Exogenous glutathione enhances cadmium accumulation and alleviates its toxicity in *Populus × canescens*

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Glutathione (GSH) plays an important role in cadmium (Cd) tolerance in woody plants, but the underlying mechanisms remain largely unknown. To elucidate the physiological and transcriptional regulation mechanisms of GSH-mediated Cd tolerance in woody plants, we exposed *Populus × canescens* (Ait.) Smith saplings to either 0 or 75 μM Cd together with one of three external GSH levels. Glutathione treatments include buthionine sulfoximine (BSO, an inhibitor of GSH biosynthesis), no external GSH and exogenous GSH. External GSH resulted in higher Cd²⁺ uptake rate in the roots, greater Cd amount in poplars, lower Cd-induced H₂O₂ levels in the roots, and higher contents of endogenous GSH in Cd-treated roots and leaves. Furthermore, external GSH led to upregulated transcript levels of several genes including zinc/iron regulated transporter related protein 6.2 (*ZIP6.2*) and natural resistance-associated macrophage protein 1.3 (*NRAMP1.3*), which probably take part in Cd uptake, glutathione synthetase 2 (*GS2*) implicated in Cd detoxification, metal tolerance protein 1 (*MTP1*) and ATP-binding cassette transporter C3 (*ABCC3*) involved in Cd vacuolar accumulation in the roots, γ-glutamylcysteine synthetase (*ECS*) and phytochelatin synthetase family protein 1 (*PCS1*) involved in Cd detoxification, and oligopeptide transporter 7 (*OPT7*) probably implicated in Cd detoxification in the leaves of Cd-exposed *P. × canescens*. In contrast, BSO often displayed the opposite effects on Cd-triggered physiological and transcriptional regulation responses in poplars. These results suggest that exogenous GSH can enhance Cd accumulation and alleviate its toxicity in poplars. This is probably attributed to external-GSH-induced higher net Cd²⁺ influx in the roots, greater Cd accumulation in aerial parts, stronger scavenging of reactive oxygen species, and transcriptional overexpression of several genes involved in Cd uptake, detoxification and accumulation.

**Keywords:** cadmium, net Cd²⁺ influx, *Populus*, reactive oxygen species, reduced glutathione, transcriptional regulation.

Introduction

Cadmium (Cd) is a toxic heavy metal in soil, where it can be absorbed by plant roots, enter the food chain and finally accumulate in human bodies, thereby leading to serious health problems. In the last few decades, anthropogenic activities including mining, the production of Cd-containing batteries and the application of phosphate fertilizers have led to marked increases in the areas with Cd-polluted soil. Currently, it is estimated that 3.5 million sites in the European Union and 600,000 sites in USA have been polluted by heavy metals including Cd (Coninx et al. 2017). In China, the data of national soil surveys have showed that ~7% of the soil samples are mainly polluted by Cd (equivalent to ~440,000 km² if we assume that the area is proportional to the number of investigated samples) (Zhao et al. 2015). Phytoremediation has been proposed to remediate Cd-polluted soil (Brown et al. 1994), where plants and associated microorganisms can take up Cd from the soil (Kramer 2005, Luo et al. 2014). Many Cd hyperaccumulating

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herbaceous plants have been identified for this purpose (Kramer 2010, Luo et al. 2016). However, these Cd hyperaccumulators often have a small aboveground biomass, which limits the accumulation of Cd in the aerial parts of these plants (van der Ent et al. 2013). Therefore, fast-growing woody plants including *Populus* species have been proposed for phytoremediation because of their high growth rates, large aboveground biomass, deep and highly branched root systems, and efficient Cd uptake and translocation to the aerial parts (Robinson et al. 2000, Ali et al. 2013).

*Populus* species are non-hyperaccumulators for Cd (Capuana 2011). However, some poplar genotypes can accumulate considerable amounts of Cd in their aerial parts. For instance, *Populus × canescens* (Ait.) Smith (*Populus tremula × P. alba*) can accumulate Cd at above 100 μg g⁻¹ dry mass in the leaf and bark tissues, which is the threshold for Cd hyperaccumulation in plants (He et al. 2011, 2013a). Thus, *P. × canescens* is a promising candidate tree for use in the phytoremediation of Cd-polluted soil. The physiological and molecular mechanisms that underlie Cd accumulation have been partially elucidated in *P. × canescens* (He et al. 2011, 2013a). In particular, it has been demonstrated that transgenic *P. × canescens* plants with higher glutathione (GSH) concentrations have higher Cd²⁺ uptake rates in the roots and greater Cd accumulation in the aerial parts (He et al. 2015), which suggests that GSH plays a pivotal role in enhancing Cd uptake and accumulation in poplars.

Glutathione is a low molecular weight thiol compound, which plays a central role in Cd detoxification (Seth et al. 2012). First, GSH can act as a Cd chelator because Cd has a high affinity for the thiol group (–SH) of GSH, which leads to the formation of Cd–GSH complexes and decreases in the toxic Cd²⁺ contents of plant cells. Second, GSH serves as a precursor for the biosynthesis of phytochelatins (PCs), which are essential for chelating Cd²⁺ in the cytosol and vacuoles of plant cells. Third, GSH can act as an important antioxidant by scavenging overproduced reactive oxygen species (ROS) in Cd-stressed plant cells. In addition, GSH and its oxidized form GSSG can act as a redox couple via changes in the ratio of GSH relative to GSSG, thereby transmitting specific information to modulate cellular signaling pathways in plants in response to Cd stress. Since GSH is critical for Cd detoxification in plants, exogenous GSH has been applied to Cd-stressed herbaceous plants to enhance their Cd tolerance (Cai et al. 2010, 2011, Chen et al. 2010, Wójcik and Tukiendorf 2011, Nakamura et al. 2013, Daud et al. 2016, Hasan et al. 2016). These results demonstrate that the physiological mechanisms underlying exogenous GSH-mediated Cd tolerance in plants still need to be elucidated.

