_202R	*TCCGT_8R	−0.037	0.028
cd3	Leaf dry weight C	XVI	56.812	2.9	5.9	*CCCCT_246R	*CTCAG_356R	0.033	−0.016
cd3	Total dry weight C–Cd	XVI	56.812	3.61	7.4	*CCCCT_246R	*CTCAG_356R	0.059	−0.028
cd3	Leaf dry weight C–Cd	XVI	56.812	5.41	11	*CCCCT_246R	*CTCAG_356R	0.072	−0.036
cd4	Leaf dry weight Cd	XVI	68.558	4.61	11.3	*CGCCA_308R	*CTCTC_100R	−0.040	0.025

[1]The relative effect of the allele originating from *P. trichocarpa*.
[2]The relative effect of the allele originating from *P. deltoides*.
[3]Difference in BLUP between control and Cd conditions (Cd effect).

Heritability estimates

Our broad-sense heritability estimate for total dry weight was somewhat lower than those for growth and yield traits measured for *Populus* in recent field studies (Marron et al. 2006, 2010, Rae et al. 2008, Dillen et al. 2009), though certainly within the range of estimates in earlier studies (Riemenschneider et al. 1996). The broad-sense heritabilities calculated for total dry weight and its components under control conditions were not substantially different from the heritabilities calculated for these traits under Cd exposure. Root volume had the lowest heritabilities under both conditions, whereas root dry weight had heritabilities that were comparable to those for other dry weight components. This discrepancy may reflect difficulties in accurately measuring root volume through water displacement.

Quantitative trait loci

Although we detected 16 QTL in this study, these occurred at only four different chromosomal locations. Co-localization of QTL for total dry weight and dry weight components was not surprising, given that these traits were highly intercorrelated and were co-segregating in the pedigree. However, it is potentially more interesting and informative that dry weight under control and Cd conditions mapped to different QTL, and had opposing relationships to the Cd response QTL (C–Cd dry weight). Dry weight under control conditions was positively correlated with Cd effect and allelic effects were in the same direction for these traits when they were co-located, suggesting that these QTL were driven by the size of plants under control conditions (i.e., large plants showed more sensitivity to the Cd treatment). In contrast, dry weight under Cd conditions was negatively correlated with Cd effect, and allelic effects were opposite for these traits at co-located QTL, suggesting that Cd tolerance at this locus was associated with growth in the presence of Cd irrespective of performance under control conditions.

Quantitative trait loci for different traits may also be co-located even when they are not correlated (Wu et al. 1997,

Table 4. Genetic (above diagonal) and phenotypic correlations among the variables measured.

Traits all correlations	Total dry weight C	Root dry weight C	Leaf dry weight C	Stem dry weight C	Root volume C	Total dry weight Cd	Root dry weight Cd	Leaf dry weight Cd	Stem dry weight Cd	Root volume Cd	Total dry weight C–Cd	Root dry weight C–Cd	Leaf dry weight C–Cd	Stem dry weight C–Cd	Root volume C–Cd
Total dry weight C	1	0.8838	0.9765	0.9463	0.7703	0.268	0.2562	0.2242	0.2822	0.1859	0.9197	0.7918	0.8783	0.8825	0.5893
Root dry weight C	0.9022	1	0.796	0.8016	0.7862	0.2106	0.2084	0.1769	0.2149	0.1401	0.8235	0.9271	0.7186	0.757	0.6323
Leaf dry weight C	0.973	0.8317	1	0.8841	0.7102	0.2648	0.2556	0.2259	0.2669	0.1816	0.8968	0.7036	0.901	0.8232	0.537
Stem dry weight C	0.9431	0.8152	0.8895	1	0.7313	0.2674	0.2454	0.2148	0.3103	0.1941	0.8646	0.7131	0.7899	0.9278	0.5485
Root volume C	0.6294	0.626	0.6059	0.5817	1	0.2165	0.205	0.1793	0.2338	0.1961	0.7043	0.7131	0.6315	0.6758	0.7931
Total dry weight Cd	–0.3557	–0.3091	–0.2795	–0.2959	–0.0793	1	0.8005	0.961	0.8843	0.5168	–0.1319	–0.0947	–0.1627	–0.0663	–0.1229
Root dry weight Cd	–0.2765	–0.3656	–0.1976	–0.2234	–0.0563	0.8157	1	0.655	0.6965	0.4847	–0.0628	–0.1733	–0.0356	–0.0157	–0.1135
Leaf dry weight Cd	–0.3188	–0.2572	–0.3007	–0.2286	–0.0624	0.9129	0.6886	1	0.7598	0.4666	–0.161	–0.0729	–0.219	–0.0727	–0.1258
Stem dry weight Cd	–0.3146	–0.2638	–0.2208	–0.356	–0.0569	0.8559	0.721	0.689	1	0.4719	–0.0701	–0.0505	–0.071	–0.0667	–0.0792
Root volume Cd	–0.079	–0.1164	–0.0457	–0.0305	–0.1963	0.4798	0.4724	0.4145	0.46	1	–0.0194	–0.0446	–0.0259	0.0186	–0.4417
Total dry weight C–Cd	0.8291	0.7409	0.7669	0.7582	0.4363	–0.8175	–0.6584	–0.7426	–0.706	–0.3353	1	0.8533	0.9699	0.935	0.6565
Root dry weight C–Cd	0.7198	0.8329	0.6294	0.6347	0.4196	–0.6752	–0.8196	–0.5678	–0.591	–0.3515	0.8475	1	0.7372	0.7683	0.6802
Leaf dry weight C–Cd	0.7977	0.6723	0.803	0.69	0.412	–0.7424	–0.5519	–0.8099	–0.5664	–0.2869	0.9358	0.742	1	0.8569	0.5939
Stem dry weight C–Cd	0.7824	0.6716	0.6941	0.8424	0.405	–0.682	–0.558	–0.5429	–0.8034	–0.2839	0.8902	0.7452	0.7662	1	0.6068
Root volume C–Cd	0.474	0.4948	0.4376	0.4119	0.796	–0.3495	–0.3295	–0.2978	–0.3221	–0.7498	0.5016	0.5003	0.4553	0.4488	1

