Poplar maintains zinc homeostasis with heavy metal genes \textit{HMA4} and \textit{PCS1}

Joshua P. Adams\textsuperscript{1,*}, Ardeshir Adeli\textsuperscript{2}, Chuan-Yu Hsu\textsuperscript{1}, Richard L. Harkess\textsuperscript{3}, Grier P. Page\textsuperscript{4}, Claude W. dePamphilis\textsuperscript{5}, Emily B. Schultz\textsuperscript{1} and Cetin Yuceer\textsuperscript{1}

\textsuperscript{1} Department of Forestry, Mississippi State University, Mississippi State, MS 39762, USA
\textsuperscript{2} USDA-ARS, Mississippi State, MS 39762, USA
\textsuperscript{3} Department of Plant and Soil Sciences, Mississippi State University, Mississippi State, MS 39762, USA
\textsuperscript{4} RTI International, Atlanta, GA 30341-5533, USA
\textsuperscript{5} Department of Biology, Pennsylvania State University, University Park, PA 16802, USA

* To whom correspondence should be addressed. E-mail: jpa18@msstate.edu

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Abstract

Perennial woody species, such as poplar (\textit{Populus} spp.) must acquire necessary heavy metals like zinc (Zn) while avoiding potential toxicity. Poplar contains genes with sequence homology to genes \textit{HMA4} and \textit{PCS1} from other species which are involved in heavy metal regulation. While basic genomic conservation exists, poplar does not have a hyperaccumulating phenotype. Poplar has a common indicator phenotype in which heavy metal accumulation is proportional to environmental concentrations but excesses are prevented. Phenotype is partly affected by regulation of \textit{HMA4} and \textit{PCS1} transcriptional abundance. Wild-type poplar down-regulates several transcripts in its Zn-interacting pathway at high Zn levels. Also, overexpressed \textit{PtHMA4} and \textit{PtPCS1} genes result in varying Zn phenotypes in poplar; specifically, there is a doubling of Zn accumulation in leaf tissues in an overexpressed \textit{PtPCS1} line. The genomic complement and regulation of poplar highlighted in this study supports a role of \textit{HMA4} and \textit{PCS1} in Zn regulation dictating its phenotype. These genes can be altered in poplar to change its interaction with Zn. However, other poplar genes in the surrounding pathway may maintain the phenotype by inhibiting drastic changes in heavy metal accumulation with a single gene transformation.

Key words: Heavy metal, heavy metal transporter, phytochelatin synthase, poplar nutrition.

Introduction

Plant intake of essential heavy metals involves complex regulation that ensures adequate availability for various biochemical reactions while avoiding toxicity from excesses. Zinc (Zn) is one of the most prevalent essential heavy metals used by plants and plays important functions in >300 enzymes (Vallee and Falchuk, 1993; Coleman, 1998). However, excesses of Zn can be antagonistic to absorption of other essential elements due to transport and competition for storage space (Clemens \textit{et al.}, 1999; Wang \textit{et al.}, 2009a). Furthermore, metals can directly cause cellular damage through corruption of DNA integrity (Michaelis \textit{et al.}, 1986; Hartwig \textit{et al.}, 2002).

Plants appear to use the same basic mechanisms to regulate heavy metals. A common transporter associated with metal accumulation is an 1186-amino acid P-type heavy metal ATPase (HMA4) that plays a role in heavy metal transport efflux of many metals including Zn, copper (Cu), and iron (Fe) (Bernard \textit{et al.}, 2004; Andres-Colas \textit{et al.}, 2006). Furthermore, increases in \textit{HMA4} expression have been shown to increase the tolerance to and accumulation of metals (Hanikenne \textit{et al.}, 2008; Grispen \textit{et al.}, 2011). Detoxification of heavy metals, especially non-essential heavy metals, is often attributed to post-translationally synthesized thiol-rich short metal binding proteins.
termed phytochelatins (PCs) (Grill et al., 1985), which are synthesized by phytochelatin synthase (PCS), which is one of the most frequently identified genes during a cDNA library screen for cadmium (Cd) tolerance (Bernard et al., 2004). The role of PCs in heavy metal tolerance has been observed in many species including common velvetgrass (Holcus lanatus) (Hartley-Whitaker et al., 2001), Azuki bean (Vigna angularis) (Inouhe et al., 2000), wheate (Triticum aestivum) (Clemens et al., 1999), Indian mustard (Brassica juncea) (Gasic and Korban, 2007), rapeseed (Brassica napus) (Mendoza-Cozatl et al., 2008), and Arabidopsis thaliana (Vatamaniuk et al., 1999) in which the metal binds with sulphur to form a large PC complex (Freedman et al., 2004; Di Baccio et al., 2005; Blum et al., 2007). More recently, Zn excesses have been specifically shown to be mediated by an active role of PC production in A. thaliana (Tennstedt et al., 2009).

Plants have evolved three main strategies for heavy metal interactions including indicators, excluders, and hyperaccumulators (Baker, 1981). These phenotypes are controlled by both the molecular constituency of the plant and its ecological patterns. Species such as Oenothera biennis, Commelina communis, Silene vulgaris, and Rumex acetosella are excluders at the molecular/physiological level that exclude metal (Wenzel et al., 2003; Wei et al., 2005), while celery (Apium graveolens), pakchoi (Brassica chinensis), and Chinese cabbage (Brassica rapa) exhibit signs of phytotoxicity at relative low ranges (e.g. <200 mg Zn kg⁻¹) (Long et al., 2003) and exhibit an ecological exclusion phenotype by not existing on metalliferous sites. A second indicator phenotype allows the plant to grow on elevated-metal sites by excluding intake of heavy metal excesses. This allows species to subsist on a range of sites with various metal loads. The final hyperaccumulating phenotype is exhibited by species, generally with small-biomass, such as Thlaspi caerulescens and Arabidopsis halleri that have found a niche in over-abundant metal environments (Chaney et al., 2000) and can accumulate as much as 10-fold the Zn relative to non-hyperaccumulating species (Lasat et al., 1998). High-biomass tree species, such as poplar, are generally thought to have a metal indicator phenotype with Zn accumulation in stems occurring at low levels [<200 mg kg⁻¹ (Sebastiani et al., 2004; Laureysens et al., 2005)] and at extremely high levels [>3000 mg kg⁻¹ (Pierzynski et al., 2002; Unterbrunner et al., 2007)] depending on the site. However, the specific manner by which poplar regulates heavy metal accumulation is unknown.