Cadmium uptake, translocation, detoxification and accumulation are key processes in plants exposed to Cd, which are controlled by a number of genes encoding proteins involved in these processes (Luo et al. 2016). For instance, zinc/iron regulated transporter related protein 6.2 (ZIP6.2) and natural resistance-associated macrophage protein 1.3 (NRAMP1.3) are considered to be located in the plasma membrane where they control the entry of Cd²⁺ into the cytosol of root cells (Migeon et al. 2010). Both plant cadmium resistance protein 2 (PCR2) and P-type heavy metal ATPase 4 (HMA4) are located in the plasma membrane where they translocate cytosolic divalent cations including Cd²⁺ for xylem loading (Hanikenne et al. 2008, Song et al. 2010). Both γ-glutamylcysteine synthetase (ECS) and glutathione synthetase 2 (GS2) encode important enzymes involved in the catalyzation of GSH biosynthesis in plants (Jez and Cahoon 2004, Jez et al. 2004). Phytochelatin synthetase family protein 1 (PCS1) catalyzes the biosynthesis of PCs. Glutathione and PCs are important chelators for cytosolic Cd²⁺. The chelates of Cd-GSH and/or Cd-PCs can be transported to the vacuoles by tonoplast-localized ATP-binding cassette transporter C1 (ABCC1) and/or ABC3 (Zientara et al. 2009, Song et al. 2014). Metal tolerance protein 1 (MTP1) encodes a member of the zinc transporter families, which is located in the vacuolar membrane, where it transports divalent cations such as Zn²⁺ and Cd²⁺ into vacuoles (Weber et al. 2013). In addition to these genes involved in Cd accumulation, some other genes are probably involved in uptake and translocation of GSH and its derivatives in plants, which may also play a role in Cd detoxification. For example, oligopeptide transporter 3 (OPT3), OPT6 and OPT7 can function as plasma-membrane-localized transporters for the uptake of GSH and/or Cd-GSH chelates in *Arabidopsis* (Cagnac et al. 2004, Pike et al. 2009, Zhai et al. 2014). A schematic model has illustrated some of these key genes involved in Cd uptake, translocation, detoxification and accumulation in plants (Figure 1). Moreover, the exogenous GSH-induced alleviation of Cd toxicity in tomato was found to be associated with the transcriptional overexpression of several genes involved in stress responses (Hasan et al. 2016). These studies indicate that the transcriptional regulation of the key genes probably plays a role in GSH-mediated Cd toxicity in herbaceous plants. However, no information is available on the transcriptional regulation of genes involved in GSH-mediated Cd tolerance in woody plants.

*Populus* species are promising fast-growing woody plants for the remediation of Cd-polluted soil, but no information is currently available about the regulatory mechanisms that underlie the effects of exogenous GSH addition on Cd-exposed poplars. Since *P. × canescens* is a good candidate tree for phytoremediation of
External GSH stimulates Cd accumulation in poplar

Figure 1. A schematic model of Cd uptake, translocation, detoxification and accumulation in plants. In the uptake process, Cd$^{2+}$ enters the cytosol via ZIP6.2 and NRAMP1.3. Cytosolic Cd$^{2+}$ in cells near to the cylinder can be translocated out of the cytosol for xylem loading by HMA4 and PCR2. In the detoxification process, cytosolic Cd$^{2+}$ can be chelated by GSH and PC. Exogenous GSH can enter the cytoplasm through OPT3/6/7, and the endogenous GSH can be biosynthesized with the catalysis of ECS and GS2. Phytochelatin (PC) is synthesized using GSH with the catalysis of PCS1. In the accumulation process, Cd$^{2+}$ and Cd-chelates can enter the vacuole via MTP1 and ABC1/3, respectively. ZIP6.2 and NRAMP1.3 are related to Cd$^{2+}$ uptake (shown in blue). HMA4 and PCR2 are involved in Cd translocation (shown in gray). OPT3/6/7, ECS, GS2 and PCS1 are related to Cd detoxification (shown in yellow). ABC1/3 and MTP1 participate in Cd accumulation (shown in green).

Cd-polluted soil (He et al. 2011, 2013a, 2015), we exposed P. × canescens saplings to either 0 or 75 μM Cd together with one of three exogenous GSH levels including an inhibitor of GSH biosynthesis, BSO, no external GSH and exogenous GSH for 27 days. The aim of this study is to elucidate the physiological and transcriptional regulation mechanisms underlying GSH-mediated Cd tolerance in poplars. We hypothesized that BSO addition may inhibit Cd$^{2+}$ uptake rate and accumulation, and exacerbate Cd toxicity, whereas external GSH application will have the opposite effects on Cd-exposed P. × canescens sapling. We analyzed growth characteristics, Cd uptake and accumulation, and in transport of GSH and its derivatives in P. × canescens saplings. Our results suggest that external GSH can stimulate Cd accumulation and alleviate Cd toxicity in poplars. These results are significant for efficient phytoremediation by the stimulation of Cd accumulation in fast-growing woody plants with external GSH.

Materials and methods

Plant material and growth conditions

Plantlets of P. × canescens (P. tremula × P. alba) were produced by micropropagation as suggested by Leple et al. (1992) in a culture room (day/night temperature: 25/18 °C; relative air humidity: 50–60%; light per day: 14 h; photosynthetic photon flux: 150 μmol m$^{-2}$ s$^{-1}$). After 4 weeks, the rooted poplar plantlets were transferred to aerated Hoagland nutrient solution (3 mM KNO$_3$, 2 mM Ca(NO$_3$)$_2$·4 H$_2$O, 1 mM NH$_4$H$_2$PO$_4$, 0.5 mM MgSO$_4$·7 H$_2$O, 25 mM KCl, 12.5 mM H$_2$BO$_3$, 1 μM MnSO$_4$·H$_2$O, 1 μM ZnSO$_4$·7 H$_2$O, 0.25 μM CuSO$_4$·5 H$_2$O, 0.25 μM Na$_2$MoO$_4$·2 H$_2$O, 26.85 μM EDTA-FeNa·3 H$_2$O) in pots (diameter: 52 cm; height: 30 cm; six plants in each pot) in a greenhouse (day/night temperature: 35/25 °C; relative air humidity: 50–60%; natural light). After cultivation in the hydroponic system for 12 weeks, 108 plants with similar height and growth performance were selected, and divided equally into six groups. Subsequently, poplar plants in the six groups were exposed to either 0 or 75 μM CdCl$_2$ together with one of three exogenous GSH levels which include 100 μM BSO (B2515, Sigma, St Louis, MO, USA; an inhibitor of GSH biosynthesis), and 0 and 100 μM exogenous GSH (G4251, Sigma). Exogenous BSO or GSH were applied after being dissolved in Hoagland nutrient solution. The Cd and GSH treatments were initiated at the same time and they lasted for 27 days. The Hoagland solution was refreshed every 3 days. For 18 plants in each group, six plants (two plants from each pot) were randomly selected for measuring the net Cd$^{2+}$ flux and the remaining 12 plants were harvested after gas exchange measurements. The heights of plants were ~55–70 cm prior to harvest.

Determination of net Cd$^{2+}$ fluxes in the roots

Net Cd$^{2+}$ fluxes were determined in the roots of P. × canescens as described previously (He et al. 2011, Ma et al. 2014). Briefly, six fine white roots (~1.5 mm in diameter) were selected from each poplar plant. The net Cd$^{2+}$ fluxes in the roots were measured using the non-invasive micro-test technique (the NMT...
system BIO-IM; Younger Corp., Amherst, MA, USA). Before performing the measurements, an ion-selective microelectrode with an external tip (~2–4 μm in diameter) was fabricated and silanized with tributylchlorosilane, and the tip was then back-filled with an ion-selective cocktail (XY-Si-Cd; Younger Corp.).

