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Research

Overexpression of bacterial γ-glutamylcysteine synthetase mediates changes in cadmium influx, allocation and detoxification in poplar

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Summary

• Overexpression of bacterial γ-glutamylcysteine synthetase in the cytosol of *Populus tremula* × *P. alba* produces higher glutathione (GSH) concentrations in leaves, thereby indicating the potential for cadmium (Cd) phytoremediation. However, the net Cd^{2+} influx in association with H^+/Ca^{2+}, Cd tolerance, and the underlying molecular and physiological mechanisms are uncharacterized in these poplars.

• We assessed net Cd^{2+} influx, Cd tolerance and the transcriptional regulation of several genes involved in Cd^{2+} transport and detoxification in wild-type and transgenic poplars.

• Poplars exhibited highest net Cd^{2+} influxes into roots at pH 5.5 and 0.1 mM Ca^{2+}. Transgenics had higher Cd^{2+} uptake rates and elevated transcript levels of several genes involved in Cd^{2+} transport and detoxification compared with wild-type poplars. Transgenics exhibited greater Cd accumulation in the aerial parts than wild-type plants in response to Cd^{2+} exposure. Moreover, transgenic poplars had lower concentrations of $O_2^{\cdot -}$ and H_2O_2; higher concentrations of total thiols, GSH and oxidized GSH in roots and/or leaves; and stimulated foliar GSH reductase activity compared with wild-type plants.

• These results indicate that transgenics are more tolerant of 100 μM Cd^{2+} than wild-type plants, probably due to the GSH-mediated induction of the transcription of genes involved in Cd^{2+} transport and detoxification.

Introduction

Cadmium (Cd, but Cd^{2+} is used when it is relevant to a biological process) is a toxic heavy metal in plants, which can be absorbed from the soil via the roots, thereby entering the food chain, possibly leading to human intake and severe health problems. To reduce the risk of Cd poisoning in humans, the phytoremediation of Cd-contaminated soils has been proposed (Clemens *et al.*, 2013). Several herbaceous species, such as *Noccaea* (*Thlaspi*) *caerulescens* and *Arabidopsis halleri*, have been identified as suitable for Cd hyperaccumulation (Milner & Kochian, 2008; Kramer, 2010). Based on the physiological and molecular mechanisms of Cd hyperaccumulation in these plants (Maestri *et al.*, 2010; Mendoza-Cozatl *et al.*, 2011; Clemens *et al.*, 2013), a number of genes have been isolated and used to create transgenic herbaceous plants for phytoremediation (Zhu *et al.*, 1999; Li *et al.*, 2004; Park *et al.*, 2012). However, the biomass of these herbaceous species is low, thus the total amount of Cd accumulated in the biomass is limited. Recently, *Populus* species have been proposed for phytoremediation because poplars are fast-growing woody plants with a large aboveground biomass and they are able to accumulate intermediate Cd concentrations in their aerial tissues (Peuke & Rennenberg, 2005; Merkle, 2006; Di Lonardo *et al.*, 2011; Castagna *et al.*, 2013; Luo *et al.*, 2014).

In recent years, studies have addressed the physiological and molecular mechanisms of poplars that respond to Cd^{2+} exposure. In response to Cd^{2+} exposure, poplars exhibit decreased photosynthesis, altered carbohydrate and nutrient concentrations, shifted reactive oxygen species (ROS) and antioxidant balance, and differentially regulated transcript and protein abundance (Kieffer *et al.*, 2008; Durand *et al.*, 2010; He *et al.*, 2011, 2013a; Elobeid *et al.*, 2012; Marmiroli *et al.*, 2013). The net Cd^{2+} flux in poplar roots is regulated in space and time (He *et al.*, 2011; Ma *et al.*, 2014), and it is coupled with the activities of plasma

membrane (PM) H^+-ATPases (Ma *et al.*, 2014). In the roots of *Suaeda salsa*, a halophytic plant, the net Cd^{2+} fluxes are inhibited by Ca^{2+} channel blockers, suggesting that some Cd^{2+} can be transported into cells through Ca^{2+} channels (L. Z. Li *et al.*, 2012). These results indicate that the H^+/Ca^{2+} status of the soil solution is a key factor that constrains Cd^{2+} uptake by plant roots. However, no information is available on how H^+/Ca^{2+} affects the net Cd^{2+} fluxes in poplar roots. Therefore, to improve the Cd^{2+} uptake capacity of poplar roots for phytoremediation, it is essential to clarify the influence of H^+/Ca^{2+} in soil solution on Cd^{2+} influx into poplar roots.

In organisms, γ-glutamylcysteine synthetase (γ-ECS) is a key enzyme in the biosynthesis pathway of glutathione (GSH), the precursor of phytochelatins (PCs) (Noctor *et al.*, 1996; Arisi *et al.*, 2000). It has been demonstrated that the overexpression of *γ-ECS* from *Escherichia coli* in Indian mustard (*Brassica juncea*) can enhance Cd tolerance and accumulation in plants (Zhu *et al.*, 1999). Moreover, a number of transgenic poplar lines were obtained by overexpressing *γ-ECS* from *E. coli* in the cytosol of *Populus tremula* × *P. alba* (Noctor *et al.*, 1996; Arisi *et al.*, 1997). The transgenic poplars exhibited higher γ-ECS activities and foliar GSH concentrations, which may enhance Cd tolerance and the accumulation of more Cd in the aerial parts compared with the wild-type during short-term (2–4 wk) Cd^{2+} exposure (Arisi *et al.*, 2000). Furthermore, the sulfur metabolism of these transgenic poplars has been studied in detail (Noctor *et al.*, 1998). However, the molecular and physiological mechanisms that underlie enhanced Cd uptake and tolerance by these poplars remain uncharacterized.

The overexpression of bacterial *γ-ECS* in the cytosol of *P. tremula* × *P. alba* leads to elevated GSH and PC contents (Noctor *et al.*, 1996, 1998), which can detoxify Cd^{2+} by chelation (Cobbett, 2000), and transgenic plants exhibit higher Cd^{2+} uptake into the roots and greater Cd tolerance compared with wild-type poplars (Koprivova *et al.*, 2002; Ivanova *et al.*, 2011). To elucidate the mechanisms responsible for the effects of bacterial *γ-ECS* overexpression in poplars, we tested wild-type and transgenic *P. tremula* × *P. alba* plants, which were grown using hydroponics. Specifically, we aimed to elucidate: the molecular and physiological mechanisms that underlie elevated Cd^{2+} uptake in relation to H^+/Ca^{2+} in the roots of transgenic poplars; and the mechanisms of GSH-mediated Cd tolerance in transgenic poplars.

Materials and Methods

Plant material and Cd^{2+} exposure

Wild-type and transgenic lines (ggs11 and ggs28) of *Populus tremula* × *P. alba* (Aiton) Smith (INRA female clone 717 1-B4) were used in the present study. The transgenic lines (ggs11 and ggs28) were produced by overexpressing γ-ECS from *E. coli* in the cytosol (Noctor *et al.*, 1996; Arisi *et al.*, 1997). The poplar lines ggs11 and ggs28 contained two and one copies of the transferred gene, respectively (Arisi *et al.*, 1997). Plantlets were produced by micro-propagation (Leple *et al.*, 1992) and cultivated in a climate chamber (day : night temperature, 25°C : 18°C; relative air humidity, 50–60%; light per day, 14 h; and photosynthetic photon flux, 150 μmol m^{-2} s^{-1}). After 4 wk, the rooted plantlets were transferred to an aerated Hoagland nutrient solution in a growth room, which had the same climate conditions as the climate chamber. The nutrient solution was renewed every 3 d. Plants were selected from the wild-type or transgenic lines (36 seedlings for each genotype) with similar heights, which were then marked at the shoot apex, divided into two groups (18 plants in each group), and cultivated for 80 d in modified Hoagland solution (modified: Ca^{2+}: 0.1 mM, pH 5.5) with either 0 or 100 μM Cd^{2+}. After gas exchange measurements, the plants were harvested (Supporting Information Methods S1). Subsequently, their root viability and characteristics were determined (Methods S1).

Measurements of net Cd^{2+} fluxes in association with H^+ and Ca^{2+}

In order to monitor the net Cd^{2+} fluxes in association with H^+ and Ca^{2+}, three fine roots (*c.* 1.5 mm in diameter) from each plant were selected from the wild-type or transgenic lines that received Cd^{2+} exposure. The net Cd^{2+} fluxes were measured noninvasively using the noninvasive micro-test technique (NMT system BIO-IM; Younger Corp., Falmouth, MA, USA) at Xuyue Science & Technology Co. Ltd (Beijing, China), as described previously (He *et al.*, 2011; Xu *et al.*, 2012a; Luo *et al.*, 2013a,b; Ma *et al.*, 2014). An ion-selective microelectrode with an external tip (*c.* 2–4 μm in diameter) was manufactured and silanized with tributylchlorosilane, and the tip was backfilled with a commercially available ion-selective cocktail (for Cd^{2+}, LIX, XY-SJ-Cd; for Ca^{2+}, LIX, XY-SJ-Ca; for H^+, LIX, XY-SJ-H; Younger, Amherst, MA, USA).