Table 5. Cd tolerance candidate genes in QTL intervals that also showed altered expression levels in the microarray experiment. Corresponding *Arabidopsis* genes were identified based on best reciprocal BLAST hits and the putative functions were derived from the TAIR 9 annotation. Fold change is the ratio of normalized expression levels under Cd versus control conditions. ns, not significant with a false discovery rate of 0.05.

Populus gene	*Arabidopsis* gene	QTL LG	Gene symbol	Fold change	Pfp	Clone	Time (h)	Putative function
gw1.XVI.2928.1	AT1G70280.2	XVI	None	12.3	3E-04	Both	24	NHL repeat-containing membrane protein
fgenesh4_pg.C_LG_III001134	AT3G59140.1	III	*MRP14*	4.7	0.003	183	72	Multidrug resistance-associated protein; ATP-type transporter
gw1.XVI.2910.1	AT2G28305.1	XVI	*LOG1*	3.3	0.013	182	72	Unknown function, expressed during 4 leaf senescence stage
eugene3.00031003	AT4G11450.1	III	*F25E4.70*	3.1	0.021	183	24,72	Unknown function, expressed during leaf senescence
estExt_fgenesh4_pg.C_LG_III1085	AT4G23496.1	III	*SP1L5*	0.32	0.016	182	72	Unknown function; potentially involved in anisotropic cell expansion
estExt_fgenesh4_pg.C_LG_III1187	AT4G24015.1	III	None	0.27	0.041	182	24	Zinc finger/unknown function
fgenesh4_pg.C_LG_III001060	No Hits	III	None	0.27	0.028	182	24	None
gw1.XVI.2786.1	AT5G03150.1	XVI	*JKD*	0.24	0.017	182	24,72	Zinc finger transcription factor; root development, regulation of cell division, cell differentiation, meristem growth
fgenesh4_pm.C_LG_XVI000330	AT2G37220.1	XVI	*F3G5.1*	0.13	0.001	182	24	Chloroplast RNA binding protein, response to ABA stimulus, response to cold

Zhang et al. 2006). For example, in this study leaf dry weight Cd and leaf dry weight C–Cd were mapped to the same position on LG XVI, but were not significantly correlated. The same was observed between root dry weight Cd and root dry weight C–Cd and between total dry weight Cd and total dry weight C–Cd (Tables 3 and 4). This could be due to a single gene or a regulatory element having pleiotropic effects on these traits and/or different genes within the QTL interval that might be independently controlling these traits, as one QTL encompasses hundreds of candidate genes (Wullschleger et al. 2005, Novaes et al. 2009). Identification of different QTLs on different linkage groups under control and Cd conditions for total dry weight and root dry weight suggested that the genes governing those traits were differentially controlled (Rae et al. 2006), and that the control exerted by these QTLs or genomic regions was dependent on Cd exposure. Finally, it is important to note that QTLs identified in this study account for a relatively small proportion of the total variation in Cd responses. Furthermore, since the population used for the current study was relatively small, QTL effects were probably overestimated (Beavis 1998), so much work remains to identify further mechanisms of Cd tolerance in *Populus*.