The fine roots cut from the plants were transferred immediately to a Petri dish containing 10 ml of the measuring solution (0.075 mM CdCl₂, 0.1 mM KCl, 0.5 mM NaCl, 0.3 mM MES, 0.2 mM Na₂SO₄; pH 5.5). Each fine root was equilibrated in the measuring solution for 10 min and then transferred to fresh measuring solution where the net Cd²⁺ fluxes were determined. To determine the position with the maximal Cd²⁺ influx along the root tip, a preliminary experiment was performed where an initial measurement was made at the root tip followed by measurements at 300 μm steps up to 1.5 mm, and then at distances 2, 3, 4 and 5 mm from the root tip (see Figure S1A available as Supplementary Data at Tree Physiology Online). Cd²⁺ gradients were measured close to the root surface (~2–5 μm) by moving the Cd²⁺-selective microelectrode between two positions (at a distance of 30 μm) in a direction perpendicular to the root surface. The net Cd²⁺ influxes were recorded for a period of 7 min at the position with the maximal Cd²⁺ influx. Data processing was performed to determine the net Cd²⁺ fluxes using the im-Flux software attached to the NMT system.

Gas exchange measurement and harvest

Gas exchange was measured using a mature leaf (leaf chron index = 8) on each plant before harvest, as suggested by He et al. (2011). The CO₂ assimilation rate (A), stomatal conductance (gs) and transpiration rate (E) were determined by using a portable photosynthesis system (LI-6400; LI-COR Inc., Lincoln, Nebraska, USA) with an attached LED light source (6400-02). The measurements were performed from 8:00 to 11:00 am with a light intensity of 1000 μmol photon m⁻² s⁻¹. In the sample chamber the air flow was set at 500 μmol s⁻¹ and the CO₂ concentration was 400 μmol mol⁻¹. The leaf temperature was 25 ± 0.8 °C.

After obtaining the photosynthesis measurements, the root system of each plant was washed carefully using 50 mM CaCl₂ for 3 min to remove Cd²⁺ from the root surface. Next, each plant was separated into the roots, wood, bark and leaves. The fresh weight of each sample was recorded before wrapping the samples with tinfoil and freezing immediately in liquid nitrogen. The weight of each sample was recorded before wrapping the samples with tinfoil and freezing immediately in liquid nitrogen. The fresh samples were ground into a powder from the roots, wood, bark and leaves. The fresh weight of each sample was recorded before wrapping the samples with tinfoil and freezing immediately in liquid nitrogen. The fresh weight of each sample was recorded before wrapping the samples with tinfoil and freezing immediately in liquid nitrogen.

Analyses of photosynthetic pigments, soluble sugars and starch

The fine powder obtained from fresh leaves (~60 mg) was extracted in 5 ml of 80% acetone. The concentrations of chlorophylls and carotenoids in the leaves were then determined using a spectrophotometer (UV-3802, Unico Instruments Co. Ltd, Shanghai, China) at 663, 646 and 470 nm, respectively (Wellburn 1994).

Soluble sugars and starch in the roots and leaves were determined using the anthrone method, as described by Yemm and Willis (1954). Briefly, the fine powder obtained from roots and leaves (~100 mg) was extracted in 3 ml of 80% ethanol and incubated in an ultrasonic bath for 30 min at 80 °C. The supernatant was collected after centrifugation (6000g, 25 °C, 10 min). Subsequently, the pellet was extracted again as mentioned above and the supernatant was combined with the previous one. Next, 2 ml of anthrone reagent was added to the supernatant and the mixture was heated in a boiling water bath for 7 min. After cooling to room temperature, the absorbance of the mixture was recorded spectrophotometrically at 620 nm. To determine the starch concentration, the pellet obtained after extracting the soluble sugars was extracted again using HClO₄. The starch concentration (expressed as the glucose equivalent) in the supernatant was analyzed as the soluble sugars.

Analysis of Cd concentrations and amounts

The fine powder (~100 mg) samples obtained from the roots, wood, bark and leaves were digested in a mixture containing 7 ml concentrated HNO₃ and 1 ml concentrated HClO₄ at 170 °C, as described previously (Schützendubel et al. 2001). The Cd content was determined in the extract by flame atomic absorption spectrometry (Hitachi 180-80, Hitachi Ltd, Tokyo, Japan). The amounts of Cd in the root, wood, bark and leaf samples were calculated based on the Cd concentration in the corresponding tissue multiplied by the dry mass of each specific tissue.

Determination of O₂⁻/− and H₂O₂

The superoxide (O₂⁻/) concentrations in the roots and leaves of P. × canescens were determined spectrophotometrically at 530 nm as described by Lei et al. (2006). Briefly, the fine powdered samples (~100 mg) were extracted in 2 ml potassium phosphate buffer (50 mM, pH 7.8). After centrifugation (10,000g, 4 °C, 10 min), the supernatant was used for the assay. An aliquot of supernatant (1 ml) was mixed with potassium phosphate buffer (0.9 ml, 50 mM, pH 7.8) and hydroxyamine hydrochloride (0.1 ml, 10 mM). The mixture was then incubated for 20 min at 25 °C, before adding p-aminobenzene sulfonic acid (1 ml, 17 mM) and α-naphthylamine (1 ml, 7 mM). The absorbance of the mixture was recorded after incubation for 20 min at 25 °C.
The H$_2$O$_2$ concentrations in the roots and leaves were determined as suggested by Lei et al. (2007) with minor modifications. The fine powder (~100 mg) was extracted in acetocoustan. After centrifugation (10,000g, 4 °C, 10 min), the supernatant was mixed with 0.1 ml of 20% TiCl$_4$ and 0.2 ml of 25% aqueous ammonia, and centrifuged again as described above. Subsequently, the supernatant was discarded and the pellet was dissolved in H$_2$SO$_4$ (3 ml, 1 M). The absorbance of the solution was determined spectrophotometrically at 410 nm.

**Analysis of endogenous GSH, GSSG and total thiols**

Endogenous GSH and GSSG were analyzed using the 5,5′-dithio-bisnitrobenzoic acid (DTNB)-glutathione reductase (GR) recycling procedure suggested by Loggini et al. (1999) with minor modifications (Chen et al. 2011). Briefly, GSSG was reduced to GSH via catalysis with GR and NADPH, and GSH was oxidized to GSSG by DTNB. The fine powdered samples (~100 mg) of the roots and leaves were extracted in ice-cold 5% sulfosalicylic acid. After centrifugation (10,000g, 4 °C, 10 min), the supernatant was used to assay GSSG and the total GSSG and GSH. GSSG was analyzed in the supernatant after removing GSH by 2-vinylpyridine derivatization. The reaction mixture (1 ml) included 0.5 M sodium phosphate buffer (pH 7.5), 2.5 mM EDTA, 0.25 mM NADPH, 6 mM DTNB and GR (1 unit). The absorbance was monitored spectrophotometrically at 412 nm. To calculate the GSSG concentration, a standard curve was constructed in the range of 0–100 μM GSSG. Glutathione was determined by subtracting GSSG from the sum of GSSG and GSH.