In order to determine the positions where the maximal Cd^{2+} flux occurred along the root, a preliminary experiment was performed by taking measurements at the root apex, at 600 and 2000 μm in the region from 0 to 2 mm (He *et al.*, 2011; Ma *et al.*, 2014), at 1 mm intervals in the region from 2 to 5 mm, and at 5 mm intervals in the region from 5 to 20 mm along the root tip (Fig. S1a). The Cd^{2+} gradients near to the root surface (*c.* 2–5 μm) were measured by moving the Cd^{2+}-selective microelectrode between two positions (at a distance of 30 μm) perpendicular to the root axis.

In order to determine the optimum pH for maximum Cd^{2+} uptake by the root tips, the net Cd^{2+} fluxes were monitored for 5 min at the position where the maximum Cd^{2+} flux occurred by using three pH values in the measuring solution (0.1 mM Cd^{2+}, 0.1 mM KCl, 0.5 mM NaCl, 0.3 mM MES, and 0.2 mM Na_2SO_4 with pH 4.0, 5.5 or 7.0, respectively). Each fine root was equilibrated in the measuring solution for 30 min and subsequently transferred to a fresh measuring solution where the net Cd^{2+} fluxes were determined. After determining the optimum pH to assess the net Cd^{2+} influx, the association of the net Cd^{2+} flux with the activity of PM H^+-ATPases was examined. Thus, the net Cd^{2+} influxes were monitored at the optimum pH for 5 min in the measuring solution and the net H^+ fluxes were measured subsequently using the H^+-selective microelectrode. Next,

the same root was transferred to a Petri dish containing 0.5 mM orthovanadate, a specific inhibitor of PM H^+-ATPases (Luo *et al.*, 2013b), and incubated for 25 min. The root was equilibrated in the measuring solution and used to record the net Cd^{2+} and H^+ fluxes again, as described above.

In order to determine the optimum Ca^{2+} concentration for maximum Cd^{2+} uptake by the roots, the net Cd^{2+} fluxes were monitored for 5 min at the optimum pH in the position where the maximum Cd^{2+} flux occurred in the measuring solutions (0.1 mM Cd^{2+}, 0.1 mM KCl, 0.5 mM NaCl, 0.3 mM MES and 0.2 mM Na_2SO_4), which were supplemented with 0.01, 0.1 or 1 mM $CaCl_2$, respectively. After determining the optimum Ca^{2+} concentration for Cd^{2+} uptake, the significance of the calcium channels for the net Cd^{2+} fluxes was investigated. Thus, the net Cd^{2+} fluxes were measured for 5 min at the optimum Ca^{2+} concentration in the measuring solution and the net Ca^{2+} fluxes were monitored subsequently using the Ca^{2+}-selective microelectrode. Next, the same root was transferred to a Petri dish containing 20 μM verapamil, a calcium channel blocker (Li *et al.*, 2010), and incubated for 25 min. The root was equilibrated and used to measure the net Cd^{2+} and Ca^{2+} fluxes again, as described above.

Analysis of the levels of gene transcripts

Total RNA extraction and purification, and quantitative reverse transcription (RT)-PCR were performed based on the method of Chang *et al.* (1993), with minor modifications (H. Li *et al.*, 2012; Cao *et al.*, 2014). Total RNA was isolated from powdered roots (*c.* 500 mg) and purified using a plant RNA extraction kit (R6827, Omega Bio-Tek, Norcross, GA, USA). Subsequently, the first strand cDNA was synthesized using 1 μg total RNA in a total volume of 20 μl, which contained 0.5 μg oligo d(T)18–primer and 200 U RevertAid Moloney murine leukemia virus reverse transcriptase (DRR037A; Takara, Dalian, China). Quantitative PCR was performed using 10 μl 2× SYBR Green Premix Ex Taq II (DRR820A; Takara), 0.5 μl cDNA, and 0.2 μM primer for each plant gene (Table S1) with a CFX96 Real Time system (CFX96; Bio-Rad, Hercules, CA, USA). Tubulin was used as a reference gene (Table S1). To ensure specificity, the PCR products were sequenced and aligned with homologues from other model plants (Fig. S2). PCR was performed in triplicate together with a dilution series of the reference gene.

Histochemical staining of Cd

In order to characterize the localization of Cd at the tissue level, sections or intact tissues from fresh samples of the fine roots, stems and leaves were rinsed in de-ionized H_2O. Subsequently, the samples were exposed to a staining solution (30 mg diphenylthiocarbazone in 60 ml acetone, 20 ml H_2O, and 100 μl glacial acetic acid) for 1 h and rinsed briefly in de-ionized H_2O (He *et al.*, 2013a). Well-stained samples containing Cd-dithizone precipitates with a red–black coloration were photographed under a light microscope (Eclipse E200; Nikon, Tokyo, Japan) using a CCD (DS-Fi1; Nikon) connected to a computer, as described by Cao *et al.* (2012).

Determination of the concentrations of elements, Cd bioconcentration (BCF), and translocation factor (T_f), foliar pigments, soluble sugars and starch

The concentrations of Cd, calcium (Ca), zinc (Zn) and iron (Fe) in root, wood, bark and leaf tissues were determined by flame atomic absorbance spectrometry (Hitachi 180-80; Hitachi Ltd, Tokyo, Japan), according to He *et al.* (2013b). The total amount of Cd in each tissue was calculated by multiplying the Cd concentration in the tissue by the biomass of the tissue.

BCF was defined as the ratio of the metal concentration in the plant roots or aerial tissues relative to that in the soil or nutrient solution (He *et al.*, 2013b). T_f indicated the ability of plants to translocate cadmium from the roots to the shoots. T_f was calculated as the cadmium concentration in the aerial tissues of a plant divided by the Cd concentration in the roots (He *et al.*, 2013b).

The concentrations of foliar pigments were determined spectrophotometrically, according to Wellburn (1994). The concentrations of total soluble sugars and starch were assessed using the anthrone method of Yemm & Willis (1954).

Analysis of $O_2^{\cdot-}$, H_2O_2 and malondialdehyde (MDA)

Histochemical assays of $O_2^{\cdot-}$ and H_2O_2 in the fine roots and leaves were conducted according to Xu *et al.* (2012b). To detect $O_2^{\cdot-}$, fresh fine root and leaf samples were vacuum infiltrated for 30 min and 3 h, respectively, in phosphate buffer (20 mM, pH 6.1) containing 2 mM nitroblue tetrazolium (NBT; N6876, Sigma-Aldrich) and 10 mM Na-azide (NaN_3; S2002, Sigma-Aldrich). The reaction was stopped with distilled water after blue formazan precipitates appeared. To eliminate interference from chlorophyll, the leaves were bleached by immersing in boiling 95% ethanol. Photographs were obtained using a Canon digital SLR camera.

In the roots, H_2O_2 was detected using the fluorescent probe 2,7-dichlorofluorescin diacetate (DCFH-DA; D6883, Sigma-Aldrich). The fresh roots were immersed and vacuum infiltrated in phosphate buffer (20 mM, pH 6.1) containing 10 μM DCFH-DA at 37°C in the dark for 30 min. After washing three times with phosphate buffer, the roots were viewed under a fluorescent microscope (Nikon Eclipse 3000, Melville, NY, USA) with a green fluorescent filter (excitation 415 nm, emission 500–530 nm). To detect H_2O_2 in leaves, the samples were immersed in 1 mg ml^{-1} diaminobenzidine (DAB; D5637, Sigma-Aldrich; pH 3.8), vacuum infiltrated for 4 h until brown spots appeared, and bleached by immersing in boiling 95% ethanol.

The concentrations of $O_2^{\cdot-}$, H_2O_2 and MDA in samples were determined spectrophotometrically, as described by He *et al.* (2013b).

Analysis of antioxidants and anti-oxidative enzyme activities

The concentrations of soluble phenols and free proline were determined spectrophotometrically, according to He *et al.* (2011), whereas ascorbate and dehydroascorbate were analysed as

described by Kampfenkel *et al.* (1995), reduced GSH and oxidized GSH (GSSG) according to Loggini *et al.* (1999), and total thiols (T-SH) according to Tamas *et al.* (2008).

The soluble proteins were extracted from samples and quantified according to Luo *et al.* (2008). The enzyme activities of guaiacol peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX) and GSH reductase (GR) were determined as described by He *et al.* (2011).

Statistical analysis

The net ion flux data were processed using Mageflux v1.0, which was attached to the NMT system. Statistical tests were performed with Statgraphics (STN, St Louis, MO, USA). Unless stated otherwise, genotype and Cd^{2+} exposure were regarded as the main factors. To reduce the chance of type I errors, all of the *P*-values obtained from multiple comparisons were corrected using Tukey's HSD method. The data were tested for normality before statistical analyses. Differences between means were considered significant when the *P*-value of the two-way ANOVA *F*-test was < 0.05. The Cq values obtained from quantitative PCR were normalized using the program proposed by Pfaffl *et al.* (2002) and the fold changes in the levels of transcripts were calculated using the REST program (Pfaffl *et al.*, 2002). The gene expression heatmap was generated using the command heatmap.2 () in the package 'gplots' in R (http://www.r-project.org/). For the principal components analysis (PCA), the data were standardized and then computed using the command prcomp () in R.

