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

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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 sulphhydryl groups (Vanasseche 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 F1 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 submersed beneath a dilute, continuously aerated nutrient solution, 0.1 × Johnson’s solution (Siddique et al. 1990). The nutrient solution was composed of 400 μM NH4NO3, 400 μM KNO3, 200 μM Ca(NO3)2·4H2O, 100 μM MgSO4·7H2O, 50 μM K2HPO4, 20 μM KCl, 25 μM H3BO3, 0.5 μM Na2MoO4·2H2O, 2 μM MnSO4·4H2O, 0.5 μM CuSO4·5H2O, 2 μM ZnSO4·7H2O, 0.5 μM CoCl2, 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/m2/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 CdCl2 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 CdCl2 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

\[ Y_{ij} = \mu + T_i + B_j + G_k + \varepsilon_{ijk} \]

where \( Y_{ij} \) 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 \( i \), \( \mu \) 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 \( \varepsilon_{ijk} \) 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 \( H^2 = V_G/(V_G + V_P) \) based on the variance components for genotype (\( V_G \)) and error (\( V_P \)) 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 \( \mu M \) CdCl\(_2\) and the remaining three containers were under control conditions. Three ramets from each genotype \( \times \) 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 \(-80^\circ C\).

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\textsuperscript{TM} 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
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 1. Summary statistics and heritability estimates for total dry weight and other measured traits along with their variance components.

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

\(^1\)Standard deviation.  
\(^2\)Standard error.  
\(^3\)Genetic variance.  
\(^4\)Phenotypic variance \((V_G + V_P)\).  
\(^5\)Broad-sense heritability.
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...
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 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.
Heritability estimates

Our broad-sense heritability estimate for total dry weight was somewhat lower than those for growth and yield traits measured for *Populus* (*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, 2006).
Table 4. Genetic (above diagonal) and phenotypic correlations among the variables measured.