The mechanisms regulating plant–zinc interactions have been extensively studied for small, annual species especially in species with hyperaccumulating phenotypes (e.g. T. caerulescens). However, these mechanisms are relatively unexplored in large, perennial species such as the high biomass Populus trichocarpa, which is the model forest species. The overall objective of this study is to elucidate the effect of poplar heavy metal genes HMA4 and PCS1 as they relate to Zn interaction. This is first accomplished through genomic comparisons with phylogenetic analysis allowing gene identification and assessment of evolutionary relationships between species with varying phenotypes. Quantification of transcript abundance modulations in poplar relative to other species further delineates how poplar interacts with Zn. Finally, the effect on the phenotype and physiology of poplar is quantified after overexpression of HMA4 and PCS1. The results of these studies lead to a further understanding of the poplar phenotype and are a first step towards identification of avenues for creation of a hyperaccumulating poplar phenotype for use in phytoremediation.

Materials and methods

Phylogenetic and pathway analyses

Phylogenetic and pathway analyses were used to identify the best HMA4 and PCS1 homologues in poplar and place them in both an evolutionary and a pathway context. Poplar genes with sequence homology to TcHMA4 and TcPCS1 in T. caerulescens were retrieved from keyword searches, eudicot gene cluster searches, and sequence blasts (i.e. BlastP) of the NCBI database (www.ncbi.nlm.nih.gov) and the Phytozome v.5.0 database (www.phytozone.net/poplar.php), which includes the P. trichocarpa genome v.2.0. Transcript sequences were retrieved and imported into MEGA 4 (Tamura et al., 2007), translated into amino acid residues, and aligned with ClustalW (Thompson et al., 1997) using the Gonnet protein weight matrix. Using the maximum composite likelihood substitution model, a phylogenetic tree was generated via the neighbor-joining method with bootstrapping (1000 replicates). Each tree was rooted on a rice (Oryza sativa) orthologue annotated as a functional ZIP gene. This process was conducted for both T. caerulescens genes and resulted in two independent phylogenic trees. HMA4 and PCS1 homologues were further analysed for similarity by comparing predicted HMA4 transmembrane helices using TMMTOP (Tusnady and Simon, 1998) and by counting conserved cysteine motifs found in PCS (Clemens, 2006).

Pathway analysis was conducted to search for a broader network of genes with which HMA4 and PCS1 may interact to affect Zn regulation. A heavy metal pathway was identified using a microarray dataset of overexpressed genes from heavy metal-hyperaccumulating A. halleri relative to homologues in non-hyperaccumulating A. thaliana (Becher et al., 2004) and a study comparing heavy metal-hyperaccumulating T. caerulescens and non-hyperaccumulating Thlaspi arvense (Hammond et al., 2006). Transcripts differentially expressing >2.0-fold or <0.5-fold were selected (respectively, 499+5985 transcripts) and imported with their respective poplar expression ratios (detailed in the following section) into Pathway Studio v 7.0 (Ariadne Genomics, Rockville, MD, USA). Genes were selected from the imported transcripts that were related to gene ontology (GO) groups that included cation, Zn, and Cd interactions. From these selections, a pathway was established using ResNet 2.0 database (Ariadne Genomics), which included transcripts not necessarily in the previous microarray studies. A section of the large pathway was selected that contained transcripts shown through previous studies to be related to physiological traits associated with plant–metal interactions. The final pathway was tested with Fisher’s exact test to determine whether the genes in this condensed pathway significantly enriched specific GO annotation groups. Poplar homologues for the genes were found by blasting (i.e. BlastP) this protein sequence into the poplar database.

Zn challenge assays and nutrient/transcript analyses

Poplar plants were established and assayed with varying Zn concentrations for transcript and nutrient analyses. Six- to eight-inch
amplification points (Ct values) with the standard curves to

and 60

ments were an unmodified Hoagland’s solution containing 1

decision making. The three Zn treat-

prevented excess solution leaching while providing adequate nutrients

for the plant. Plants were then randomly assigned to three metal
treatment with on-column DNase I treatment (Promega, Madison,

WI, USA) and RNA clean-up via RNeasy Plant Mini Kit (Qiagen,

Valencia, CA, USA). RNA (1 μg) was reverse-transcribed using
M-MLV reverse transcriptase (Promega). Transcripts were
analysed via qPCR using Power SYBR-Green (Applied Biosystems,
Carlsbad, CA, USA). Ubiquitin transcript (UBQ) amplification was used as an internal control for both qPCR
analyses (Infante et al., 2008). Primers used in Power SYBR-Green
reactions were verified for specificity via ABI Prism dissociation
curve analysis software and also by separating the PCR products
by electrophoresis on a 1% agarose gel. All primers used
throughout this study were preliminarily tested for specificity
before use and can be found in Supplementary Table S1
(Supplementary data available at JXB online). Amplification
reactions were performed using ABI 7500 Fast Real-Time PCR
System (Applied Biosystems) relative transcript quantification.
Each reaction mixture contained 0.5 μl of cDNA template, 5.0 μl
of SYBR-Green Master Mix, 0.25 μl of both 10 μM primers, and
4.0 μl of ddH2O. The qPCR programme consisted of an initial
incubation (95 °C for 10 min) followed by 35 cycles of 95 °C for 15
s and 60 °C for 1 min. Each reaction was independently repeated
at least three times. Standard curves were constructed using serial
dilutions of cDNA. Transcripts were analysed by using threshold
amplification points (Ct values) with the standard curves to
calculate transcript concentration. Subsequently, the ratio of target
transcript concentration to UBQ transcript concentration was

Zn and other nutrients in poplar were compared across the Zn
treatments. Remaining tissues from the metal challenge were analysed
for nutrient concentrations. At least three samples of each tissue
(root, stem, and leaf) at each metal exposure level (1 μM, 10 μM,
and 1 mM) were used. Samples were taken from –80 °C storage, dried for
48 h at 50 °C, and ground into a fine powder with a mortar and
pestle. Samples were then processed with a dry ash procedure (Isaac,
1983) to obtain nutrient concentrations [Ca, Cu, Fe, potassium (K),
magnesium (Mg), sodium (Na), phosphorus (P), and manganese
(Mn) mg kg⁻¹] and with a nitric acid extraction procedure to obtain
Zn concentrations (Ebbs and Kochian, 1998). All nutrients from the
leaf, stem, and root tissues were determined spectrophotometrically
using an inductively coupled plasma (ICP) emission spectrophotom-
eter (Thermo Jarrel Ash Iris Advantage ICP, Houghton, MI, USA).
A mixed general linear model was used to compare the nutrient
covariances between the fixed and metal exposure effects
and the random biological (plant) effect using SAS v 9.1. Nutrient
accumulation and compartmentalisation were compared by
rescaling all nutrient concentrations and calculating Euclidean
distances between each tissue, sample time, and Zn treatment from
dichograms was built using R software (R Development
Core Team, 2008).