Results

Growth and root characteristics

After 14 wk of cultivation in the climate chamber, the wild-type and transgenic poplars (ggs11 and ggs28) exhibited similar morphology (data not shown). The presence of the transgene in poplar lines ggs11 and ggs28, and the absence of this gene in wild-type plants were confirmed (Fig. S3). The CO_2 assimilation rates (*A*) were similar in the wild-type and transgenic lines, but most of the other photosynthetic parameters such as the transpiration rate (*E*) and concentrations of chlorophyll *a* and *b* differed between the wild-type and transgenic lines (Table S2). The root, wood and bark biomasses did not differ between the wild-type and transgenics, whereas the leaf biomass of the transgenics was *c.* 30% lower compared with the wild-type plants in control conditions without Cd^{2+} (Table S3). The root viability of transgenic plants was 45–56% higher than that of wild-type poplars in control conditions without Cd^{2+}, whereas the total root length and root volume of the transgenics were less than those of the wild-type plants (Table S3).

In the transgenic and wild-type poplars, photosynthesis, biomass and the root characteristics were negatively affected by Cd^{2+} exposure, but the inhibitory effects of Cd^{2+} exposure were often significantly more pronounced in the wild-type plants than the transgenics (Tables S2, S3).

Net Cd^{2+} fluxes in association with the fluxes of H^+ and Ca^{2+}

The apical region of fine roots in the wild-type and transgenic poplars exhibited spatial variation in Cd^{2+} uptake (Fig. S1b). The maximum net Cd^{2+} influx occurred at 600 μm from the root tips in wild-type or transgenic poplars (Fig. S1b). Higher net Cd^{2+} influxes were observed in the roots of the transgenics than the wild-type poplars (Fig. S1b), which was supported by the results of the short-term Cd^{2+} flux experiment (Methods S1; Fig. S1c). To determine the influence of the pH in the measuring solution on Cd^{2+} uptake by the roots of wild-type and transgenic poplars, the net Cd^{2+} fluxes were monitored at 600 μm from the root tip (Fig. 1). In addition to pH 5.5, which was the pH of the Hoagland solution applied to poplars, pH 4.0 and pH 7.0 were also tested. The net Cd^{2+} influx was higher (48–311%) in the roots of transgenics compared with the wild-type poplars at all three pH values (Fig. 1). At pH 5.5, the net Cd^{2+} influx increased by *c.* 0.9- to 12-fold compared with that at pH 4.0 or 7.0 for both the wild-type and transgenics (Fig. 1). To investigate the dependency of Cd^{2+} uptake on proton transport, the net Cd^{2+} and H^+ fluxes were measured in the roots of wild-type and transgenic poplars (Fig. 2). The net Cd^{2+} influx (Fig. 2A) and net H^+ efflux (Fig. 2B) were determined in the roots of wild-type and transgenic plants, but net Cd^{2+} efflux (Fig. 2A) and net H^+ influx (Fig. 2B) were detected after the inactivation of PM H^+-ATPases by vanadate, thereby suggesting that activated H^+-ATPases are required to drive Cd^{2+} uptake.

To assess the impact of Ca^{2+} in the measuring solution on Cd^{2+} uptake by the roots of wild-type and transgenic poplars, the net Cd^{2+} fluxes were monitored at 600 μm from the root tip in the presence of 0.01, 0.1 and 1 mM Ca^{2+} (Fig. 3). The net Cd^{2+} influxes into the roots of ggs11 and ggs28 plants were *c.* 18–127% higher than those into the roots of wild-type poplars at the three Ca^{2+} concentrations tested (Fig. 3). In the roots of wild-type poplars, the net Cd^{2+} influxes were 27.6 ± 4.9, 63.1 ± 3.1,

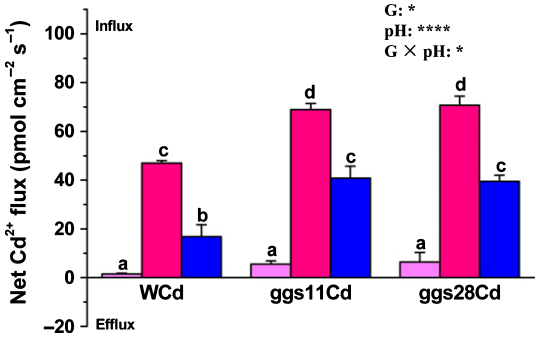

Fig. 1 Means of net Cd^{2+} fluxes in 5 min in different measuring solutions at 600 μm distance from the root tips of wild-type (W) or transgenic lines (ggs11, ggs28) of *Populus tremula* × *P. alba* exposed to 100 μM Cd^{2+} (Cd) for 80 d. Pink bars, pH 4.0; red bars, pH 5.5; blue bars, pH 7.0. Data indicate means ± SE ($n = 6$). Different letters on the bars indicate significant difference between the treatments. *P*-values of the two-way ANOVAs of genotype (G), pH and their interaction (G × pH) are indicated: *, $P < 0.05$; ****, $P < 0.0001$.

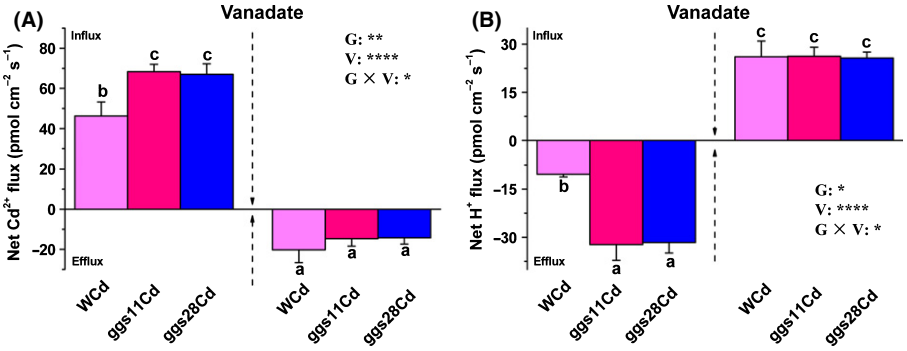

Fig. 2 Net fluxes of Cd^{2+} (A) and H^+ (B) in 5 min in the measuring solution at 600 μm from the root tips of wild-type (W) or transgenic lines (ggs11, ggs28) of *Populus tremula* × *P. alba* exposed to 100 μM Cd^{2+} (Cd) for 80 d. Data indicate means ± SE ($n = 6$). Different letters on the bars indicate significant difference between the treatments. *P*-values of the two-way ANOVAs of genotype (G), vanadate (V) and their interaction (G × V) are indicated. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

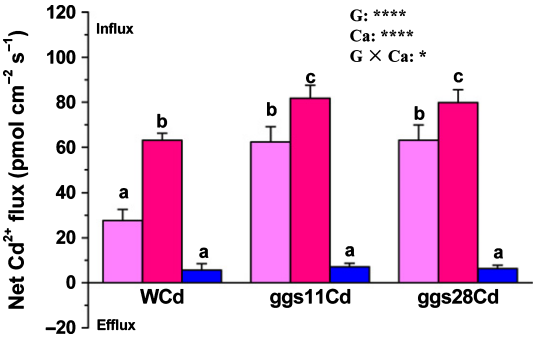

Fig. 3 Net Cd^{2+} fluxes in 5 min in different measuring solutions at 600 μm from the root tips of wild-type (W) or transgenic lines (ggs11, ggs28) of *Populus tremula* × *P. alba* exposed to 100 μM Cd^{2+} (Cd) for 80 d. Pink bars, 0.01 mM Ca^{2+}; red bars, 0.1 mM Ca^{2+}; blue bars, 1 mM Ca^{2+}. Data indicate means ± SE ($n = 6$). Different letters on the bars indicate significant difference between the treatments. *P*-values of the two-way ANOVAs of genotype (G), Ca and their interaction (G × Ca) are indicated. *, $P < 0.05$; ****, $P < 0.0001$.

and 5.7 ± 2.8 pmol cm^{-2} s^{-1} at 0.01, 0.1 and 1 mM Ca^{2+}, respectively (Fig. 3). Similarly, the net Cd^{2+} influxes in ggs11 and ggs28 plants were highest at 0.1 mM Ca^{2+} and lowest at 1 mM Ca^{2+} (Fig. 3). These results indicate that Cd^{2+} uptake by the roots of wild-type and transgenic poplars is modulated by Ca^{2+}. We hypothesized that some Cd^{2+} in the measuring solution enters the root cells via calcium channels located at the PM. To test this hypothesis, the activity of calcium channels was inhibited specifically by verapamil (Fig. 4). As expected, the net Cd^{2+} influx into roots was inhibited by 70% in the wild-type and by *c.* 80% in transgenics (Fig. 4A) when the calcium channels were blocked by verapamil (Fig. 4B).