<table>
<thead>
<tr>
<th>Traits all correlations</th>
<th>Total dry weight C</th>
<th>Root dry weight C</th>
<th>Leaf dry weight C</th>
<th>Stem dry weight C</th>
<th>Root volume C</th>
<th>Total dry weight C</th>
<th>Root dry weight C</th>
<th>Leaf dry weight C</th>
<th>Stem dry weight C</th>
<th>Root volume C</th>
<th>Total dry weight C–Cd</th>
<th>Root dry weight C–Cd</th>
<th>Leaf dry weight C–Cd</th>
<th>Stem dry weight C–Cd</th>
<th>Root volume C–Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total dry weight C</td>
<td>1</td>
<td>0.8838</td>
<td>0.9765</td>
<td>0.9463</td>
<td>0.7703</td>
<td>0.268</td>
<td>0.2562</td>
<td>0.2242</td>
<td>0.1859</td>
<td>0.9197</td>
<td>0.7918</td>
<td>0.8783</td>
<td>0.8825</td>
<td>0.5893</td>
<td></td>
</tr>
<tr>
<td>Root dry weight C</td>
<td>0.9022</td>
<td>1</td>
<td>0.796</td>
<td>0.8106</td>
<td>0.7862</td>
<td>0.2106</td>
<td>0.2084</td>
<td>0.1769</td>
<td>0.2149</td>
<td>0.1401</td>
<td>0.8235</td>
<td>0.9271</td>
<td>0.7186</td>
<td>0.757</td>
<td>0.6323</td>
</tr>
<tr>
<td>Leaf dry weight C</td>
<td>0.973</td>
<td>0.8317</td>
<td>1</td>
<td>0.8841</td>
<td>0.7102</td>
<td>0.2648</td>
<td>0.2556</td>
<td>0.2259</td>
<td>0.2669</td>
<td>0.1816</td>
<td>0.8968</td>
<td>0.7036</td>
<td>0.901</td>
<td>0.8232</td>
<td>0.537</td>
</tr>
<tr>
<td>Stem dry weight C</td>
<td>0.9431</td>
<td>0.8152</td>
<td>0.8895</td>
<td>1</td>
<td>0.7313</td>
<td>0.2674</td>
<td>0.2454</td>
<td>0.2148</td>
<td>0.3103</td>
<td>0.1941</td>
<td>0.8646</td>
<td>0.7131</td>
<td>0.7899</td>
<td>0.9278</td>
<td>0.5485</td>
</tr>
<tr>
<td>Root volume C</td>
<td>0.6294</td>
<td>0.626</td>
<td>0.6059</td>
<td>0.5817</td>
<td>1</td>
<td>0.2165</td>
<td>0.205</td>
<td>0.1793</td>
<td>0.2338</td>
<td>0.1961</td>
<td>0.7043</td>
<td>0.7131</td>
<td>0.6315</td>
<td>0.6758</td>
<td>0.7931</td>
</tr>
<tr>
<td>Total dry weight Cd</td>
<td>−0.3557</td>
<td>−0.3091</td>
<td>−0.2795</td>
<td>−0.2959</td>
<td>−0.0793</td>
<td>1</td>
<td>0.8005</td>
<td>0.961</td>
<td>0.8843</td>
<td>0.5168</td>
<td>−0.1319</td>
<td>−0.0947</td>
<td>−0.1627</td>
<td>−0.0663</td>
<td>−0.1229</td>
</tr>
<tr>
<td>Root dry weight Cd</td>
<td>−0.2765</td>
<td>−0.3656</td>
<td>−0.1976</td>
<td>−0.2234</td>
<td>−0.0563</td>
<td>0.8157</td>
<td>1</td>
<td>0.655</td>
<td>0.6965</td>
<td>0.4847</td>
<td>−0.0628</td>
<td>−0.1733</td>
<td>−0.0356</td>
<td>−0.0157</td>
<td>−0.1135</td>
</tr>
<tr>
<td>Leaf dry weight Cd</td>
<td>−0.3188</td>
<td>−0.2572</td>
<td>−0.3007</td>
<td>−0.2286</td>
<td>−0.0624</td>
<td>0.9129</td>
<td>0.6886</td>
<td>1</td>
<td>0.7598</td>
<td>0.4666</td>
<td>−0.161</td>
<td>−0.0729</td>
<td>−0.219</td>
<td>−0.0727</td>
<td>−0.1258</td>
</tr>
<tr>
<td>Stem dry weight Cd</td>
<td>−0.3146</td>
<td>−0.2638</td>
<td>−0.2208</td>
<td>−0.356</td>
<td>−0.0569</td>
<td>0.8559</td>
<td>0.721</td>
<td>0.689</td>
<td>1</td>
<td>0.4719</td>
<td>−0.0701</td>
<td>−0.0505</td>
<td>−0.071</td>
<td>−0.0667</td>
<td>−0.0792</td>
</tr>
<tr>
<td>Root volume Cd</td>
<td>−0.079</td>
<td>−0.1164</td>
<td>−0.0457</td>
<td>−0.0305</td>
<td>−0.1963</td>
<td>0.4798</td>
<td>0.4724</td>
<td>0.4145</td>
<td>0.46</td>
<td>1</td>
<td>−0.0194</td>
<td>−0.0446</td>
<td>−0.0259</td>
<td>0.0186</td>
<td>−0.4417</td>
</tr>
<tr>
<td>Total dry weight C–Cd</td>
<td>0.8291</td>
<td>0.7409</td>
<td>0.7669</td>
<td>0.7582</td>
<td>0.4363</td>
<td>−0.8175</td>
<td>−0.6584</td>
<td>−0.7426</td>
<td>−0.706</td>
<td>−0.3353</td>
<td>1</td>
<td>0.8533</td>
<td>0.9699</td>
<td>0.935</td>
<td>0.6565</td>
</tr>
<tr>
<td>Root dry weight C–Cd</td>
<td>0.7198</td>
<td>0.8329</td>
<td>0.6294</td>
<td>0.6347</td>
<td>0.4196</td>
<td>−0.6752</td>
<td>−0.8196</td>
<td>−0.5678</td>
<td>−0.591</td>
<td>−0.3515</td>
<td>0.8475</td>
<td>1</td>
<td>0.7372</td>
<td>0.7683</td>
<td>0.6802</td>
</tr>
<tr>
<td>Leaf dry weight C–Cd</td>
<td>0.7977</td>
<td>0.6723</td>
<td>0.803</td>
<td>0.69</td>
<td>0.412</td>
<td>−0.7424</td>
<td>−0.5519</td>
<td>−0.8099</td>
<td>−0.5664</td>
<td>−0.2869</td>
<td>0.9358</td>
<td>0.742</td>
<td>1</td>
<td>0.8569</td>
<td>0.5939</td>
</tr>
<tr>
<td>Stem dry weight C–Cd</td>
<td>0.7824</td>
<td>0.6716</td>
<td>0.6941</td>
<td>0.8424</td>
<td>0.405</td>
<td>−0.682</td>
<td>−0.558</td>
<td>−0.5429</td>
<td>−0.8034</td>
<td>−0.2839</td>
<td>0.8902</td>
<td>0.7452</td>
<td>0.7662</td>
<td>1</td>
<td>0.6068</td>
</tr>
<tr>
<td>Root volume C–Cd</td>
<td>0.474</td>
<td>0.4948</td>
<td>0.4376</td>
<td>0.4119</td>
<td>0.796</td>
<td>−0.3495</td>
<td>−0.3295</td>
<td>−0.2978</td>
<td>−0.3221</td>
<td>−0.7498</td>
<td>0.5016</td>
<td>0.5003</td>
<td>0.4553</td>
<td>0.4488</td>
<td>1</td>
</tr>
</tbody>
</table>
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 1,571 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 che- Hen, including glutathione with Cd and Pb by ATP-driven processes (Lu et al. 1998, Tommasini et al. 1998, Kolukisaoglu et al. 2002, Martinio 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,