Overexpression of PtPCS1 and PtHMA4 genes in poplar
PtPCS1 and PtHMA4 were isolated, modified with an
overexpression promoter, and transformed back into poplar
plants. Total RNA was extracted from P. trichocarpa cv. Ngualy
using RNeasy Plant Mini Kit. Total RNA (1 μg) was reverse-
transcribed using M-MLV reverse transcriptase (Promega).
Forward and reverse primers were designed based on each
database cDNA sequence (Supplementary Table S2 at JXB online).
The full-length coding sequence of each gene was
amplified using PCR with Pfu DNA polymerase (Stratagene, La
Jolla, CA, USA) using a three-step (94 °C for 20 s, 60 °C for 20 s,
and 72 °C for 145 s) 30-cycle programme. PCR products were
separated on a 1% agarose gel, extracted, cloned into the pGEM-T
Easy vector (Promega), and sequenced with a CEQ 8000 Genetic
Analysis System (Beckman-Coulter, Fullerton, CA, USA).
Additional restriction site sequences (Supplementary Table S2 at
JXB online) were designed for the termini of each gene and added
using a second PCR amplification reaction. This PCR reaction used
the cloned gene as the template, primer pairs (with added restriction
sites), and Pfu DNA polymerase in a 30-cycle programme (94 °C for
20 s, 60 °C for 20 s, and 72 °C for 145 s). Each PCR product was
purified from agarose gel, cloned into pGEM-T Easy vector, and
resequenced to ensure absence of sequence polymorphisms.
Each gene construct was digested with appropriate restriction enzymes
and cloned into the binary vector pBI121 (Clontech, Palo Alto, CA,
USA). In both constructs, the gene replaced the β-glucuronidase
(GUS) reporter gene downstream of the cauliflower mosaic virus
(CaMV) 35S constitutive expression promoter and upstream of the
nopaline synthase promoter (NOS) terminal sequence. The binary
vectors PSSS-PtPCS1 and PSSS-PtHMA4 were transformed into
Agrobacterium tumefaciens strain C58, which subsequently was used
to transform the hybrid poplar clone INRA 717-1B4 (Populus
tremula × Populus alba).

Poplar transformation was performed using an established
transformation procedure (Han et al., 2000). Between 5 and 10
independent lines were generated and transgene insertion was
confirmed with genomic PCR using the respective gene’s forward
primer and the NOS terminal reverse primer. Expression of the
transgene was tested with qPCR using the previously detailed
procedures.

Zinc challenge of plants overexpressing PtHMA4 and PtPCS1
Overexpression lines were assayed with various concentrations of
Zn to assess gene function. An average overexpressing poplar line
of both constructs and two controls (wild-type and pBI101 lines)
were used to assay rooting ability as a proxy for tolerance in
poplar line contained the empty vector pBI101 with no promoter
elevated Zn conditions (Gasic and Korban, 2007). The pBI101
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by 4 (line) factorial design with four replications was used in which lines were tested across three metal concentrations, and five line replications were nested within each plate. They were grown for 30 d in growth chambers with environmental conditions: 8/16 h (night/day) day length, 25 °C, and 45% relative humidity. Throughout the course of the study, rooting emergence, leaf emergence, and time to rooting emergence were recorded and root weight was measured after completion of the 30 d.

The three selected PtPCS1 lines, representing high, medium, and low overexpression levels, and two control lines were assayed for Zn accumulation. Twelve plants from these three lines and two control lines were grown for 2-4 months from March to July in potting soil under controlled greenhouse conditions as previously described. Each plant was transplanted into sterile, inert sand substrate in a 12.25 cm pot with an attached water-catching basin and supplemented with Hoagland’s Solution, which was renewed every other day for 1 week prior to starting the metal assay. Again, a water misting system that misted plants and soil surface every 5 min for 30 s at a rate of 15 l h⁻¹ was used.

These plants were randomly assigned to the metal assay using a 4 (line) by 3 (Zn treatment) factorial design. Four plants from each line were randomly assigned to three Zn levels. The Zn treatments were conducted by applying 25 ml of three different rates of ZnSO₄ line were randomly assigned to three Zn levels. The Zn treatments (lines) by 3 (Zn treatment) factorial design. Four plants from each

In the pathway analysis, 6484 transcripts from previous studies represented 1545 GO annotation groups. Groups related to heavy metals included cation transport, cation transmembrane transporter activity, Cd ion transport, Cd ion transmembrane transport, Zn ion transport, Zn-mediated transmembrane transport, Zn ion transporter activity, and Zn ion binding. The condensed pathway (Supplementary Table S4 at JXB online) contained five transcripts that contributed significantly to ATPase activity, Cd ion transmembrane transporter activity, Zn ion transmembrane transporter activity, and Zn ion transport with P<0.05 for these GO annotation groups (Supplementary Table S5 at JXB online). Two genes in the pathway were PtHMA4 and PtPCS1. The other genes included two related to ATPase activity (APY2 and AHA3) and one dehydrase (ELI3-1).