Levels of the transcripts of genes involved in Cd^{2+} transport, detoxification and interaction with H^+ and Ca^{2+}

The wild-type and transgenic poplars exhibited differences in Cd^{2+} uptake in association with H^+/Ca^{2+} by the roots, thus we expected that there was differential transcriptional regulation of the genes involved in Cd^{2+} transport, detoxification and interaction with H^+ and Ca^{2+}. Based on previous studies and our preliminary experiments, our transcriptional analysis focused on nine genes involved in Cd^{2+} transport and detoxification, three genes encoding PM H^+-ATPases, and three genes involved in the Ca^{2+} transport and signalling pathway (Fig. 5). In poplars, natural resistance-associated macrophage protein 1.3 (NRAMP1.3), zinc/iron-regulated transporter-like protein 2 (ZIP2) and ZIP6.2 are considered to be located at the PM where they control Cd^{2+} entry into the cytosol of root cells (Migeon *et al.*, 2010; Ma *et al.*, 2014). The transcript levels of *NRAMP1.3* and *ZIP6.2* in the roots of ggs11 and ggs28 poplars were already upregulated compared with those in the wild-type plants in the absence of Cd^{2+} (Fig. 5). After Cd^{2+} exposure, the mRNA levels of both genes were enhanced in the roots of the wild-type and transgenics, but a greater increase was detected in the roots of ggs11 and ggs28 (Fig. 5). In *A. thaliana*, cytosolic Cd^{2+} can be bound by PCs, which are synthesized from GSH by PC synthase (PCS) (Howden *et al.*, 1995a,b). Subsequently, the PC–Cd complexes can be transported into vacuoles by ATP-binding cassette (ABC) transporters, such as ABCC1 and ABCC2 (Park *et al.*, 2012; Song *et al.*, 2014). Alternatively, cytosolic Cd^{2+} can be translocated to vacuoles by metal tolerance protein 1 (MTP1), which is located in the tonoplast membrane (Lin & Aarts, 2012). In poplar roots, the transcript levels of the homologous genes of *AtPCS*, *AtABCC1* and *AtMTP1* exhibit differential expression in response to Cd^{2+} exposure (Ma *et al.*, 2014). The mRNA levels of homologous *PCS* increased *c.* 2.6- to 2.8-fold in the roots of ggs11 and ggs28 compared with the roots of the wild-type when Cd^{2+} was absent. When plants were exposed to Cd^{2+}, the levels of the *PCS* transcripts were enhanced further in the roots of the transgenics compared with the wild-type (Fig. 5). In addition, the transcript levels of the poplar homologue *ABCC1* were upregulated in the roots of ggs11 and ggs28 compared with the wild-type in the absence of Cd^{2+}, whereas no changes in these transcript levels were detected in the wild-type and transgenics after Cd^{2+} exposure (Fig. 5). By contrast, decreased mRNA levels of the poplar orthologue *MTP1* were detected in the transgenics compared with the wild-type in the absence of Cd^{2+} and after Cd^{2+} treatment, the transcript levels of this gene were reduced further in all poplar lines (Fig. 5). In *A. thaliana*, ABC transporter of the mitochondria 3 (ATM3) is located in the mitochondrial membrane where it exports Cd-GSH into the cytosol (Kim *et al.*, 2006). The mRNA levels of a gene analogous to *AtATM3* were similar in the wild-type and transgenics in the absence of Cd^{2+}, whereas Cd^{2+} exposure increased the transcript levels of this gene in the wild-type and transgenics (Fig. 5). In *A. thaliana*, heavy metal ATPase4 (HMA4) and plant cadmium

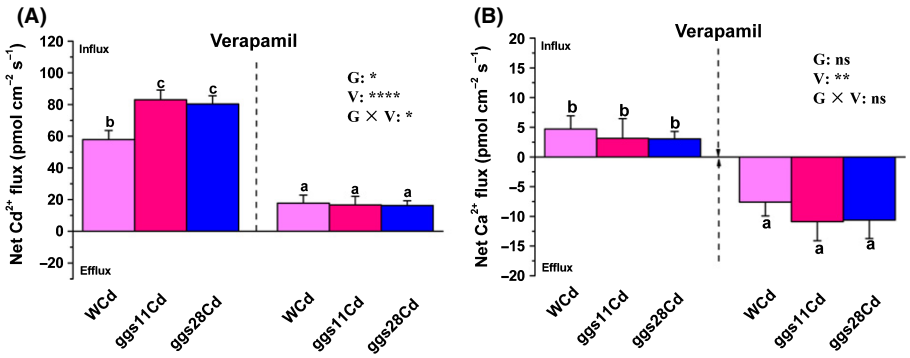

Fig. 4 Net fluxes of Cd^{2+} (A) and Ca^{2+} (B) in 5 min in the measuring solution (pH 5.5) contained 0.1 mM Ca^{2+} at 600 μm from the root tips of wild-type (W) or transgenic lines (ggs11, ggs28) of *Populus tremula* × *P. alba* exposed to 100 μM Cd^{2+} (Cd) for 80 d. Data indicate means ± SE ($n = 6$). Different letters on the bars indicate significant difference between the treatments. *P*-values of the two-way ANOVAs of genotype (G), verapamil (V) and their interaction (G × V) are indicated. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; ns, not significant.

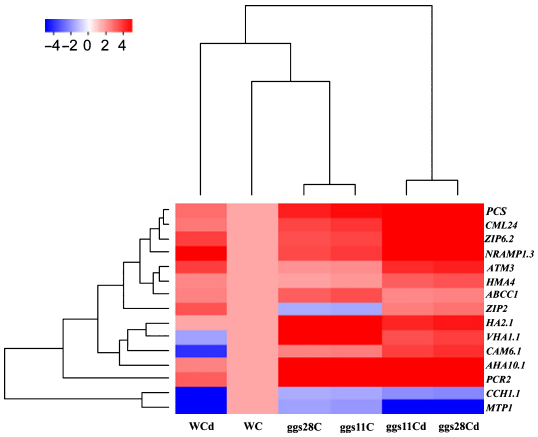

Fig. 5 Fold changes of transcripts of key genes involved in Cd^{2+} uptake, transport and detoxification in fine roots of wild-type (W) or transgenic lines (ggs11, ggs28) of *Populus tremula* × *P. alba* exposed to 0 (C) or 100 μM Cd^{2+} (Cd) for 80 d. The colour scale indicates fold changes of mRNAs. For each gene, the expression level was set to 1 in roots of wild-type (W) *P. tremula* × *P. alba* exposed to 0 μM Cd^{2+} (C), and the corresponding fold changes were calculated under other treatments.

resistance protein 2 (PCR2) are located in the PM where they export bivalent cations (e.g. Cd^{2+}/Zn^{2+}) from the cytosol, and they facilitate the translocation of these ions into the central cylinder for xylem transport (Mills *et al.*, 2003; Song *et al.*, 2010). In poplar, the mRNA level of *HMA4* is altered in response to heavy metal exposure (Adams *et al.*, 2011; Ma *et al.*, 2014). The transcript levels of *HMA4* were similar in the wild-type and transgenics in the absence of Cd^{2+} but after Cd^{2+} exposure, enhanced transcript levels of this gene were detected in the roots of the transgenics compared with wild-type poplars (Fig. 5). The transcript levels of the homologous gene of *AtPCR2* in the roots of transgenics were *c.* 3.5- to 3.7-fold higher than those in the roots of wild-type poplars in the absence of Cd^{2+}, whereas the mRNA levels of this gene were increased by *c.* 10-fold in transgenics and by *c.* 2-fold in wild-type roots in response to Cd^{2+} exposure (Fig. 5).

The uptake of Cd^{2+} into the roots of wild-type and transgenic poplars may be related to the activities of PM H^+-ATPases, thus the transcriptional regulation of three genes encoding PM H^+-ATPases was studied, that is *VHA1.1*, *HA2.1* and *AHA10.1* (Fig. 5). A previous study demonstrated that the transcript levels of *HA2.1* and *AHA10.1* changed in poplar roots treated with Cd^{2+} (Ma *et al.*, 2014). The mRNA levels of *HA2.1* and *AHA10.1* were increased in the roots of the transgenics compared with the wild-type in control conditions without Cd^{2+}, whereas they remained unchanged in the roots of Cd^{2+}-treated wild-type poplars. However, marked increases in the mRNA levels of these genes were detected in the roots of Cd^{2+}-exposed transgenics (Fig. 5).

The net Cd^{2+} influx into poplar roots was also affected by the presence of Ca^{2+} in the solution. Therefore, the expression levels of three genes involved in the Ca^{2+} signalling pathway and transport, that is *Calmodulin-like protein24* (*CML24*), *Calmodulin6.1* (*CAM6.1*) and *Calcium channel1.1* (*CCH1.1*), were studied in the roots of wild-type and transgenic poplars. In *A. thaliana*, *CML24* encodes a protein that shares 40% similarity with the calmodulin that functions in Ca^{2+} binding and it is located in the PM and the Golgi apparatus (Ma *et al.*, 2008). In sweet potato (*Ipomoea batatas*), *CAM6.1* encodes a calmodulin located in the cytoplasm (Chen *et al.*, 2012) and *AtCCH1.1* encodes a depolarization-activated Ca^{2+} channel in the tonoplast membrane (Dadacz-Narloch *et al.*, 2011). The transcript levels of homologous *AtCML24* increased in the roots of the transgenics compared with the wild-type poplars in control conditions without Cd^{2+}. The mRNA levels of this gene remained unaltered in the Cd^{2+}-treated wild-type, whereas they were markedly increased in Cd^{2+}-exposed transgenics (Fig. 5). The transcript levels of the homologous potato gene *CAM6.1* were similar in the wild-type and transgenics in the absence of Cd^{2+}, whereas the mRNA levels of this gene were repressed in the Cd^{2+}-treated wild-type but upregulated in the Cd^{2+}-exposed transgenics (Fig. 5). Reduced transcript levels of the orthologous gene of *AtCCH1.1* were found in the roots of the transgenics compared with the wild-type in control conditions without Cd^{2+} and repression of the *CCH1.1* transcript levels was detected in the wild-type and transgenics exposed to Cd^{2+} (Fig. 5).