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**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.**

<table>
<thead>
<tr>
<th>Populus gene</th>
<th>Arabidopsis gene</th>
<th>QTL LG</th>
<th>Gene symbol</th>
<th>Fold change</th>
<th>Pfp</th>
<th>Clone</th>
<th>Time (h)</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>gw1.XVI.2928.1</td>
<td>AT1G70280.2</td>
<td>XVI</td>
<td>None</td>
<td>12.3</td>
<td>3E-04</td>
<td>Both</td>
<td>24</td>
<td>NHL repeat-containing membrane protein</td>
</tr>
<tr>
<td>fgenesh4_pg.C_LG_III001134</td>
<td>AT3G59140.1</td>
<td>III</td>
<td>MRP14</td>
<td>4.7</td>
<td>0.003</td>
<td>183</td>
<td>72</td>
<td>Multidrug resistance-associated protein; ATP-type transporter</td>
</tr>
<tr>
<td>gw1.XVI.2910.1</td>
<td>AT2G28305.1</td>
<td>XVI</td>
<td>LOG1</td>
<td>3.3</td>
<td>0.013</td>
<td>182</td>
<td>72</td>
<td>Unknown function, expressed during 4 leaf senescence stage</td>
</tr>
<tr>
<td>eugene3.00031003</td>
<td>AT4G11450.1</td>
<td>III</td>
<td>F25E4.70</td>
<td>3.1</td>
<td>0.021</td>
<td>183</td>
<td>24,72</td>
<td>Unknown function, expressed during leaf senescence</td>
</tr>
<tr>
<td>estExt_fgenesh4_pg.C_LG_III1085</td>
<td>AT4G23496.1</td>
<td>III</td>
<td>SPl5</td>
<td>0.32</td>
<td>0.016</td>
<td>182</td>
<td>72</td>
<td>Unknown function; potentially involved in anisotropic cell expansion</td>
</tr>
<tr>
<td>estExt_fgenesh4_pg.C_LG_III1187</td>
<td>AT4G24015.1</td>
<td>III</td>
<td>None</td>
<td>0.27</td>
<td>0.041</td>
<td>182</td>
<td>24</td>
<td>Zinc finger/unknown function</td>
</tr>
<tr>
<td>fgenesh4_pg.C_LG_III001060</td>
<td>No Hits</td>
<td>III</td>
<td>None</td>
<td>0.27</td>
<td>0.028</td>
<td>182</td>
<td>24</td>
<td>Zinc finger transcription factor; root development, regulation of cell division, cell differentiation, meristem growth</td>
</tr>
<tr>
<td>gw1.XVI.2786.1</td>
<td>AT5G03150.1</td>
<td>XVI</td>
<td>JKD</td>
<td>0.24</td>
<td>0.017</td>
<td>182</td>
<td>24,72</td>
<td>Chloroplast RNA binding protein, response to ABA stimulus, response to cold</td>
</tr>
<tr>
<td>fgenesh4_pm.C_LG_XVI000330</td>
<td>AT2G37220.1</td>
<td>XVI</td>
<td>F3G5.1</td>
<td>0.13</td>
<td>0.001</td>
<td>182</td>
<td>24</td>
<td>Chloroplast RNA binding protein, response to ABA stimulus, response to cold</td>
</tr>
</tbody>
</table>
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 ToIB, 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).

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. 1995b) 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-Cozat et al. 2010). Cadmium sensitive 2 is a γ-glutamylcysteine synthetase, which is involved in glutathione biosynthesis, a precursor for PCs (Howden et al. 1995a). 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. 1995a, 1995b). 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, HMA1 through 4 transport Zn/Cd/Pb/Cu 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 AtGST1 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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References