Total thiols were determined according to the method suggested by Tamas et al. (2008) with minor modifications (He et al. 2013a). The fine powdered samples (~100 mg) of the roots and leaves were extracted in 20 mM EDTA on ice. After centrifugation (12,000g, 4 °C, 10 min), an aliquot (0.25 ml) of the supernatant was mixed with Tris buffer (0.2 M, pH 8.2, 2.5 ml) and DTNB (10 mM, 50 ml), and incubated at room temperature for 15 min. Subsequently, the absorbance was determined spectrophotometrically at 412 nm.

**Determination of soluble phenolics and free proline**

The concentrations of soluble phenolics in the roots and leaves were analyzed as described previously (Luo et al. 2008). The fine powdered samples (~100 mg) were homogenized in 3% sulfosalicylic acid in a boiling water bath for 15 min. After centrifugation (12,000g, 25 °C, 10 min), the supernatant was collected and mixed with glacial acetic acid and ninhydrin reagent (2.5 g ninhydrin in 60 ml glacial acetic acid and 40 ml of 2 M phosphoric acid). After incubation (98 °C, 30 min), the absorbance of the mixture was determined spectrophotometrically at 518 nm when the solution had cooled to room temperature.

**Assays of enzyme activities**

The soluble proteins in the roots and leaves were extracted as reported previously (Luo et al. 2008). Briefly, frozen fine powder of the roots or leaves (~100 mg) was homogenized in cold extraction buffer (100 mM potassium phosphate (pH 7.8), 200 mg polyvinylpolypyrrolidone and 0.5% (v/v) Triton X-100). The mixture was incubated for 15 min on ice and centrifuged (15,000g, 4 °C, 30 min). The supernatant was eluted through Sephadex G-25 columns (PD-10 column, Pharmacia, Freiburg, Germany). The soluble proteins in the eluent were determined according to the Bradford method, using bovine serum albumin (Interchim, Montluçon, France) as the standard. The soluble protein extracts were used for the assays of enzyme activities.

The activity of GR (EC 1.6.4.2) was determined in a reaction system containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na$_2$EDTA, 0.15 mM NADPH, 0.5 mM GSSG, and the protein extract as described by Chen et al. (2011). The GR activity was measured using a spectrophotometer (UV-3802, Unico Instruments Co. Ltd) at 340 nm according to the rate of decrease in NADPH using an extinction coefficient (6.2 mM$^{-1}$ cm$^{-1}$). One unit of GR was referred to the amount of enzymes necessary to oxidize 1 nmol of NADPH min$^{-1}$ mg$^{-1}$ protein at 25 °C.

The activity of peroxidase (POD, EC 1.11.1.7) was determined spectrophotometrically at 436 nm in a reaction system containing 50 mM potassium phosphate buffer (pH 6.5), 40 mM guaiacol, 10 mM H$_2$O$_2$ and the protein extract at 25 °C as proposed by Chen et al. (2011). The POD activity was calculated according to the rate of tetraguaiacol production using an extinction coefficient of 25.5 mM$^{-1}$ cm$^{-1}$. One unit of the enzyme was defined as the amount of POD that is needed to oxidize 1 mmol of guaiacol min$^{-1}$ mg$^{-1}$ protein.

The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured spectrophotometrically at 290 nm in the assay containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM sodium ascorbate, 2.5 mM H$_2$O$_2$ and the protein extract as suggested by He et al. (2011). The APX activity was calculated based on the rate of oxidized ascorbate production using an extinction coefficient (2.8 mM$^{-1}$ cm$^{-1}$). One unit of APX was referred to the amount of the enzyme necessary to degrade 1 μmol of ascorbate min$^{-1}$ mg$^{-1}$ protein.
The activity of catalase (CAT, EC 1.11.1.6) was determined spectrophotometrically at 240 nm in a reaction solution containing 50 mM potassium phosphate buffer (pH 7.0), 40 mM H$_2$O$_2$ and the protein extract as described by He et al. (2011). The CAT activity was calculated according to the rate of decrease in H$_2$O$_2$ using an extinction coefficient (0.036 mM$^{-1}$ cm$^{-1}$). One unit of CAT was the amount of the enzyme that is needed to break down 1 mmol of H$_2$O$_2$ per min at 25 °C.

**Determination of the transcript levels of genes involved in Cd transport and detoxification**

Total RNA was isolated from the roots and leaves according to the method proposed by Chang et al. (1993) with minor modifications (Li et al. 2012). Total RNA was extracted from the fine powdered samples (~200 mg fresh weight) and purified with a plant RNA extraction kit (R6827, Omega Bio-Tek, Norcross, GA, USA). Aliquots of 1 μg total RNA were used for first strand cDNA synthesis in a total volume of 20 μl containing 0.5 μg oligo d(T) 18-prime and 200 U RevertAid Moloney murine leukemia virus reverse transcriptase (DRR037A, Takara, Dalian, China), according to the manufacturer’s instructions. Quantitative PCR was conducted using a CFX96 Real Time System (CFX96, Bio-Rad, Hercules, CA, USA), as described by Ma et al. (2014). The PCR solution contained 10 μl 2 × SYBR Green Premix Ex Taq II (DRR820A, Takara), 0.5 μl cDNA, and 0.2 μM of primers, which were designed specifically for each gene (Table S1 available as Supplementary Data at *Tree Physiology* Online). To ensure the specificity, the PCR products were sequenced and aligned with homologs in other model plants (see Figure S2 available as Supplementary Data at *Tree Physiology* Online). The *TUB4.1* gene encoding beta tubulin was used as a reference gene (Table S1 available as Supplementary Data at *Tree Physiology* Online) because the transcript level of this gene in *P. × canescens* remained quite stable across several stress conditions including Cd exposure according to our previous studies (He et al. 2015, Shi et al. 2015). PCR was performed in triplicate together with a dilution series for the reference gene.

**Statistical analysis**

Statistical tests were performed with Statgraphics (STN, St Louis, MO, USA). To test for significant changes in the net Cd$^{2+}$ flux in the roots, the main effects of CdCl$_2$ (Cd), GSH and distance to the root tip were analyzed by three-way analysis of variance (ANOVA). For the other experimental variables, two-way ANOVAs were employed with Cd and exogenous GSH as the two factors. If interactions were significant, a posteriori comparisons of means were performed. Data were tested to confirm normality before statistical analyses. Differences between means were considered significant when the $P$-value according to the ANOVA F-test was <0.05. Before principal component analysis (PCA), data were standardized and subsequently computed using the prcomp() command in R (http://www.r-project.org/). The Cq values obtained from quantitative PCR were normalized using the program proposed by Pfaff et al. (2002) and the fold changes in the levels of transcripts were calculated using the REST program (Pfaff et al. 2002). Cluster analysis of gene expression was performed using heatmap.2 in the ‘gplots’ package in R (http://www.r-project.org/), as suggested by Luo et al. (2013).