Cd localization and accumulation, and its effects on nutrients and carbohydrates

The Cd localization had a similar pattern in the wild-type and transgenic poplars (Fig. 6). In roots, Cd was located mainly in the epidermal cells and root hairs (Fig. 6a1–a3). In stems, Cd was located mainly in the cell walls of vessels and cortical cells, as well as in collenchyma cells (Fig. 6b1–b3). In leaves, Cd was found mainly in leaf veins (Fig. 6c1–c3) and mesophyll cells (Fig. 6d1–d3).

At the tissue level, Cd accumulation was detected in the roots, wood, bark and leaves of wild-type and transgenic poplars (Figs 7, S4). The Cd concentrations were lower in the roots of transgenics than the wild-type poplars, whereas significantly higher Cd concentrations (26–35%) were found in the wood, bark and leaf tissues of transgenics compared with the wild-type after Cd^{2+} exposure (Fig. S4). As a consequence, total Cd amounts were 69–82% higher in the aerial parts of the transgenics compared with the wild-type plants (Fig. 7A). Cd accumulation was not detected in the wild-type or the transgenic controls (Fig. 7A). The BCF was higher in the roots of the wild-type than the transgenics, whereas the opposite pattern applied to the aerial parts (Fig. 7B). The Cd translocation factor was 3.7- to 4.6-fold higher in the transgenics than the wild-type poplars (Fig. 7C).

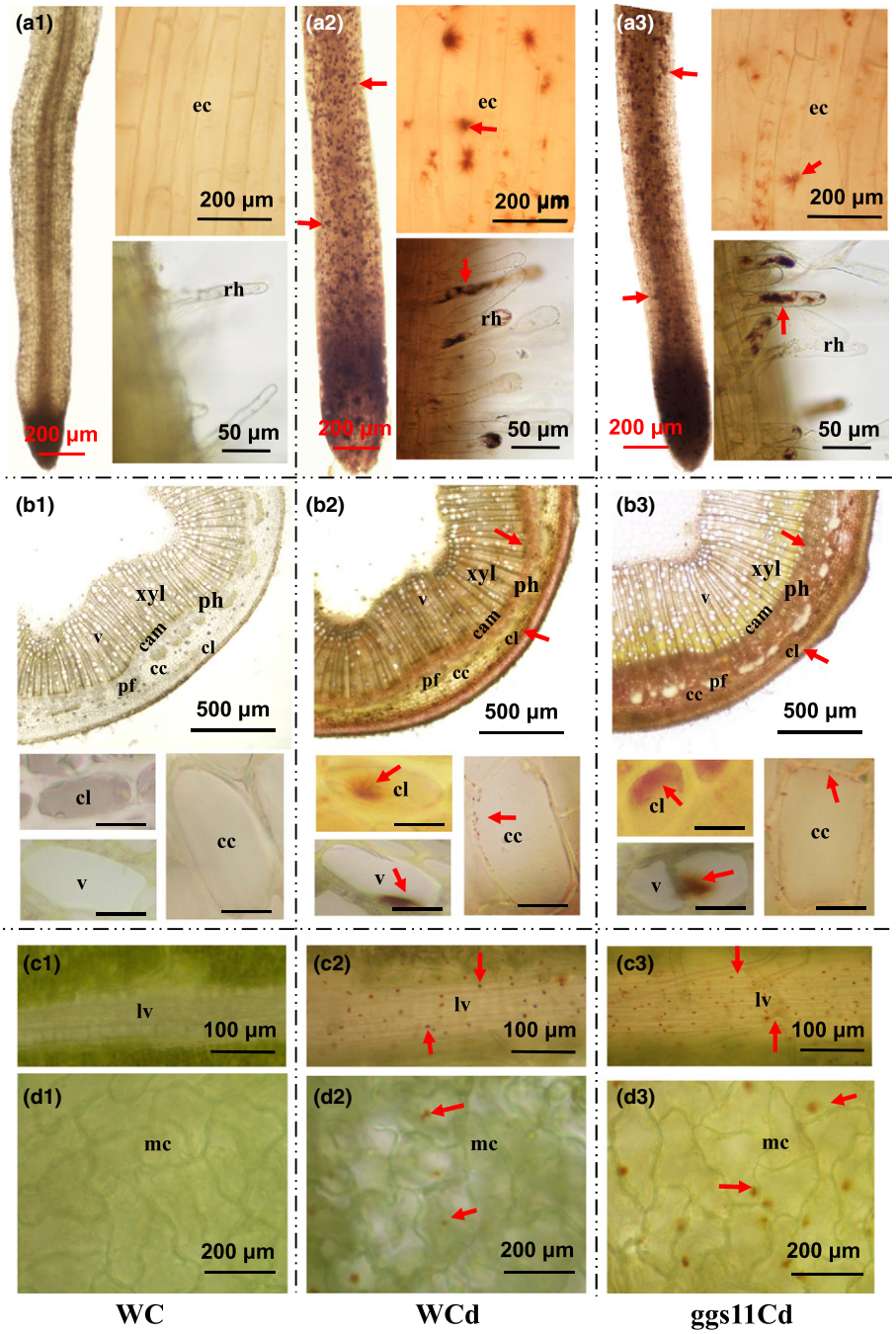

Fig. 6 Cadmium (Cd) localization in the root (a1–a3), stem (b1–b3) and leaf tissues (c1–c3, d1–d3) of wild-type (W) or transgenic line (ggs11) of *Populus tremula* × *P. alba* exposed to 0 (C) or 100 μM Cd^{2+} (Cd) for 80 d. Arrows point to precipitates of Cd-dithizone. The histochemical detection of Cd in root, stem and leaf tissues of transgenic lines (ggs11, ggs28, not shown) under 0 μM Cd^{2+} (C) treatment was similar to wild-type (W) *P. tremula* × *P. alba*, and Cd-dithizone localization in all tissues of line ggs28 (not shown) was similar to that of line ggs11 under 100 μM Cd^{2+} (Cd). (a2, a3) Cd-dithizone precipitates in the roots, the inserts highlighted deposited Cd in epidermal cells and root hairs; (b2, b3) Cd-dithizone precipitates in stem, the inserts highlighted deposited Cd in collenchyma, vessel and cortical cell, bars in inserts, 20 μm; (c2, c3) Cd-dithizone precipitates in leaf veins; (d2, d3) Cd-dithizone precipitates in mesophyll cells. ec, epidermal cells; rh, root hairs; cl, collenchyma; cc, cortical cells; pf, phloem fiber; ph, phloem; cam, cambium; v, vessel; xyl, xylem; lv, leaf vein; mc, mesophyll cells.

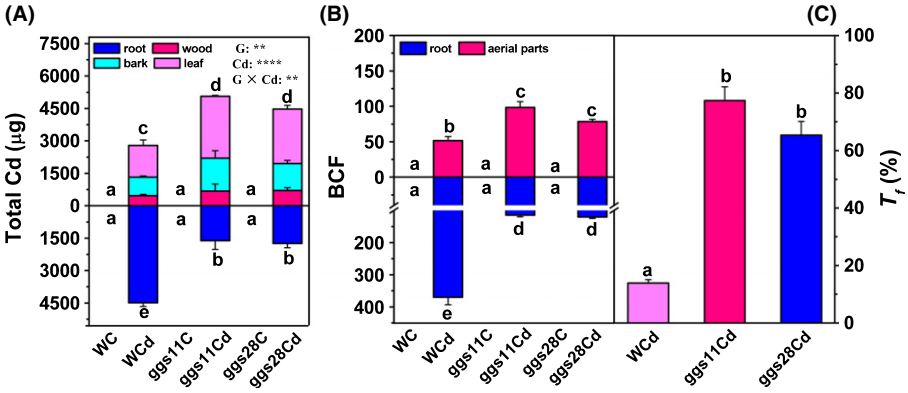

Fig. 7 (A) Total cadmium (Cd) amount in root, wood, bark and leaf tissues, (B) bio-concentration factor (BCF) in root and aerial parts, and (C) translocation factor (T_f) of wild-type (W) or transgenic lines (ggs11, ggs28) of *Populus tremula* × *P. alba* exposed to 0 (C) or 100 μM Cd^{2+} (Cd) for 80 d. Bars indicate means ± SE ($n = 6$). Different letters on the bars for the same tissue indicate significant difference between the treatments. *P*-values of the two-way ANOVAs of genotype (G), Cd^{2+} (Cd) and their interaction (G × Cd) are indicated. **, $P < 0.01$; ****, $P < 0.0001$.