Candidate genes from QTL and microarray analyses

Transcriptional responses to experimental stimuli are one possible indicator of gene function. We therefore used microarray analyses to gain further insights into the potential roles of the many candidate genes within the QTL intervals. Of the 1571 genes contained within the Cd response QTL intervals, only 12 showed differential expression under Cd treatment. Among these were homologs of AT3G59140.1 (also known as multidrug resistance-associated protein 14), which were significantly overexpressed in the Cd-tolerant genotype (1–183) relative to the control after 72 h of treatment. Two closely related sequences matching this gene occur in the Cd tolerance QTL on LG III, presumably the result of recent tandem duplication. This gene belongs to the MRP (multidrug resistance-associated protein) subfamily of the superfamily of ATP binding cassette (ABC) transporters, which has been implicated in Cd sequestration (Klein et al. 2006). ATP binding cassette transporters facilitate the translocation of chelates including glutathione with Cd and Pb by ATP-driven processes (Lu et al. 1998, Tommasini et al. 1998, Kolukisaoglu et al. 2002, Martinoia et al. 2002, Klein et al. 2003). Interestingly, expressions of AtMRPs 3, 4, 6, 7 and 14 were up-regulated by Cd in *Arabidopsis* (Kolukisaoglu et al. 2002,

Bovet et al. 2003, Kim et al. 2007). Wojas *et al.* (2009) demonstrated the role of AtMRP7 in Cd tolerance and vacuolar sequestration in tobacco. Many yeast mutant complementation studies have demonstrated the role of AtMRPs in Cd tolerance, and these MRPs could be used as targets of genetic engineering to increase Cd tolerance and accumulation (Wojas et al. 2009). This MRP is therefore an excellent candidate for further functional characterization in *Populus*.

The remaining genes with differential expression in the QTL intervals have no known role in Cd homeostasis or transport. Four of these genes showed significant up-regulation, and five showed down-regulation (Table 6). Six of these have no known function for their closest homologs in model plant species. Interestingly, the gene that was most strongly up-regulated (12.3-fold) in response to 24-h Cd treatment in both genotypes encodes a putative membrane protein. The predicted protein sequence contains an NHL-repeat domain, which is a six-bladed propeller structure characteristic of a large number of proteins across all major kingdoms (Chaudhuri et al. 2008), including 35 predicted proteins in *Populus* and 30 in *Arabidopsis*. For example, the beta propeller is a prominent feature of receptor proteins like TolB, which is involved in antibiotic translocation in *Escherichia coli* (Kleanthous 2010) and of phosphate-chelating phytases found in a wide range of bacteria and fungi (Lung et al. 2005). The vast majority of plant proteins containing this domain have no known function, although some may be involved in alkaloid biosynthesis (Ma et al. 2006). Although no known connection with Cd tolerance has been demonstrated, it is intriguing that this protein is localized in cell membranes and is known to function in pore formation in microbes. Functional characterization could well lead to the identification of a novel metal tolerance mechanism in *Populus* and other plants.

Candidate genes from the QTL intervals

It is quite possible that genes responsible for interspecific differences in Cd tolerance would not show transcriptional responses that would be detectable in our experiments. In fact, some candidate genes with potential roles in Cd tolerance were present in the QTL intervals, but did not show differential expression in the microarray experiment. These include CAD2 (cadmium sensitive 2), HMA5 (heavy metal ATPase5), CHX18 (cation/hydrogen exchanger 18), GST10 (glutathione-S-transferase1) and GPX6 (glutathione peroxidase 6) (Table 6).

Table 6. Candidate genes identified from the QTL intervals with no detected change in expression in response to Cd treatment.

Populus gene	*Arabidopsis* gene	QTL LG	Gene symbol	Annotation
estExt_fgenesh4_pm.C_LG_III0405	AT4G23100.1	III	GSH1, CAD2	Glutamate-cysteine ligase
estExt_Genewise1_v1.C_LG_III2138	AT1G63440.1	III	HMA5	Heavy metal ATPase
fgenesh4_pm.C_LG_III000450	AT5G41610.1	III	CHX18	Monovalent cation:proton antiporter
estExt_fgenesh4_pg.C_LG_III1026	AT5G41210.1	III	GST10	Glutathione-S-transferase
fgenesh4_kg.C_LG_III000037	AT4G11600.1	III	GPX	Glutathione peroxidase