Poplar reacts to Zn changes both in metal accumulation and in transcriptional abundance of the two identified genes, PtHMA4 and PtPCS1, and their broader interacting pathway. Metal and nutrient accumulation by tissue, day, and Zn treatment demonstrate that localization of most nutrients occurs in poplar roots or leaves (Fig. 2A; Supplementary Table S6 at JXB online), while stems had relatively depressed loads of all nutrients except for Na, P, and K. Leaves also had very high concentrations of these three nutrients as well as Mg, Mn, and Ca, causing these six nutrients to cluster together. The other three nutrients, Zn, Cu, and Fe, cluster together and were primarily concentrated in root tissues. Within the cluster containing Zn, differences were seen between Zn and the other two metals. Zn accumulation in these wild-type poplars was

Results

Poplar contains heavy metal-related genes

Two genes closely related to TcHMA4 and TcPCS1 were identified in poplar and are part of a broader pathway of genes. Poplar genes POPTR_0006s07650.1 and POPTR_0014s18420.1 share sequence homology with two established T. caerulescens genes, TcHMA4 and TcPCS1, respectively. Poplar has 11 HMA4 genes that are more closely related to TcHMA4 than the rice outgroup. However, HMA1 (POPTR_0006s07650) was more closely related to TcHMA4 than the other 10 genes (Fig. 1A). This transcript (PtHMA4) was the best hit during the protein BLAST and was the only poplar gene that was in the clade close to HMA4 genes of species in the Brassicaceae family. Predicted topologies of the proteins show a wide array of transmembrane loops from five to nine (Supplementary Table S3 at JXB online). The most conserved area is among the HMA4s of the mustard species with nine transmembrane loops. The closest poplar homologue only has seven loops. The PCS phylogeny only included two possible poplar homologues. The locations of these two genes in the tree indicate that PCS1 (POPTR_0014s18410.1) is closest to TcPCS1 (Fig. 1B). However, PCS1 (POPTR_0014s18420.1) was the best TcPCS1 BLAST hit in the poplar genome, had the greatest percentage protein identity (67% versus 58%), had the same number of conserved cysteine motifs, and thus, was selected as the PCS homologue.

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significantly ($P<0.001$) affected by plant tissue type (leaf, stem, and root) and by Zn exposure concentration (Fig. 2B), but not affected ($P=0.85$) by exposure time (24 h and 48 h). Greatest accumulation differences occurred in roots under 1 mM Zn exposure, which accumulated more Zn than the other treatments. These differences decreased in the aerial tissues and between the 1 µM and 10 µM Zn exposures. An opposing trend was observed with closely related Cu. There was a significant tissue–Zn treatment interaction effect ($P=0.005$), in which high Zn levels negatively affected Cu absorption by roots (Fig. 2C).

Preliminary transcript assays in leaves mirrored the insignificant Zn accumulation changes in the leaf and were not studied further. *PtHMA4* and *PtPCS1* were assayed in stem and root tissues. Stem tissues had significant *PtHMA4* and *PtPCS1* changes in transcript abundance across the Zn gradient ($P<0.001$) (Fig. 2D; Supplementary Table S7 at *JXB* online). These differences were greatest between the 1 mM treatment level and lower 1 µM and 10 µM treatments. The transporter *PtHMA4* had a 0.63-fold decrease in expression between the highest and lowest concentrations with greatest abundance in roots (2.4-fold the abundance in stems). *PtPCS1* was also significantly decreased when exposed to 1 mM Zn with a 4.8-fold drop in transcript abundance in stems. Overall, stems contain the highest *PtPCS1* abundance with an average 17.2 times the amount in roots.

In roots, where metal differences were greatest, three additional genes identified through the pathway analysis were analysed. Overall, the transcript abundance pattern was the same as that seen in stems in which expression decreased significantly with increases in Zn exposure (Fig. 2E). The notable exception was *PtPCS1*, which did not have a significant ($P=0.18$) increase as metal concentration changed.

**Overexpression of HMA4 and PCS1 affect poplar–Zn interaction during early development**

Altering expression of *PtHMA4* and *PtPCS1* affected Zn interaction with regard to tolerance and accumulation. Independent lines of poplar containing *PtPCS1* (Fig. 3A) and *PtHMA4* (Fig. 3B) under constitutive control were successfully created. The connection between *PtPCS1* and *PtHMA4* in Fig. 2D was quantitatively investigated for a transcriptional link via qPCR using tissues from both corresponding to the gene names are located in Supplementary Table S3 at *JXB* online. Species include: Ah, *A. halleri*; Al, *Arabidopsis lyrata*; At, *A. thaliana*; Tj, *Thlaspi japonicum*; Tc, *T. caerulescens*; Bj, *B. juncea*; Bn, *B. napus*; Cp, *Carica papaya*; Rc, *Ricinus communis*; Me, *Manihot esculenta*; Vv, *Vitis vinifera*; Cs, *Cucumis sativus*; Sr, *Sesbania rostrata*; Gm, *Glycine max*; Mt, *Medicago truncatula*; Mg, *Mimulus guttatus*; Nt, *Nicotiana tabacum*; Ng, *Nicotiana glauca*; Pt, *Populus deltoides*; and Os, *Oryza sativa*.
Fig. 2. Nutrient accumulation of *P. trichocarpa* cv. Nisqually sampled under Zn treatments of 1 μM, 10 μM, and 1 mM (*n* = 59). (A) Relationships based on average Euclidian distance of nutrient accumulation across 2 d, metal exposure, and tissue type and their relative concentrations. (B) Zn and (C) Cu accumulation in root, stem, and leaf tissues across both sampling periods. (D) Transcriptional abundance changes of *PtHMA4* and *PtPCS1* in stem tissue where ratios of gene/ubiquitin, ranging between 1.6- and 45.4-fold, are indicated by colour. (E) The pathway represents the connections of these same two genes and closely connected poplar genes to external stimuli and physiological effects with red to blue coloration denoting relative, high to low, magnitude change in gene/ubiquitin
con structs and controls. As expected, PtPCS1 expression was significantly ($P=0.02$) higher (3.8-fold) in the P35S:PtPCS1 lines relative to either the P35S:PtHMA4 lines or the controls (Fig. 3C). Expression of PtPCS1 in the P35S:PtHMA4 construct was not significantly different from the control plants. PtHMA4 expression among the two lines and control behaved differently. As expected, there was an overall difference ($P=0.03$) with PtHMA4 expression levels in the P35S:PtHMA4 construct nearly 21 times that found in the wild-type plants. However, the P35S:PtPCS1 construct had elevated PtHMA4 levels as well. The P35S:PtPCS1 construct expressed PtHMA4 an average seven times more than the control plants, and was statistically different from neither the P35S:PtHMA4 lines nor the wild-type lines. This co-increase in both transcripts may indicate that PtPCS1 has a downstream positive effect on PtHMA4 in poplar.