**Results**

**Net Cd$^{2+}$ fluxes in the roots**

The maximal net Cd$^{2+}$ fluxes occurred at 600 μm from the root tip irrespective of preceding Cd exposure and exogenous GSH treatments (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). Under each treatment, net Cd$^{2+}$ fluxes were quite stable (Figure 2A). Cadmium pre-exposure decreased net Cd$^{2+}$ fluxes by 29–40% compared with those...
in the roots without Cd pre-exposure prior to the measurement (Figure 2B). Net Cd\(^{2+}\) influxes were 140 ± 3 pmol cm\(^{-2}\) s\(^{-1}\) in poplar roots without Cd pre-exposure and external GSH addition, and 84 ± 8 pmol cm\(^{-2}\) s\(^{-1}\) in the roots with Cd pre-exposure and no external GSH application, respectively (Figure 2B). Net Cd\(^{2+}\) influx in the roots without Cd pre-exposure was inhibited 33% by BSO addition, but stimulated 15% by external GSH application, compared with that without exogenous GSH addition (Figure 2B). Similarly, BSO-induced reductions and external GSH-caused increases in net Cd\(^{2+}\) influxes were found in Cd pre-exposed roots (Figure 2B).

**Growth characteristics, soluble sugars and starch**

In the mature leaves of *P. × canescens*, CO\(_2\) assimilation rate (A) was markedly inhibited by Cd exposure (Table 1). In Cd-stressed poplar leaves, A was enhanced by the addition of exogenous GSH (Table 1), thereby suggesting that the exogenous application of GSH can alleviate Cd-induced stress in *P. × canescens*. Stomatal conductance (g\(_s\)) and transpiration rate (E) were significantly inhibited by Cd exposure, and the Cd-induced inhibition was not mitigated by exogenous GSH addition (Table 1). The concentrations of photosynthetic pigments were also decreased in the mature leaves of *P. × canescens* when treated with Cd, but these Cd-induced negative effects were not relieved by exogenous GSH addition (Table 1). In agreement with the Cd-induced inhibition of photosynthesis, the dry mass of the roots, wood, bark and leaves of *P. × canescens* was reduced by Cd exposure, and it was not relieved by exogenous GSH addition (Table 2).

The concentrations of soluble sugars and starch were markedly elevated in the roots and leaves under Cd exposure (see Figure S3 available as Supplementary Data at Tree Physiology Online). Specifically, the levels of soluble sugars were increased by 31–168% in the roots and 16–53% in the leaves under Cd treatments, and the starch concentrations were also enhanced by 12–81% in the roots and 25–154% in the leaves of Cd-treated *P. × canescens*. In most cases, the effects of Cd on the concentrations of soluble sugars and starch in the roots and leaves of *P. × canescens* were exaggerated by BSO addition, but these effects were ameliorated by exogenous GSH application (see Figure S3 available as Supplementary Data at Tree Physiology Online).

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**Table 1.** CO\(_2\) assimilation rate (A, μmol CO\(_2\) m\(^{-2}\) s\(^{-1}\)), stomatal conductance (g\(_s\), mol H\(_2\)O m\(^{-2}\) s\(^{-1}\))), transpiration rate (E, mmol H\(_2\)O m\(^{-2}\) s\(^{-1}\)) and photosynthetic pigments (mg g\(^{-1}\) dry weight) in the leaves of *P. × canescens* exposed to either 0 (–Cd) or 75 μM Cd (+Cd) together with one of three exogenous GSH levels (with GSH inhibitor (+BSO), without exogenous GSH (–GSH) or with exogenous GSH (+GSH)) for 27 days. Data indicate means ± SE (n = 12). Different letters following the values in the same column indicate significant difference between the treatments. Chl a: chlorophyll a; Chl b: chlorophyll b; Chl (a + b): sum of chlorophyll a and b; Car: carotenoid. P-values of the ANOVAs of Cd, GSH and their interaction (Cd × GSH) are indicated. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not significant.

<table>
<thead>
<tr>
<th>Cd</th>
<th>GSH</th>
<th>A</th>
<th>g(_s)</th>
<th>E</th>
<th>Chl a</th>
<th>Chl b</th>
<th>Chl (a + b)</th>
<th>Car</th>
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</thead>
<tbody>
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<td>−Cd</td>
<td>+BSO</td>
<td>10.27±0.04 c</td>
<td>0.24±0.02 a</td>
<td>8.70±0.43 d</td>
<td>13.78±0.58 c</td>
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<tr>
<td>−GSH</td>
<td></td>
<td>12.82±0.07 e</td>
<td>0.37±0.02 b</td>
<td>11.23±0.41 e</td>
<td>13.87±0.55 c</td>
<td>6.86±0.31 c</td>
<td>20.73±0.63 c</td>
<td>2.17±0.10 b</td>
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<td>12.06±0.51 b</td>
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<td>8.62±0.41 a</td>
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**Table 2.** The dry mass (g) of roots, wood, bark and leaves of *P. × canescens* exposed to either 0 (−Cd) or 75 μM Cd (+Cd) together with one of three exogenous GSH levels (with GSH inhibitor (+BSO), without exogenous GSH (−GSH) or with exogenous GSH (+GSH)) for 27 days. Data indicate means ± SE (n = 12). Different letters following the values in the same column indicate significant difference between the treatments. P-values of the ANOVAs of Cd, GSH and their interaction (Cd × GSH) are indicated. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not significant.

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<td>8.8±0.4 c</td>
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</table>

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Cadmium accumulation

Cadmium accumulation was not detected in the poplars without Cd exposure, whereas the Cd concentrations in the root, wood, bark and leaf tissues of Cd-stressed poplars were 936–1803, 214–707, 205–378 and 121–291 μg g⁻¹ dry weight, respectively (Figure 3). Moreover, the Cd concentrations were decreased in poplar tissues by BSO addition, but elevated in the bark and leaf tissues by exogenous GSH application in Cd-exposed P. × canescens (Figure 3).

Based on the tissue dry mass and its Cd content, the total amount of Cd was estimated for the corresponding tissue in P. × canescens (Figure 4). In the roots, the total Cd amounts were 1685–2939 μg, and the total Cd amounts were 1039–3296 μg in the aerial parts of Cd-treated poplars. The total amounts of Cd were reduced by ca 43% and 61%, respectively, in the roots and aerial parts of poplars with BSO addition compared with those without exogenous GSH application (Figure 4). By contrast, the total amounts of Cd were increased by ~22% in the aerial parts with external GSH addition compared with those without exogenous GSH application (Figure 4).

Oxidative stress

The O₂⁻ concentrations increased significantly in the roots of Cd-exposed P. × canescens compared with those under the control conditions (Figure 5A). The Cd-induced production of O₂⁻ was elevated in poplar roots by either BSO or exogenous GSH application (Figure 5A). Unexpectedly, the O₂⁻ levels were lower in the leaves of poplars treated with Cd compared with those under the control conditions (Figure 5B). Interestingly, in poplar leaves the Cd-induced reduction in the O₂⁻ concentration was alleviated by BSO addition, but it was exacerbated by the exogenous application of GSH (Figure 5B).

The H₂O₂ concentrations were 15–195% higher in the roots and leaves of Cd-treated poplars compared with those in the control plants (Figure 5C and D). The Cd-induced production of H₂O₂ was exacerbated by BSO addition, but it was alleviated by
the exogenous application of GSH in the roots of *P. × canescens* (Figure 5C). However, the Cd-induced production of H$_2$O$_2$ in poplar leaves was mitigated by the addition of either BSO or exogenous GSH (Figure 5D).