Phytochelatins (PCs) are one of the major chelators produced by plants upon exposure to heavy metals. These peptides chelate heavy metals and facilitate their sequestration in vacuoles, thus limiting heavy metal toxicity (Koprivova et al. 2002). Phytochelatins are synthesized from glutathione by phytochelatin synthase in the presence of heavy metal ions (Grill et al. 1986, Rauser 1999). Phytochelatins are induced most strongly by Cd, and plants without PCs showed hypersensitivity toward Cd (Howden et al. 1995*b*) and other heavy metals. The Cd–PC complexes are stored primarily in the root vacuoles, although translocation to shoots also occurs (Kim et al. 2007, Saathoff et al. 2011). The transporter responsible for vacuolar sequestration of Cd–PC complexes is yet to be definitively identified in plants, but ABC transporters like the MRPs have been implicated in plants (Klein et al. 2006, Wojas et al. 2009) and yeast (Mendoza-Cozatl et al. 2010). Cadmium sensitive 2 is a γ-glutamylcysteine synthetase, which is involved in glutathione biosynthesis, a precursor for PCs (Howden et al. 1995*a*). In *Arabidopsis*, *cad2-1* seedlings were more sensitive to Cd than wild-type plants, presumably because the mutants were unable to synthesize PCs (Howden et al. 1995*a*, 1995*b*). In this study, the presence of CAD2 in the Cd tolerance QTL on LG III suggests a role for this gene in Cd tolerance in *Populus*.

Populus contains three homologs of HMA5 (heavy metal ATPase5) (Migeon et al. 2010), including the one present within the LG III QTL. Some members of this family have been shown to transport Cd and enhance Cd tolerance when overexpressed. For example, HMA 1 through 4 transport Zn/Cd/Pb/Co in *Arabidopsis* and other plants (Cobbett et al. 2003). AtHMA4 enhanced Cd and Zn tolerance, transport, accumulation and root–shoot partitioning when overexpressed in tobacco (Siemianowski et al. 2011). Furthermore, overexpression of HMA3 enhanced Cd accumulation and tolerance in rice (*Oryza sativa* L.) (Miyadate et al. 2010). Although HMA5 is not known to transport Cd (Andres-Colas et al. 2006), it is possible that the specificities of these transporters are different in *Populus*, especially since the expression profiles of the putative homologs differ markedly between *Populus* and *Arabidopsis* (Migeon et al. 2010). This gene is therefore a viable candidate for functional characterization for Cd transport in *Populus*.

Another gene from the QTL interval on LG III has homology to CHX18, a cation transporter that belongs to a group of vacuolar membrane proteins in the cation exchanger (CAX) subfamily of proteins. These transport cations utilizing an H+/ATPase pump

that generates proton gradients across the vacuolar membrane, and are involved in calcium transport and metal homeostasis (Hirschi et al. 2000, Mei et al. 2009, Migeon et al. 2010). Cation exchangers have a broad range of metal substrates in plants, including significant roles in transporting Cd in roots and shoots (Korenkov et al. 2009). Overexpression of CAX genes enhances Cd tolerance and accumulation in *Arabidopsis* (Hirschi et al. 2000, Mei et al. 2009, Migeon et al. 2010), tobacco (Korenkov et al. 2007) and petunia (Wu et al. 2011).

A third gene from the QTL interval on LG III has homology to GST10, which belongs to the theta group of the superfamily of glutathione-S-transferases (GSTs). Glutathione-S-transferases are broadly important in protection against and detoxification of harmful xenobiotics, including heavy metals (Dixon et al. 2005, Brentner et al. 2008). GPX6 (glutathione peroxidase), together with reduced glutathione, reduces hydrogen peroxide and other organic peroxides to water. AtGPX6 was up-regulated under Cd exposure in *Arabidopsis* (Sarry et al. 2006), and is strongly induced under a wide range of abiotic stresses (Milla et al. 2003). Thus, homologs of AtGSTT1 and AtGPX6 might contribute to Cd tolerance in *Populus* through glutathione-mediated oxidative stress tolerance.

Conclusion

This study is the first to map QTL loci for Cd tolerance in the commercially and ecologically important genus *Populus*. This is an important initial step toward enhancing Cd tolerance in this genus for phytoremediation applications, either through future marker-assisted selection strategies, or through genetic engineering. We refined the list of potential candidate genes from the QTL intervals using whole-genome microarray analysis. This revealed putative metal transporters and genes of unknown function that can now be further characterized for their role in Cd transport and vacuolar sequestration. Further work should be geared toward establishing finer-scale associations for these genes by assaying polymorphisms in association populations within *P. trichocarpa* and *P. deltoides*, and by assaying informative candidate polymorphisms that differentiate these species. Furthermore, expression data should be gathered for a larger number of genotypes, and QTLs should be validated in different genetic and environmental backgrounds under both greenhouse and field conditions. Ultimately, this research could lead to the development of heavy metal-tolerant and/or hyperaccumulating *Populus* clones for reclaiming and remediating the extensive marginal lands contaminated with heavy metals.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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