Controls and Line 1 of both the P35S:PtPCS1 and P35S:PtHMA4 constructs were assayed to study early development. After only a few (3–5) days, visual negative effects, including reduced stem or root development and chlorosis of leaves, were evident for all lines exposed to 1 mM Zn (Fig. 3D). After 30 d, rooting was rare for any plantlets exposed to the 1 mM Zn treatment (~2% across all lines), and those few that did root only rooted in the final days of the study. Therefore, analysis was only conducted on the 1 μM and 10 μM Zn concentrations. Visual differences were slightly evident between the 1 μM and 10 μM Zn treatments with plants receiving the lower concentrations having healthier, darker green leaves. P35S:PtHMA4 was visually the healthiest with dark green leaves and vibrant, spreading roots. Wild-type plants were not as vigorous but grew slightly better than the P35S:PtPCS1 lines. P35S:PtPCS1 plants did survive and root, but their roots were generally smaller and were less apt to spread.

Leaf emergence from the stem segment supports the visual differences seen between lines. The percentage per plate of stems that formed leaves was significantly different ($P=0.003$) between lines, with P35S:PtHMA4 having the greatest percentage (89%) and P35S:PtPCS1 and controls (28–30%) not exhibiting any significant difference (Fig. 3E). Rooting percentage was significantly affected by an interaction ($P<0.001$) between Zn concentration and lines. Rooting percentage was stable between the two metal concentrations (Fig. 3F). However, the two transgenic lines showed opposing Zn effects in which the P35S:PtPCS1 line had a large percentage increase (47%) at the higher Zn concentration and P35S:PtHMA4 line had a large decrease (32%) at the higher concentration. Plantlets that rooted did so at significantly different times by line ($P=0.01$) (Fig. 3G). On average, the P35S:PtPCS1 line and controls (19–21 d) rooted at the same time, while the P35S:PtHMA4 line rooted much earlier (13 d).

Overexpressed PCS1 increases Zn concentration in poplar

The metal assay on older plants was conducted on three lines of P35S:PtPCS1. Lines 1, 2, and 3 were selected to represent respectively medium, low, and high concentrations of the overexpressed PtPCS1 (Fig. 3A). In this long-term study, both transgenic and control poplars were resilient to 1 mM and 10 mM Zn exposure with no mortality. This is in contrast to the near complete suspension of development (i.e. rooting and leafing) at 1 mM in the rooting study. Still, biomass partitioning was affected by line and Zn concentration exposure independently (Supplementary Table S8 at JXB online). The effect on partitioning was not observed in the root-to-stem ratio ($P=0.169$), but was significant with regard to the root-to-(stem+leaf) ratio ($P=0.002$) indicating that a shift was occurring in foliar weight (Fig. 4A). In this case, the ratio for the control plants was significantly reduced compared with all the transgenic lines; thus, the P35S:PtPCS1 lines had less foliar biomass relative to the weight of other tissues. Zn exposure affected both ratios significantly [$P=0.05$ and 0.04 for root-to-stem and root-to-(stem+leaf), respectively] (Fig. 4B).

Since both ratios were significantly affected by metal concentration, there was a decrease in root biomass growth at the elevated Zn concentrations.

The physiological effect evident during visual inspection was a change in leaf and stem coloration in which plants exposed to high Zn levels began to express brown/red coloration in foliage compared with the normal green (Fig. 4C) However, these differences were only visually evident among the Zn treatments and not among lines. Thus, leaf differences among lines and levels of Zn exposure were quantified by measuring leaf SR and TR through the course of the assay. Both SR and TR were significantly affected by metal concentration on older plants was conducted on three lines of P35S:PtPCS1. Lines 1, 2, and 3 were selected to represent respectively medium, low, and high concentrations of the overexpressed PtPCS1 (Fig. 3A). In this long-term study, both transgenic and control poplars were resilient to 1 mM and 10 mM Zn exposure with no mortality. This is in contrast to the near complete suspension of development (i.e. rooting and leafing) at 1 mM in the rooting study. Still, biomass partitioning was affected by line and Zn concentration exposure independently (Supplementary Table S8 at JXB online). The effect on partitioning was not observed in the root-to-stem ratio ($P=0.169$), but was significant with regard to the root-to-(stem+leaf) ratio ($P=0.002$) indicating that a shift was occurring in foliar weight (Fig. 4A). In this case, the ratio for the control plants was significantly reduced compared with all the transgenic lines; thus, the P35S:PtPCS1 lines had less foliar biomass relative to the weight of other tissues. Zn exposure affected both ratios significantly [$P=0.05$ and 0.04 for root-to-stem and root-to-(stem+leaf), respectively] (Fig. 4B).

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The physiological effect evident during visual inspection was a change in leaf and stem coloration in which plants exposed to high Zn levels began to express brown/red coloration in foliage compared with the normal green (Fig. 4C) However, these differences were only visually evident among the Zn treatments and not among lines. Thus, leaf differences among lines and levels of Zn exposure were quantified by measuring leaf SR and TR through the course of the assay. Both SR and TR were significantly affected by an interaction of line and concentration ($P=0.03$ and 0.024, respectively). SR change fluctuated the least across time and Zn concentration in Line 3 and the control (Fig. 4D). These showed only a slight increase at the highest Zn exposure. By the end of the study there was less variation in Lines 1 and 2. TRs among the control plants were low and stable across both Zn concentration and time. However, the three lines of P35S:PtPCS1 behaved
differently (Fig. 4E). TRs for Line 1 generally increased at low levels of Zn exposure while Lines 2 and 3 exhibited decreased TRs.

Exposure to 30 d of elevated Zn affected poplar’s accumulation of nutrients and highlighted accumulation differences between the lines and controls. The accumulation patterns of other nutrients across tissues and treatments was different from Zn accumulation patterns indicated by the dendrogram showing Zn as an outgroup (Fig. 5A). Generally, increases in Zn exposure were antagonistic to accumulation of other nutrients. The length of the assay (30 d) as well as the higher concentrations used also created a different pattern of localization compared with the 48 h assay (Fig. 2A), in which all nutrients accumulated primarily in leaves and, to a lesser extent, roots (Supplementary Table S9 at JXB online).