**Non-enzymatic and enzymatic antioxidants**

In poplar roots, the endogenous GSH concentrations increased by 53–538% under Cd exposure conditions (Figure 6A). The Cd-induced endogenous GSH concentration in the roots of *P. × canescens* was unaffected by BSO addition, but it was enhanced by the exogenous application of GSH (Figure 6A). In poplar leaves, the endogenous GSH concentration was induced by Cd exposure, whereas the Cd-induced endogenous GSH levels were decreased by the addition of either BSO or exogenous GSH (Figure 6B).

In the roots, the GSSG concentrations were decreased by 73–75% due to Cd exposure (Figure 6C). In the roots of *P. × canescens*, the Cd-induced reduction in the GSSG level was relieved by BSO addition, whereas it was aggravated by the exogenous application of GSH (Figure 6C). Similarly, the GSSG concentrations were decreased by 18–44% due to Cd exposure in the leaves (Figure 6D). The Cd-induced reduction in GSSG was mitigated by BSO addition, but it was unaffected by the exogenous application of GSH in poplar leaves (Figure 6D).

In the roots, the concentrations of total thiols were increased by 90–343% due to Cd exposure (Figure 6E). The Cd-induced increases in the concentrations of total thiols were relieved by the addition of BSO or exogenous GSH in the roots of *P. × canescens* (Figure 6E). Similarly, in the leaves, the concentrations of total thiols were increased by 90–326% due to Cd exposure (Figure 6F). The Cd-induced increases in the concentrations of total thiols were relieved by the addition of BSO or exogenous GSH in the leaves of *P. × canescens* (Figure 6F).

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Figure 5. Concentrations of O$_2^-$ and H$_2$O$_2$ in the roots and leaves of *P. × canescens* exposed to either 0 (−Cd) or 75 μM Cd (+Cd) together with one of three exogenous GSH levels (with GSH inhibitor (+BSO), without exogenous GSH (−GSH) or with exogenous GSH (+GSH)) for 27 days. Bars indicate means ± SE (n = 12). Different letters on the bars indicate significant differences between the treatments. *P*-values of the ANOVAs of Cd, GSH and their interaction (Cd × GSH) are indicated. *P* < 0.05; ****P < 0.0001.
thiols were enhanced due to Cd exposure, and these Cd-induced increases in the total thiols were unaffected by the addition of BSO or exogenous GSH (Figure 6F).

In the roots and leaves of *P. × canescens*, the concentrations of soluble phenolics were increased by ~26–208% due to Cd exposure (see Figure S4 available as Supplementary Data at

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Figure 6. Endogenous GSH, oxidized glutathione (GSSG) and total thiols (T-SH) in the roots and leaves of *P. × canescens* exposed to either 0 (−Cd) or 75 μM Cd (+Cd) together with one of three exogenous GSH levels (with GSH inhibitor (+BSO), without exogenous GSH (−GSH) or with exogenous GSH (+GSH)) for 27 days. Bars indicate means ± SE (*n* = 12). Different letters on the bars indicate significant differences between the treatments. *P*-values of the ANOVAs of Cd, GSH and their interaction (Cd × GSH) are indicated. *P* < 0.05; **P** < 0.01; ****P** < 0.0001, ns, not significant.
Tree Physiology Online). The Cd-induced increases in the concentrations of soluble phenolics were exacerbated by the addition of BSO, whereas they were alleviated by the exogenous application of GSH in poplar roots (see Figure S4A available as Supplementary Data at Tree Physiology Online). However, the Cd-induced increases in the levels of soluble phenolics were unaffected by the addition of BSO or exogenous GSH in the leaves (see Figure S4B available as Supplementary Data at Tree Physiology Online). In the roots, the concentrations of free proline increased markedly due to Cd exposure (see Figure S4C available as Supplementary Data at Tree Physiology Online), but in the leaves, the free proline level was only induced by Cd exposure (see Figure S4D available as Supplementary Data at Tree Physiology Online). The Cd-induced increases in the concentration of free proline were exacerbated by the addition of BSO, but they were relieved by the exogenous application of GSH in poplar roots (see Figure S4C available as Supplementary Data at Tree Physiology Online). However, the Cd-induced increases in the free proline levels in the leaves of *P. × canescens* were alleviated by the addition of either BSO or exogenous GSH (see Figure S4D available as Supplementary Data at Tree Physiology Online).

The activities of antioxidant enzymes such as GR and CAT were often inhibited by Cd exposure in the roots and leaves of *P. × canescens* (see Figure S5 available as Supplementary Data at Tree Physiology Online). The Cd-induced decreases in the activities of antioxidant enzymes in poplars were unaffected by the addition of either BSO or exogenous GSH (see Figure S5 available as Supplementary Data at Tree Physiology Online).

**Principal component analysis of growth and physiological responses**

To identify the key parameters related to the growth and physiology of *P. × canescens* in response to Cd and exogenous GSH treatments, PCA was conducted based on the results obtained for the roots and leaves (Figure 7, see Table S2 available as Supplementary Data at Tree Physiology Online). In the roots, PC1 and PC2 accounted for 49% and 21% of the variation, respectively (Figure 7A). PC1 clearly separated the variations in the effects of Cd since data points were gathered along the two sides of the dashed line according to the conditions of 0 or 75 μM Cd exposure (Figure 7A). PC2 explained the effects of exogenous GSH treatments which was clearly demonstrated by the data points in the cycles with three levels of GSH treatments on the right side of the dashed line (Figure 7A). The concentrations of free proline and Cd in the roots, and the total amounts of Cd were key contributors to PC1, whereas the activities of APX and CAT in the roots, and the concentration of total thiols in the roots were important factors that contributed to PC2. In the leaves, PC1 and PC2 accounted for 58% and 17% of the variation, respectively (Figure 7B). PC1 clearly separated the variations in the effects of Cd, and PC2 explained the effects of exogenous GSH treatment (Figure 7B). The concentrations of foliar total thiols and Cd, and the total amounts of Cd were key contributors to PC1, whereas the activities of foliar POD and g5, and the concentration of starch were important factors that contributed to PC2. Thus, the PCA results indicate that the poplar roots exhibited greater sensitivity to Cd exposure and/or exogenous GSH treatment compared with poplar leaves, probably because of differences in the roots and leaves in terms of the concentrations of free proline and Cd in the roots, and the foliar levels of total thiols and the total amounts of Cd.