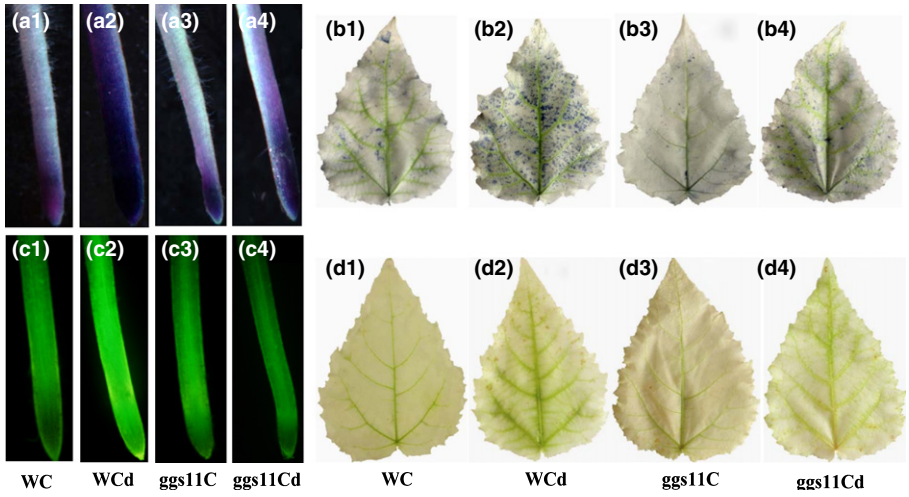

Fig. 8 Histochemical detection of $O_2^{\cdot-}$ (a1–a4, b1–b4) and H_2O_2 (c1–c4, d1–d4) in root (a, c) and leaf (b, d) tissues of wild-type (W) or transgenic line (ggs11) of *Populus tremula* × *P. alba* exposed to 0 (C) or 100 μM Cd^{2+} (Cd) for 80 d. Line ggs28 (not shown) produced similar histochemical staining of $O_2^{\cdot-}$ or H_2O_2 as line ggs11. Blue and brown deposits in leaves indicate $O_2^{\cdot-}$ and H_2O_2, respectively. Additional images of H_2O_2 staining in roots are presented in Supporting Information Fig. S6.

In addition, the concentrations of Ca, Zn and Fe were determined in the root, wood, bark and leaf tissues of wild-type and transgenic poplars (Table S4). In the roots, the Ca concentrations were higher in the transgenics than the wild-type in control conditions, whereas the opposite was observed after Cd^{2+} exposure. Cd^{2+} exposure increased the Ca concentrations in the roots of the wild-type, whereas it reduced the Ca concentrations in the roots of transgenic poplars. In the wood and bark tissues, the Ca concentrations were higher in the transgenics than the wild-type plants, and Cd^{2+} treatment increased the Ca concentrations in the wood and bark of all poplar lines. No genotype effects were found on the concentrations of Zn and Fe in the tissues examined. Cd^{2+} treatment increased the Zn concentrations in the roots of transgenic and wild-type poplars ($P < 0.0001$), but decreased the Zn concentrations in the bark of transgenics ($P < 0.01$).

Total soluble sugars and starch are important compounds for energy metabolism, particularly in stress conditions, and thus the concentrations of these compounds were examined (Fig. S5). The concentrations of soluble sugars were higher in the root, wood and leaf tissues of the transgenics than the wild-type plants. The starch concentrations were also higher in the roots and leaves of the transgenics than the wild-type poplars. Cd^{2+} exposure increased the concentrations of soluble sugars in wood and bark, and decreased the concentrations of starch in the leaves of wild-type and transgenic plants.

ROS and antioxidants

The ROS and antioxidant concentrations can be imbalanced in plants after Cd^{2+} exposure. Thus, concentrations of $O_2^{\cdot-}$, H_2O_2, MDA, antioxidants and the activities of anti-oxidative enzymes were assessed in wild-type and transgenic poplars (Figs 8, 9, S6–S8). Histochemical staining detected higher concentrations of $O_2^{\cdot-}$ and H_2O_2 in the roots or leaves of the wild-type compared with transgenic plants (Fig. 8). Cd^{2+} exposure enhanced $O_2^{\cdot-}$ accumulation in the roots and leaves of wild-type and transgenic poplars, but this effect was less pronounced in the transgenics than in the wild-type plants (Fig. 8). Similarly, H_2O_2 accumulation increased in the roots and leaves of the wild-type and transgenics after Cd^{2+} treatment, but this effect was also less pronounced in the transgenics (Fig. 8). These results were confirmed by spectrophotometric analyses (Fig. S6). Moreover, the analysis of MDA concentrations indicated oxidative stress after Cd^{2+} exposure, which supported the changes in the $O_2^{\cdot-}$ and H_2O_2 concentrations in the wild-type and transgenic poplars (Fig. S6).

The concentrations of total thiols were higher in the roots of the transgenics than the wild-type plants, irrespective of the Cd^{2+} treatments (Fig. 9). Cd^{2+} exposure increased the total thiol concentrations in the wood, bark and leaf tissues of wild-type and transgenic poplars (Fig. 9). The GSH concentrations were higher in the roots, bark and leaves of transgenics than the wild-type poplars in control conditions without Cd^{2+} (Fig. 9). Cd^{2+} exposure increased the GSH concentrations in the root, bark and leaf

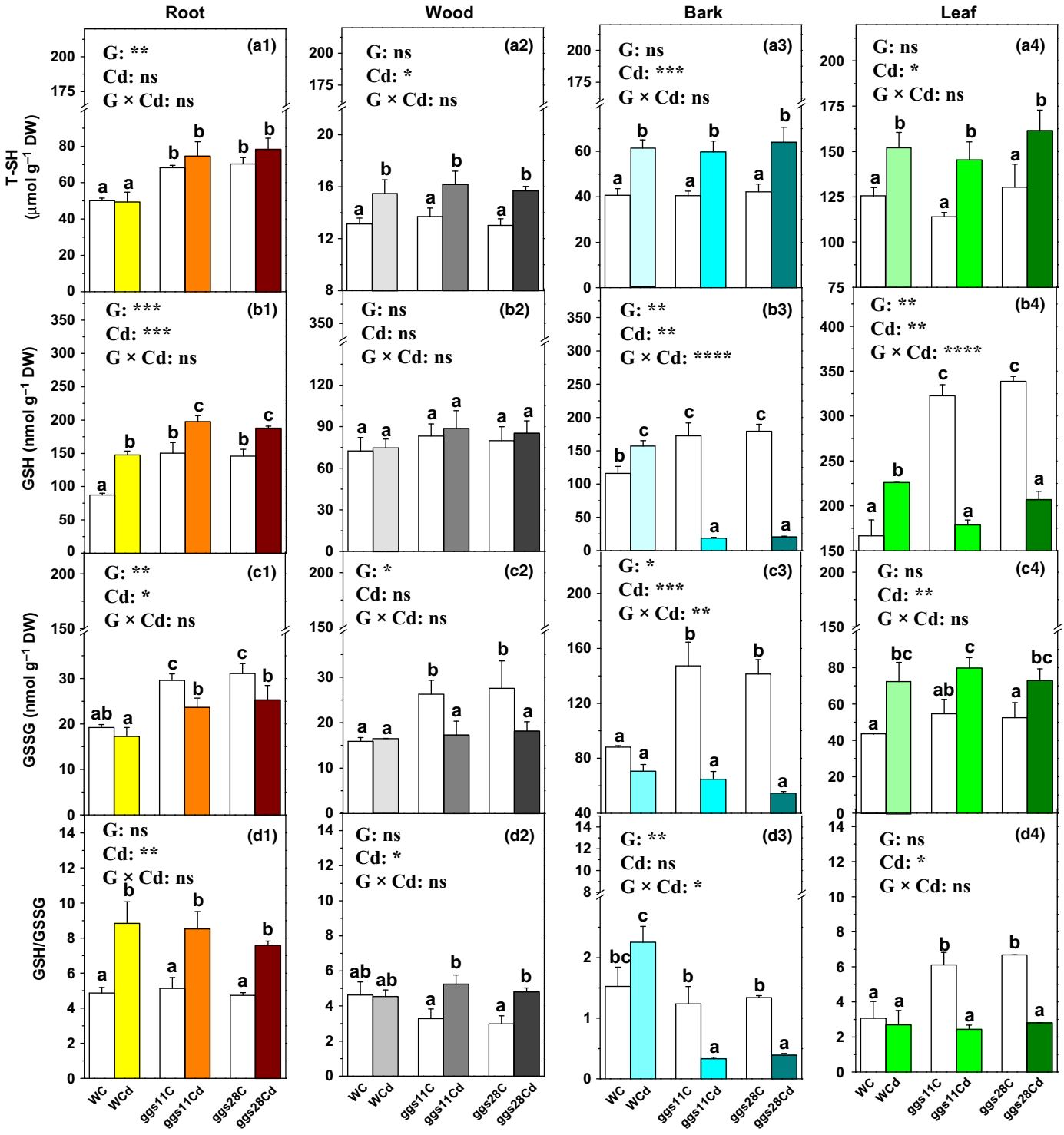

Fig. 9 Total thiols (T-SH, a1–a4), glutathione (GSH, b1–b4), oxidized glutathione (GSSG, c1–c4) and GSH/GSSG (d1–d4) in root (a1–d1), wood (a2–d2), bark (a3–d3) and leaf (a4–d4) tissues of wild-type (W) or transgenic lines (ggs11, ggs28) of *Populus tremula* × *P. alba* exposed to 0 (C) or 100 µM Cd^{2+} (Cd) for 80 d. Bars indicate means ± SE ($n = 6$). Different letters on the bars for the same tissue indicate significant difference between the treatments. *P*-values of the two-way ANOVAs of genotype (G), Cd^{2+} (Cd) and their interaction (G × Cd) are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

tissues of the wild-type, but increased the GSH concentrations in the roots and decreased the GSH concentrations in the bark and leaf tissues of the transgenics (Fig. 9). The concentrations of GSSG were higher in the root, wood and bark tissues of the transgenics compared with the wild-type plants, irrespective of the Cd^{2+} treatments. Cd^{2+} exposure reduced the GSSG concentrations in the roots, wood and bark of the transgenics, but increased the GSSG concentrations in the leaves (Fig. 9). The ratio of GSH relative to GSSG was higher in bark of the wild-type than that of transgenic poplars. Cd^{2+} exposure resulted in a higher ratio of GSH relative to GSSG in the roots of the wild-type and transgenics, as well as in the wood of the transgenics, but the GSH to GSSG ratio was lower in the bark and leaves of the transgenics (Fig. 9).