In root, stem, and leaf tissues, Zn exposure significantly affected accumulation (P<0.001 for all tissues). However,
while a line effect was not present in the root tissues ($P=0.28$), in both stem and leaf tissues there was an interaction effect between line and Zn concentration ($P=0.026$ and 0.023, respectively). This interaction effect only led to actual statistical differences between lines at the highest exposure concentration (i.e. 10 mM Zn) with Line 3 accumulating only 188 mg kg$^{-1}$, which is less than half the accumulation of either the control or the other two lines (Fig. 5B). Differences in leaf Zn accumulation among lines were also evident (Fig. 5C). Again, the lowest exposure level (1 μM Zn) led to no differences between lines. However, in leaf tissue, lines exhibited differences under both elevated concentration treatments. Line 1 accumulated the highest concentrations of Zn with an average 2.6-fold greater accumulation than the control in both of the Zn treatments.

Joint regression was used to explore the interaction between line and Zn exposure in both stem and leaf tissue. The line response to Zn exposure concentration is estimated with the coefficient $B1$. When $B1=1$, average stability is indicated and the response of the family is parallel to the
response of all families. When $B_1<1$, the family is unresponsive relative to the other families. When $B_1>1$, the family is unstable and exhibits a greater response to Zn exposure concentration than the average response for all families. Relative to the average response of all poplar lines, Line 1 accumulated Zn in stem tissues at average levels. However, Line 1 accumulated 1.62-fold more than the next highest accumulator (Line 2 in leaves) and accumulated 2.09-fold more Zn than the controls (Supplementary Fig. S1A at JXB online). Also Line 1 leaf accumulation was very responsive to the change in exposure concentration ($B_1=1.43$) and increased its accumulation at a greater rate as Zn was added to the system relative to the other tested lines (Supplementary Fig. S1B at JXB online). In contrast, Line 3 was less responsive to increased Zn concentrations for both stem and leaf tissues with $B_1$ values 0.50 and 0.86, respectively (Supplementary Fig. S1C at JXB online).

Zn accumulation differences are partly explained by the $PtPCS1$ levels of each line. Multiple regression using each

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**Fig. 5.** Nutrient accumulation of $P_{35S}:PtPCS1$ lines and control lines (pBI101 and wild-type 717) exposed to 1 μM, 1 mM, or 10 mM Zn (overall $n=136$). (A) Relationships based on average Euclidian distance of nutrient accumulation across lines, metal exposure, and tissue type and their relative concentrations. Zn accumulation (mg/kg) in stem tissues (B) and leaf tissues (C) across metal treatments, with letters representing least significant difference ranked means within each cluster (metal exposure) while NS represents no difference. Accumulation of (D) Zn, (E) Cu, and (F) Fe in leaf tissue is projected across zinc exposure and $PtPCS1$ expression using local regression analysis (model: metal accumulation $= PtPCS1$ concentration $\times$ metal exposure). In (D), (E), and (F), $PtPCS1$ concentration axis represents a range of 0.4–1.2, metal exposure axis represents a range from 0 μM to 10 mM, and Zn, Cu, and Fe accumulation represent ranges 32.5–1521.1, 92.1–732.8, and 4.9–41.6 mg/kg, respectively. Letters in the graph section represent Fisher’s PLSD mean separation where differing letters represent significantly different means at the 0.05 level. NS, no significant difference.
line’s average PtPCS1 and PtHMA4 transcript concentration and Zn exposure level demonstrated that PtPCS1 and Zn exposure level significantly affected Zn accumulation (P=0.04 and 0.005, respectively) while PtHMA4 was only influential (P=0.08). Accumulations of other nutrients did not show that they were directly affected by the two transcripts but rather were affected by metal exposure effects. Generally, increased Zn exposure and PtPCS1 both led to higher Zn accumulation (Fig. 5D). Conversely, the two metal nutrients Cu and Fe, in two differing clusters from Fig. 5A, displayed opposite trends (Fig. 5E, F) with drastic decreases in accumulation as Zn exposure increases, and stability across PtPCS1 levels.

**Discussion**

Poplar accumulation of Zn varies clonally (Sebastiani et al., 2004; Laureysens et al., 2005) as do hyperaccumulating species such as *T. caerulescens* (Roosens et al., 2003). Also, poplar is tolerant of sites with increased heavy metal levels, including Zn, but does not accumulate these metals at hyperaccumulator levels of *T. caerulescens*. While genetic variability controlling Zn homeostasis exists in poplar, specific mechanisms are unresolved in this high biomass, forest species. In this study, genomic, transcriptomic, and physiological techniques reveal a coordinated response of *HMA4* and *PCS1* genes through the root and stem to environmental Zn concentrations.

Poplar contains genes with close sequence homology to *HMA4* and *PCS1* implicated in absorption/transport and detoxification of heavy metals in an array of divergent species. In both gene families, all poplar genes are more closely related to the target gene than the monocot rice outgroup consistent with the species phylogeny (Tree of Life; http://tolweb.org/tree). The heavily studied transporter *HMA4* family contains 11 potential homologues from poplar and representatives from many other species including hyperaccumulators and non-hyperaccumulators (Fig. 1A). In the *HMA4* family, only one transcript is closely related to *HMA4* members of the Brassicaceae family including those associated with hyperaccumulation. This *HMA4* homologue, POPTR_0006s07650.1, is a member of the purported Salicoid duplication gene set (Tuskan et al., 2006). However, its paralogue POPTR_0018s14140.1 is not in the final phylogenetic tree because of severe truncation (i.e. the protein sequence is only 158 amino acids compared with 1188 amino acids). The *PCS1* family only contains two poplar members that are paralogues from the Salicoid duplication event and have diverged from each other greatly compared with paralogues in another non-hyperaccumulator *A. thaliana* (60.4% compared with 70.1% protein sequence identity between paralogues in the respective species) (Heiss et al., 2003). While both genes code for a critical Cys58 residue (Clemens, 2006), only one gene (POPTR_0018s18420.1) had many other cysteine motifs (five) conserved across PCS1 from various species.