**Transcriptional regulation of genes involved in Cd uptake and detoxification, and transport of GSH and its derivatives**

The mRNA levels of representative genes involved in Cd uptake, translocation, detoxification and accumulation were assessed in the roots and leaves of *P. × canescens* (Figure 8). In poplar
roots, ZIP6.2 and NRAMP1.3 are probably involved in Cd^{2+} uptake, PCS1 likely participates in biosynthesis of PCs for Cd detoxification, and MTP1 and ABCC3 are probably implicated in Cd vacuolar accumulation formed the first cluster (Figure 8A). The transcript levels of these genes remained unaltered in the Cd-exposed roots without the exogenous addition of GSH, but the mRNA levels of these genes were upregulated in the Cd-treated roots by the addition of either BSO or exogenous GSH. Without Cd exposure, the transcript levels of NRAMP1.3, MTP1 and ABCC3 were decreased in the roots due to the addition of BSO, whereas the transcript levels of these genes remained largely unchanged by the exogenous GSH addition. Under Cd exposure, the mRNA levels of most of these genes were overexpressed by the addition of either BSO or exogenous GSH.

The genes in the second cluster in the roots comprised PCR2 and HMA4, which are involved in Cd^{2+} translocation, OPT3, OPT6 and OPT7, which probably participate in uptake of GSH from the soil solution and detoxification of Cd, and ABCC1, which is likely involved in Cd vascular accumulation. The mRNA levels of most of these genes in this cluster were increased significantly in the roots by Cd exposure under no external GSH addition, whereas the transcript levels of most of these genes remained unaltered in the Cd-exposed roots by BSO addition or exogenous GSH application (Figure 8A). Under no Cd exposure, the transcript levels of PCR2, OPT3, OPT6 and OPT7 were decreased in the roots due to BSO addition, whereas the mRNA levels of these genes remained unaltered in the roots when external GSH was applied. In particular, the mRNA level of ABCC1 was significantly upregulated in the external GSH-applied roots without Cd exposure. Under Cd exposure, the mRNA levels of OPT6, OPT7 and ABCC1 were decreased in the roots due to BSO addition, and the transcript levels of all the genes in this cluster remained relatively stable in the roots treated by the exogenous GSH. Unexpectedly, the mRNA level of HMA4 remained relatively stable in the roots treated with either Cd or the exogenous GSH.

The third cluster of genes in the roots comprised ECS and GS2, which are implicated in GSH biosynthesis for Cd detoxification. The transcript levels of both of these genes were induced in Cd-exposed roots irrespective of exogenous GSH treatments (Figure 8A). Under no Cd exposure, the mRNA levels of ECS and GS2 remained unaltered in the roots treated with either BSO or exogenous GSH, whereas the transcript levels of these two genes were enhanced in the roots treated with either BSO or exogenous GSH addition under Cd exposure.

In the leaves, the first cluster of genes comprised NRAMP1.3, HMA4, MTP1 and ABCC3 (Figure 8B). The transcript levels of these four genes were increased in the Cd-treated P. × canescens leaves irrespective of the addition of BSO or exogenous GSH. The mRNA levels of these four genes were downregulated in poplar leaves treated with either BSO or exogenous GSH, irrespective of Cd exposure. The second cluster of genes in the leaves comprised ZIP6.2, OPT3, OPT6, OPT7, ECS, GS2, PCS1 and ABCC1 (Figure 8B). The mRNA levels of most of these genes remained relatively stable in Cd-exposed leaves with the addition of exogenous GSH or BSO. Under no Cd exposure, the transcript levels of most of these genes were downregulated in poplar leaves due to the application of either BSO or exogenous GSH. Under Cd exposure conditions, the mRNA levels of OPT3, OPT6, OPT7, ECS and GS2 were decreased in poplar leaves due to the addition of BSO, whereas the transcript levels of most of these genes remained unaltered in the leaves treated with exogenous GSH addition.
Discussion

*Populus × canescens saplings suffered from Cd toxicity*

In this study, the great net Cd\(^{2+}\) influxes in the roots, high Cd concentrations in the tissues examined and large amounts of Cd accumulated in poplar plants under the conditions without exogenous GSH addition demonstrate that *P. × canescens* has a high capacity for Cd uptake and accumulation. These findings are in agreement with the results of previous studies (He et al. 2011, 2013a). Although *P. × canescens* saplings are capable of Cd accumulation, the Cd-induced decreases in A and biomass of *P. × canescens* after Cd exposure for ∼4 weeks indicate that Cd toxicity has probably occurred in these poplars. Actually, this Cd toxicity has been demonstrated by Cd-induced overproduction of O\(_2\)\(^ {•−}\) in the roots and \(H_2O_2\) in the roots and leaves of *P. × canescens*, which can lead to oxidative stress. In herbaceous plants, Cd-induced oxidative stress can cause programmed cell death, leading to decreased growth (Garnier et al. 2006). Therefore, Cd-induced inhibition of A and decreases in the dry biomass of *P. × canescens* are probably associated with Cd-induced oxidative stress. Cadmium exposure in other woody plants has also resulted in the inhibition of photosynthesis and growth (Domínguez et al. 2011, He et al. 2013b, Pietrini et al. 2015).

Glutathione is a key compound for scavenging over-generated ROS in plant cells exposed to Cd (Foyer and Noctor 2005, Seth et al. 2012). The accumulation of endogenous GSH and total thiols in the roots and leaves of Cd-exposed *P. × canescens* without exogenous GSH addition is consistent with the results obtained in previous studies where this poplar hybrid was exposed to various concentrations of Cd or Zn (He et al. 2013a, Ma et al. 2014, Shi et al. 2015). The elevated concentrations of endogenous GSH and total thiols may play key roles in scavenging overproduced O\(_2\)\(^ {•−}\) and \(H_2O_2\) in Cd-treated *P. × canescens* saplings. Enhanced availability of \(H_2O_2\) can drive GSH accumulation in the vacuoles and chloroplasts of *Arabidopsis* cells (Queval et al. 2011). In addition, phenolics and proline can also be involved in scavenging Cd-induced ROS in plants (Sharma and Dietz 2009, Ali and Hadi 2015, Simek et al. 2016). The elevated levels of soluble phenolics and free proline in Cd-treated *P. × canescens* without external GSH addition are in agreement with the results obtained in previous studies (He et al. 2011, 2013a). These results indicate that phenolics and proline may play roles in maintaining ROS homeostasis in poplars exposed to Cd.

Since Cd is a non-essential and highly toxic element to plant cells, its uptake, translocation, detoxification and accumulation is strictly controlled at the molecular and physiological levels. In agreement with Cd-induced physiological responses including Cd influx and accumulation, and internal GSH accumulation in poplar saplings, the elevated transcript levels of a few genes involved in Cd uptake, transport, detoxification and accumulation have been observed in the roots and/or leaves of Cd-treated *P. × canescens*. Among these upregulated genes, it is important to notice the homologs of *Arabidopsis* ZIP6.2 and NRAMP1.3 involved in absorption of Cd\(^{2+}\), PCR2 and HMA4 participated in Cd translocation from cytosol to vascular cylinder, ECS and GS2 involved in GSH biosynthesis for Cd detoxification, and MTP1 and ABCC1/3 are implicated in Cd vacuolar accumulation in *P. × canescens*. Consistently, enhanced mRNA levels of some of these genes, including ZIP6.2, NRAMP1.3, PCR2, HMA4 and MTP1, have also been found in poplar plants exposed to Cd or excess Zn (Ma et al. 2014, He et al. 2015, Shi et al. 2015). These findings indicate that poplar plants control Cd uptake, translocation, detoxification and accumulation through transcriptional regulation of several key genes involved in processes of Cd accumulation.