Soluble phenolics, free proline and ascorbate are important antioxidants in plants. In transgenics, higher concentrations of soluble phenolics were detected in the roots and leaves, as well as free proline in the root and bark tissues, compared with wild-type plants (Fig. S7). In most cases, Cd^{2+} treatment resulted in higher concentrations of soluble phenolics, free proline and ascorbate in the wild-type and transgenic poplars (Fig. S7).

In order to assess the activities of anti-oxidative enzyme, GPX, CAT, APX and GR were analysed in wild-type and transgenic poplars (Fig. S8). In the transgenics, GPX and GR had higher activities in the leaves, but APX and GR had lower activities in the roots compared with the wild-type plants. In most cases, Cd^{2+} exposure reduced the activities of anti-oxidative enzymes.

PCA of physiological responses

In order to determine the response patterns of the wild-type and transgenic poplars to Cd^{2+} exposure, PCA was conducted using the physiological data related to Cd^{2+} exposure, nutrient concentrations, growth, photosynthesis parameters, soluble sugars and starch, ROS and antioxidants (Fig. 10; Table S5). PC1 clearly separated the effects of Cd^{2+} treatment and PC2 uncovered variation in the genotype effects. PC1 and PC2 accounted for 48% and 15% of the total variation, respectively. The Cd concentrations and amounts in wood, bark, and leaf tissues were key contributors to PC1, whereas the concentrations of total thiols, soluble sugars and H_2O_2 in the roots were important factors for PC2. The results of the PCA analysis indicated that the wild-type and transgenic poplars exhibited distinct physiological responses to Cd^{2+} exposure, which were attributable mainly to the differences between the wild-type and transgenics in terms of the concentrations of total thiols, H_2O_2 and soluble sugars in the roots.

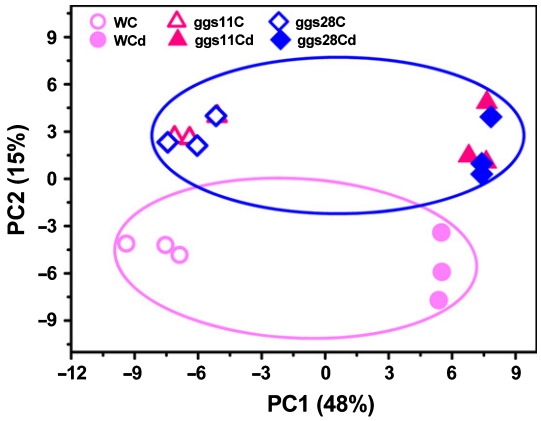

Fig. 10 Principal components analysis (PCA) plots of cadmium (Cd) concentrations and amounts, nutrient elements, growth, photosynthesis related parameters, soluble sugars and starch, and oxidants and antioxidants in the fine roots, wood, bark and leaves of wild-type (W) or transgenic lines (ggs11, ggs28) of *Populus tremula* × *P. alba* exposed to 0 (C) or 100 μM Cd^{2+} (Cd) for 80 d. PCA was conducted based on data (both values were averaged in the same tissue with the same treatment) presented in Tables S2–S4 and Figs S4–S8.

Discussion

Molecular and physiological mechanisms that underlie the elevated net Cd^{2+} influxes related to H^+/Ca^{2+} in the roots of transgenic poplars

The roots are the primary organs used by plants to absorb Cd^{2+} and they play a pivotal role in phytoremediation (Lux *et al.*, 2011), but little information is available on the effects of H^+/Ca^{2+} in the soil solution on Cd^{2+} influxes into the roots and the underlying molecular and physiological mechanisms. This study provides experimental evidence of the impacts of H^+/Ca^{2+} on Cd^{2+} uptake and the underlying molecular mechanisms in the roots of woody plants.

The maximum net Cd^{2+} influxes at 600 μm from the root tips of the wild-type and transgenic poplars were consistent with those reported in previous studies (He *et al.*, 2011; Ma *et al.*, 2014). A higher Cd^{2+} uptake capacity was also observed in the apical region (0–2 mm) of the actively growing roots of herbaceous plants compared with the more distant regions of roots (Pineros *et al.*, 1998; Farrell *et al.*, 2005; Lux *et al.*, 2011; L. Z. Li *et al.*, 2012). The apical region of roots is characterized by epidermal cells that lack cutin in the cell wall, thereby facilitating Cd^{2+} uptake (Lux *et al.*, 2011; Chen *et al.*, 2013). In addition, the root viability is often assessed using the 2,3,5-triphenyl tetrazolium chloride (TTC) reduction assay in various stress conditions (Bagniewska-Zadworna, 2008; Yamauchi *et al.*, 2014). TTC is reduced mainly by dehydrogenases, which are associated with the function of mitochondria in plants (Bagniewska-Zadworna, 2008). The root viabilities of the transgenic poplars in control conditions without Cd^{2+} exposure were higher compared with those of the wild-type plants, thereby suggesting that the transgene can result in higher physiological activities of dehydrogenases in the roots of transgenic poplars. The higher net Cd^{2+} influx into the roots of transgenic poplars corresponded to increased root viability, which indicates that transgenic poplars possess a higher capacity for Cd^{2+} uptake compared with wild-type poplars. Cd is a nonessential and highly toxic element for plants, thus this higher root capacity for Cd^{2+} uptake is probably related to the molecular and physiological regulation of Cd^{2+} detoxification in transgenic poplars. In agreement with the higher net Cd^{2+} influx into the roots of the transgenics compared with wild-type poplars, the transcript levels of homologues of *Arabidopsis* genes

involved in Cd^{2+} transport and detoxification, such as *ZIP6.2*, *NRAMP1.3*, *PCS*, *ABCC1*, and *PCR2*, were also increased in the roots of transgenic poplars. The increased transcript levels of these genes in transgenic poplars compared with the wild-type plants appeared to contribute to the greater capacity for Cd^{2+} absorption by the roots of the transgenics. In the cytosol, Cd^{2+} can be chelated with GSH and/or PCs to minimize its toxicity. PCs are biosynthesized using GSH as a precursor in a reaction that is catalysed by PCS. In the ggs11 and ggs28 lines, previous studies have shown that overexpressing bacterial *γ-ECS* in the cytosol increased the concentrations of foliar GSH (Arisi *et al.*, 1997, 2000) and elevated the sulfur fluxes via the sulfate assimilation pathway (Scheerer *et al.*, 2010) compared with wild-type poplars. In agreement, the present study found elevated concentrations of GSH in the roots of the transgenics compared with wild-type poplars. The increased concentrations of GSH and enhanced mRNA levels of *PCS* in the roots of transgenics may result in a higher capacity for Cd^{2+} detoxification. Consistent with our results, overexpression of *PCS* from *P. trichocarpa* in *P. tremula* × *P. alba* doubled the accumulation of Zn in the leaves (Adams *et al.*, 2011). Recently, it was reported that the overexpression of *PCS1* from the aquatic macrophyte *Ceratophyllum demersum* in *A. thaliana* increased Cd^{2+} accumulation in aerial tissues (Shukla *et al.*, 2013). These results indicate that the transcriptional regulation of key genes involved in Cd^{2+} uptake and detoxification play essential roles in the capacity of roots to absorb Cd^{2+}.

Our analysis of the effects of changes in the pH on net Cd^{2+} fluxes suggest that the net Cd^{2+} fluxes in the roots of transgenics were more sensitive to pH than the roots of wild-type poplars; thus, Cd^{2+} absorption is an energy-driven process in poplar roots. Protons are closely involved in the translocation of cations or anions across the PM of plant root cells (Newman, 2001). However, no previous studies have reported the effects of pH changes on the Cd^{2+} flux in plants, although differences in the pH dependency of nutrient ion fluxes have been reported in plant roots. In soybean (*Glycine max*), the net NH_4^+ influxes were lower at pH 4.0 than pH 7.0, but changes in the pH had no effect on the net NH_4^+ influxes in two conifer species, *Pinus contorta* and *Pseudotsuga menziesii* (Hawkins & Robbins, 2010). Cd^{2+} is thought to enter the cytosol *via* nutrient ion transporters located in the PM. The maximum net Cd^{2+} influxes at pH 5.5 suggest that this pH represents a more suitable environment for these ion transporters compared with pH 4.0 or 7.0.