Because the poplar genome has been duplicated, gene families have expanded in size, nearly doubling the number of *HMA*-like genes and accounting for both PCS genes. While poplar has retained many gene paralogues either from a slow evolution rate or not having strong selective pressures for genome downsizing, poplar has not conserved multiple gene copies closely related to those known to be involved in heavy metal hyperaccumulation. This has happened in the hyperaccumulating *A. halleri* where gene expression has been increased by triplication and conservation of *HMA4* (Hankenne et al., 2008) and metal tolerance protein 1 (MTP1; Shahzad et al., 2010).

**Poplar down-regulates HMA4 and PCS1 transcripts in response to Zn**

While the poplar genome has maintained *PCS1* and *HMA4* orthologues, its transcriptional control is critical in regulating Zn. Transcripts assayed in this study are both reactive (i.e. root changes correspond to accumulation changes) and proactive (i.e. modulations occur in stems where no accumulation changes have occurred) to environmental Zn demonstrating a tight holistic control of nutrient homeostasis. Transcript modulations contribute to a phenotype in which Zn enters under plant deficiency and is inhibited under surplus. A quick response, within 48 h, of transcriptional abundance occurs in roots and stems even though only roots had significant Zn accumulation differences. Similar tissue coordination of nutrient regulation is seen in the hyperaccumulator *T. caerulescens* for which metal tolerance is associated with stems and hyperaccumulation is associated with roots (de Guimaraes et al., 2009). In roots, *HMA4* and other pathway transcripts related to ATPase-driven transport decrease with the addition of only 10 μM Zn. These same genes, except *ELI-3*, were significantly down-regulated when comparing the non-hyperaccumulating *T. arvense* with the hyperaccumulating *T. caerulescens* (Hammond et al., 2006). None of these genes tested in poplar appeared in the *A. halleri* assay; though this was probably due to the relatively few transcripts that were all up-regulated available from this study (Becher et al., 2004). Decreases observed in poplar and *T. arvense* indicate that transcriptional regulation may be different from that of hyperaccumulators. Poplar, like *A. thaliana*, generally contracts its metal transporter expression as Zn exposure increases (Fig. 2E), keeping localization in roots (Mills et al., 2003; Hussain et al., 2004; Papoyan and Kochian, 2004).

Down-regulation of transporter transcripts is coupled with stable PtPCS1 expression across the metal gradient and a significant decrease in PtPCS1 in stems. This corresponds to the tissue compartmentalization seen in *B. juncea* (Heiss et al., 2003) and suggests that PCs are not being used in a traditional detoxification role as proposed by some studies (Clemens et al., 1999; Batamanik et al., 1999; Inouhe et al., 2000; Hartley-Whitaker et al., 2001). Furthermore, PtPCS1 expression does not correlate with Zn exposure as would be expected if PC production were tightly correlated with glutathione (GSH) increases in poplar leaves (Di Baccio et al., 2005). This may be indicative of a holistic approach employed by poplar in...
which down-regulation of PC production in stems may be a method by which PCS physically closer to the point of Zn interaction has adequate GSH for PC production (Cobbett, 2000; Blum et al., 2007).

**Altered PtHMA4 and PtPCS1 affect poplar during early development**

Wild-type poplar has *HMA4* and *PCS1* genes that fluctuate with nutrients in a rapid and systemic manner. When these two genes are overexpressed, the genes cause a change in poplar’s Zn interacting phenotype. The first major new feature elucidated by these constructs is a positive interaction between the upstream *PCS1* and downstream *HMA4* (Fig. 3C). This link is observed in plants grown at the same low nutrient level; thus, modulations in *HMA4* are most likely not a response to Zn increases but directly related to *PCS1* activity. A functional link between PCS1 and HMA4 has been reported (Fig. 2E) where *A. thaliana* mutants for each gene are additively more sensitive to Cd (Hussain et al., 2004; Wong and Cobbett, 2009), but no previous transcriptional links have been reported.

Gene function is demonstrated by differences in these transgenic lines at early developmental stages. Physiologically, *P35S:*PtPCS1 plants were very similar to control plantlets in leaf emergence and days till rooting, while *P35S:*PtHMA4 lines were superior (Fig. 3D–G) possibly due to stimulations to the surrounding pathway for ATP production through increased APY2 and AHA3 activity (Fig. 2E) allowing proper cell division, cell growth, and overall plant development (Palmgren and Christensen, 1994; Thomas et al., 1999; Wolf et al., 2007; Wu et al., 2007). *P35S:*PtPCS1 rooting percentage was inversely affected by Zn concentration (Fig. 3F) and had overall marginal performance pointing to GSH depletion increasing Zn sensitivity (Gasic and Korban, 2007; Wojas et al., 2008, 2010). As a precursor to other chelation agents, GSH depletion impedes production and function of other metal-interacting proteins such as the *Arabidopsis* oligopeptide transporter (OPT) shutting down meristematic growth, making plants more sensitive to heavy metals, and halting cell division (Vernoux et al., 2000; Cagnac et al., 2004; Freeman et al., 2005; Blum et al., 2007). This poor performance in the rooting assay illustrates that PCS is functional in poplar but may be an agent for increased absorption and transport efficiency as seen in *A. thaliana, Arabidopsis lesiacum*, and *B. juncea* (Ingle et al., 2005; Bleeker et al., 2006; Gasic and Korban, 2007; Tennstedt et al., 2009).

**PtPCS1 overexpression shifts the poplar phenotype towards an accumulator**

Unlike the drastic negative effects seen in young poplar plants exposed to increased levels of Zn, more mature poplar, with developed roots and leaves, interacts more successfully with Zn even at 10 times the lethal level in the rooting studies. Poplar appears to exert a holistic response to environmental Zn increases by changing physiological processes and nutrient localization. Over 30 d, both *P35S:*PtPCS1 lines and controls maintained similar root and stem biomass while leaf biomass increased in controls (Fig. 4A). This may be due to the stable and low control plant TR which allows more leaf surface area since water loss is relatively low (Parker, 1949; Sinclair et al., 2005). Hence, depressed TR may be part of a systematic strategy in which control plants absorb less nutrient solution or compensation for negative impacts on leaf chlorophyll (Ebbs and Uchil, 2008). Furthermore, *P35S:*PtPCS1 lines may be better able to maintain normal ion flow across the Zn gradient, preventing K and Na salt build-up in guard cells that could cause increased SR and decreased TR (Dietrich et al., 2001; Brag, 2006).