*External GSH stimulated Cd accumulation and ameliorated Cd toxicity in poplars*

Glutathione plays a central role in the accumulation of heavy metals in plants (Seth et al. 2012). Thus, it is expected that application of exogenous GSH can improve Cd tolerance in plants. Although external GSH addition reduced the uptake of Cd in the roots, as well as concentration and accumulation of Cd in herbaceous plants (Cai et al. 2010, Chen et al. 2010, Nakamura et al. 2013, Cao et al. 2015, Daud et al. 2016), external GSH-induced Cd accumulation was also found in tomato (Hasan et al. 2016). The higher Cd uptake rates and Cd accumulations in the aerial parts of *P. × canescens* treated with external GSH demonstrate that external GSH stimulates Cd uptake and accumulation in poplar plants. External GSH-induced Cd absorption and accumulation in the roots and leaves of *P. × canescens* is probably associated with GSH-triggered transcriptional regulation of genes involved in Cd uptake and accumulation. In other words, this can be ascribed to external GSH-induced higher mRNA levels of ZIP6.2 and NRAMP1.3 involved in Cd uptake, and MTP1 and ABCC3, which are implicated in vacuolar storage of Cd chelates. Consistently, transgenic *P. × canescens* plants with higher levels of endogenous GSH also displayed transcriptional overexpression of ZIP6.2 and NRAMP1.3 (He et al. 2015). Furthermore, GSH can form complexes with Cd\(^{2+}\). It is likely that exogenous GSH may chelate with Cd\(^{2+}\) to form GSH–Cd complexes which can then be absorbed by the roots and further transported from the roots to the aboveground parts. Additionally, exogenous GSH can be taken up by poplar roots and subsequently it can chelate with cytosolic Cd\(^{2+}\) to enhance Cd accumulation in poplars. Previous studies have showed that peptide transporters in the plasma membrane, such as OPT3, OPT6 and OPT7, are involved in the uptake of GSH and GSH–Cd conjugates from the nutrient solution to cells of herbaceous plants (Cagnac et al. 2004, Zhang et al. 2004, Pike et al. 2009, Zhai et al. 2014). Thus, these peptide transporters may also play roles in the uptake of GSH and/or GSH–Cd chelates in poplar roots. However, no external GSH-triggered increases in
transcript levels of OPT3, OPT6 and OPT7 in poplar roots and/or leaves indicate that these peptide transporters probably have no effects on the uptake of GSH and/or GSH–Cd conjugates. Anyway, we assume that other transporters may be involved in the uptake of external GSH and/or GSH–Cd chelates to poplar cells. It is intriguing to investigate these transporters in poplars in future studies.

In addition to the roles of GSH in Cd uptake and accumulation, GSH is also essential for Cd detoxification in plants. To detoxify cytosolic Cd\(^{2+}\), GSH can directly chelate with Cd\(^{2+}\). Moreover, GSH can act as a precursor for PCs, which can form Cd–PCs to ameliorate Cd toxicity in plants. Glutathione is synthesized under the catalysis of glutathione synthetase (GS) (Herrera et al. 2007). Phytochelatin synthase (PCS) (Clemens and Persão 2009). Under Cd exposure, external GSH-induced increases in transcript levels of GS2 in the roots and PCS1 in the leaves of P. × canescens indicate that external Cd toxicity may trigger transcriptional upregulation of several genes involved in biosynthesis of internal GSH and PCs to alleviate Cd toxicity. In addition, GSH can function as an antioxidant to scavenge Cd-induced ROS in plant cells (Seth et al. 2012). The finding that Cd-induced H\(_2\)O\(_2\) production was exacerbated by the addition of BSO and alleviated by external GSH application in poplar roots indicates that external GSH can affect internal GSH, which probably acts as a H\(_2\)O\(_2\) scavenger to maintain ROS homeostasis in poplar cells exposed to Cd. As a result, external GSH reduces Cd toxicity in P. × canescens. Under Cd exposure conditions, higher endogenous GSH concentrations in external GSH-applied poplars and lower internal GSH levels in BSO-treated roots and leaves of P. × canescens suggest that exogenous GSH can be converted into intracellular GSH, which may play an important role in ameliorating Cd toxicity. However, it is intriguing for the underlying mechanism of the conversion of exogenous GSH to endogenous GSH in poplars. As mentioned above, it is likely that external GSH can be taken up by plasma membrane-localized transporters in poplar roots and some of these GSH are probably transported to the leaves.

In conclusion, we showed that the Cd\(^{2+}\) uptake rates were inhibited by the addition of BSO and stimulated by the external GSH in the roots of P. × canescens. Cadmium exposure significantly increased the concentrations and amounts of Cd in poplar tissues, but Cd accumulation in these plants was reduced by BSO application and stimulated by external GSH. Cadmium accumulation resulted in elevated H\(_2\)O\(_2\) concentrations in poplar roots, but this Cd-induced H\(_2\)O\(_2\) production was exacerbated by BSO addition and decreased by external GSH application in the roots. Cadmium exposure led to higher concentrations of endogenous GSH in the roots of poplars, and this Cd-induced endogenous GSH accumulation was exacerbated by external GSH. Cadmium treatment resulted in the transcriptional upregulation of a few genes involved in Cd uptake, transport, detoxification and accumulation, including ZIP6.2, PCS2, GS2, OPT3, OPT6 and OPT7 in the roots, and NRAMP1.3, HMA4, GS2, OPT1, ABCC1 and ABCC3 in the leaves of poplars. External GSH led to increased transcript levels of several genes implicated in Cd absorption, detoxification and accumulation, such as ZIP6.2, NRAMP1.3, GS2, OPT1 and ABCC3 in the roots, and ECS, PCS1 and OPT7 in the leaves, of poplars exposed to Cd. These results suggest that exogenous GSH can stimulate Cd accumulation and ameliorate Cd toxicity in poplars, which is mainly attributed to external GSH-induced greater uptake of Cd\(^{2+}\) in the roots and translocation from the roots to aerial parts, stronger scavenging of Cd-induced ROS, higher endogenous GSH levels, and increases in transcript levels of several genes involved in Cd uptake, transport, detoxification and accumulation. These results have implications for efficient phytoremediation by enhancing the accumulation of toxic heavy metals in fast-growing woody plants with the addition of exogenous GSH.

**Supplementary Data**

Supplementary Data for this article are available at Tree Physiology Online.

**Acknowledgments**

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**Conflict of interest**

The authors declare that they have no conflicts of interest.

**Funding**

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**Abbreviations**

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<th>Abbreviation</th>
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<td>A</td>
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pools in *Populus nigra* under Free Air CO₂ Enrichment (FACE) and nitrogen fertilisation. Plant Soil 304:45–57.