Complementing the physiological changes, *P35S:*PtPCS1 leads to Zn increases in both stem and leaf tissues with a positive relationship between *PCS1* expression levels and the amount of leaf accumulation (Fig. 5B–D). Still the greatest accumulation increases were in the leaves of *P35S:*PtPCS1-Line 1 with an average of 1521 mg/kg in comparison with 30–32 000 mg/kg accumulated by *T. caerulescens* and *A. halleri* under 10 mM and 1 mM Zn treatments, respectively (Brown et al., 1995; Zhao et al., 2000). Though not at hyperaccumulator levels, Line 1 did outperform previous reports from perennial, woody species by achieving leaf Zn concentrations 1.75 times the total applied Zn concentration in the soil after 30 d. This line’s accumulation also increased at greater rates than the other lines (B1 coefficient=1.43) shifting this line to an accumulator-like phenotype. Two previous studies involving poplar hybrid and *Salix caprea* accumulated far more Zn in their leaves, but grew on sites with soil concentrations of 47,233 and 14,000 mg kg⁻¹, respectively. These do not approach the relative leaf concentration of Line 1 with concentrations only reaching 7.6% and 33% of the soil concentrations (Pierzynski et al., 2002; Unterbrunner et al., 2007). Since Zn accumulation did not approach concentrations in hyperaccumulating plants, *PCS1* may not be a central contributor to the hyperaccumulating phenotype, possibly due to a broader set of metal-related genes synergistically controlling the wild-type phenotype. This is consistent with recent transformations of *A. thaliana*, in which lines that co-overexpressed both *HMA4* and *MT2b* were significantly more tolerant to Zn and Cd than either single gene overexpression construct (Grispen et al., 2011). Another factor that may inhibit hyperaccumulation is that use of the CaMV 35S promoter provides no tissue localization. This potentially leads to disruption of tissue function, which may be a limiting factor for deducing the true function of a gene such as *PCS1* (Tennstedt et al., 2009; Grispen et al., 2011).

**Zn and transcript modulations affect the entire nutrient load**

In a broader nutrient scope, poplar accumulates high amounts of nutrients in metabolically active tissues such as leaves and, to a lesser extent, roots in both wild-type and transgenic lines (Figs 2A, 5A). The addition of extra Zn into the system affects poplar quickly. After only 48 h, the
highest concentration of Zn-containing solution resulted in a large increase in Zn in roots. This rapid increase in accumulation is at the expense of Cu and Fe, which demonstrate a competitive relationship with Zn. At 48 h, Zn accumulation in leaf and stem tissue was minimal with an ~4-fold and 4.4-fold average decrease relative to accumulation in roots. This trend was accentuated over the longer 30 day assay. As expected, Zn accumulation patterns were different from those of other nutrients. Also, Cu and Fe no longer clustered with Zn after 30 d. Across all tissues, Fe had a net decrease of 9% from the 1 μM Zn to 10 mM Zn levels, which is similar to the antagonistic relationship between these two metals displayed in maize [Zea mays (Lee et al., 1969; Rosen et al., 1977; Wang et al., 2009b)] associated with competition for the iron/zinc transporters [IRT3/ZNT1 (Cohen et al., 1998; Lombi et al., 2002)]. Alternatively, Cu had a total tissue increase of 28% after 30 d over the 1 μM Zn to 10 mM Zn levels. However, this increase was only found in roots, and in leaf and stems Cu accumulation became increasingly similar to that of Fe (Fig. 5A, E, F). Thus, high Zn is antagonistic to both Cu and Fe at different locations. Fe appears to compete with Zn at the root level, while Cu is unable to transport into the leaves, indicating a possible competitive disadvantage for long-distance transport between the root and stem associated with PCs (Gong et al., 2003; Chen et al., 2006; Mendoza-Cozatl et al., 2008).

In conclusion, poplar growth and development are highly sensitive to environmental Zn at early developmental stages but relatively resilient when tissues are fully differentiated and Zn homeostasis is maintained primarily in roots and leaves where active metabolism is occurring. Poplar seems to have an indicator phenotype providing a systemic, rapid response to changes in Zn availability using PCS1 and HMA4 family members. Down-regulation of gene transcriptional abundance generally impedes rapid, long-distance root-to-stem Zn transport in line with reports from other indicator phenotypes. Overexpression of two key genes, PCS1 and HMA4, does alter the phenotype. At early stages of development, HMA4 relieves some negative effects of Zn, and at later stages metal accumulation is increased by overexpression of PCS1. This study elucidated possible avenues of poplar regulation of essential heavy metal accumulation. A still better understanding of the genetic controls used by poplar to interact with heavy metals may be achieved with continued delineation of the larger pathway, inclusion of more expression data from other gene family members, and use of a promoter more consistent with expression localization of hyperaccumulators.

Supplementary data

Supplementary Fig. S1 shows analysis with joint regression of the interaction effects of line-metal treatment of both tissues (stem and leaf).

Supplementary Table S1 lists primers for poplar gene expression analyses via qPCR assays.

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Supplementary Table S2 lists primers for poplar gene isolation and restriction site addition with RT-PCR.

Supplementary Table S3 gives gene names, accession numbers, and predicted transmembrane loops/conserved cysteine motifs for respective HMA4 and PCS1 genes in the phylogenetic trees.

Supplementary Table S4 shows the pathway connections between treatment and downstream primary and secondary effects.

Supplementary Table S5 shows the relationship between GO annotation groups and pathway gene members.

Supplementary Table S6 lists nutrient averages and variances accumulated in wild-type leaf, stem, and root tissues across metal concentrations at 24 h and 48 h in wild-type plants.

Supplementary Table S7 shows transcription ratio (target gene/ubiquitin) averages and variances across three metal treatments at 24 h and 48 h for root and stem tissues in wild-type plants.

Supplementary Table S8 gives the dry weight averages and variances of PtPCS1 overexpression lines and controls across metal treatments for leaf, stem, and root tissues after 30 d.

Supplementary Table S9 gives nutrient averages and variances accumulated in PtPCS1 overexpression lines and controls in leaf, stem, and root tissues across metal concentrations after 30 d.
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