This different pH dependency of the roots of the wild-type and transgenic poplars is probably associated with PM H^+-ATPases, which extrude cytosolic protons outside at the expense of ATP to create electrochemical gradients across the PM that drive ion transport (Palmgren, 2001). Thus, higher Cd^{2+} transport rates require a stronger driving force, which can be obtained with more abundant PM H^+-ATPases. In agreement with the higher net Cd^{2+} influxes in transgenics at the optimum pH, we detected higher transcript levels of *VHA1.1*, *HA2.1* and *AHA10.1*, which encode PM H^+-ATPases, in the transgenics compared with the wild-type poplars, possibly leading to elevated PM H^+-ATPase activity levels in the transgenics. These results suggest that the enhanced mRNA levels of genes encoding PM H^+-ATPases may facilitate higher Cd^{2+} uptake in the roots of the transgenics compared with wild-type poplars at the optimum pH.

In addition to pH, Ca^{2+} plays a role in the net Cd^{2+} influxes into poplar roots. The maximum net Cd^{2+} influx into the roots of all poplar lines occurred at 0.1 mM Ca^{2+}, which demonstrates that this is the optimum Ca^{2+} concentration for Cd^{2+} uptake, irrespective of transformation. The higher net Cd^{2+} influxes into the roots of the transgenics compared with wild-type poplars at the optimum Ca^{2+} concentration suggests that the net Cd^{2+} uptake in transgenics is more sensitive to changes in the exogenous Ca^{2+} concentration. No information is available on the effects of changes in the Ca^{2+} concentration on the net Cd^{2+} fluxes in plant roots, but previous studies suggest that exogenous Ca^{2+} can affect Cd accumulation in plants and attenuate Cd^{2+} phytotoxicity (Kim *et al.*, 2002; Nedjimi, 2009; Hayakawa *et al.*, 2011; Wan *et al.*, 2011). For example, the addition of exogenous Ca^{2+} resulted in higher Cd concentrations in the roots but less Cd accumulation in the aerial parts of a woody species, *Gamblea innovans* (Hayakawa *et al.*, 2011).

Part of the Cd^{2+} that is taken up is thought to enter plant cells via Ca^{2+} channels located in the PM. In agreement with the higher net Cd^{2+} influx at the optimum Ca^{2+} concentration, lower repression of the transcript levels of *CCH1.1* and enhanced abundances of the mRNAs of *CAM6.1* and *CML24* were detected in roots of the transgenics compared with the wild-type plants. These findings indicate that the transcriptional regulation of genes involved in Ca^{2+} transport and binding plays an essential role in sensing changes in the Ca^{2+} concentration to facilitate Cd^{2+} uptake. The physiological and molecular functions of these genes have been documented in plants (Delk *et al.*, 2005; Dadacz-Narloch *et al.*, 2013), but little information is available regarding the effects of Cd^{2+} exposure on their transcriptional regulation. Our results indicate that the differential transcriptional regulation of these genes in transgenic and wild-type poplars contributes to the enhanced Cd^{2+} uptake in transgenics at the optimum Ca^{2+} concentration.

Overall, our results indicate that pH 5.5 and a Ca^{2+} concentration of 0.1 mM are the optimum conditions for maximizing the net Cd^{2+} influxes into the roots of wild-type and transgenic poplars, and that the higher capacity of the roots of the transgenics for Cd^{2+} uptake is probably related to the increased transcript levels of genes involved in Cd^{2+} uptake and detoxification.

Mechanisms of GSH-mediated Cd tolerance in transgenic poplars

More Cd was accumulated in the aerial parts of the transgenics, but lower reductions in *A* and the biomass, as well as higher concentrations of soluble sugars and starch were found in the transgenics exposed to Cd^{2+}. Moreover, lower concentrations of $O_2^{\cdot -}$ and H_2O_2 were detected in the roots and leaves of transgenics compared with wild-type poplars after Cd^{2+} exposure. These results suggest that transgenic poplars are more vigorous and that they experience less oxidative stress after Cd^{2+} exposure, which is probably related to the GSH-mediated Cd tolerance of these plants. The higher GSH concentrations in the root, bark and leaf

tissues of transgenic poplars under the control conditions were attributable to the overexpression of bacterial γ-*ECS* in the cytosol of these plants. It is known that GSH plays a central role in the metal tolerance of plants (Seth *et al.*, 2012). This is because: the functional groups of GSH or its derivatives such as PCs have high affinities for different metals; GSH can function as an antioxidant that scavenges ROS, which are induced by heavy metals; and GSH can act as a signal molecule via the inter-conversion of its redox coupling (GSH/GSSG) to transmit specific information in signalling networks (Seth *et al.*, 2012).

Our results show clearly that GSH exerts the three functions mentioned above to mediate Cd^{2+} uptake and detoxification in wild-type and transgenic poplars. As a chelator, GSH often becomes depleted after metal exposure because it is chelated directly with the metal, or is utilized as a precursor for PCs biosynthesis to detoxify free metal ions in plant cells (Mendoza-Cozatl *et al.*, 2008; Seth *et al.*, 2012). In agreement, lower GSH concentrations were detected in the bark and leaf tissues of transgenic poplars but not in the wild-type plants after Cd^{2+} exposure, thereby indicating that the elevated GSH due to the overexpression of bacterial γ-*ECS* in the cytosol was depleted dramatically because of the marked Cd accumulation in these tissues. Previously, it was demonstrated that Cd-GSH complexes are important carrier forms during the long-distance transport of Cd^{2+} in the phloem sap of *Brassica napus* (Mendoza-Cozatl *et al.*, 2008). Therefore, the depletion of GSH was probably caused by its conversion into Cd-GSH complexes in the phloem of transgenic poplars. Furthermore, as a precursor of PCs, GSH can be associated with PC speciation which can affect the stability of Cd–PC complexes during long-distance transport of Cd^{2+} in plants (Vacchina *et al.*, 1999). The large decreases in GSH of transgenic poplars can contribute to biosynthesis of PCs chelated with Cd^{2+}, which requires identification of PCs speciation in further studies. As a component of the GSH-ascorbate cycle, GSH may participate in the removal of excess H_2O_2 (Noctor & Foyer, 1998). In addition, GSH can be involved in H_2O_2 degradation, which is catalysed by GSH peroxidase (Szalai *et al.*, 2009). The higher GSH concentrations detected in the roots of transgenic poplars

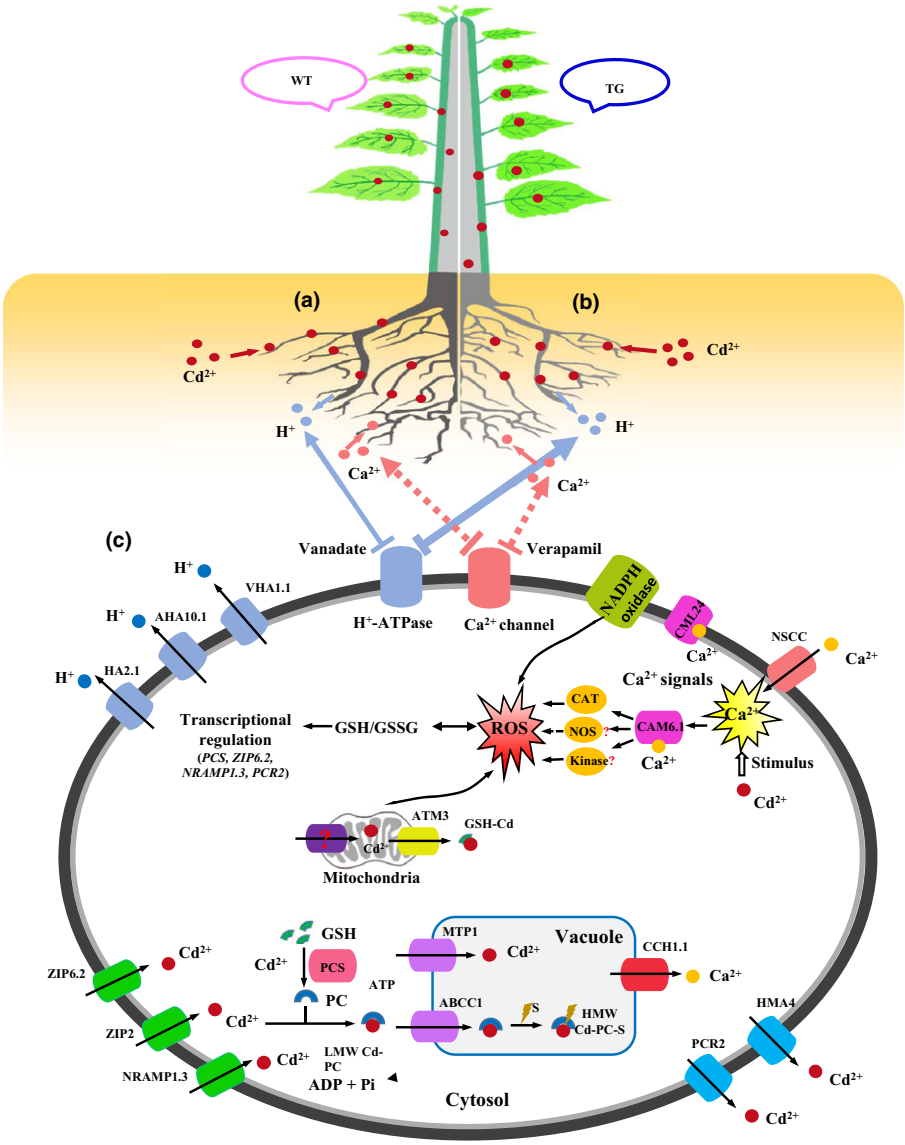

Fig. 11 A schematic model for transgenic *Populus tremula* × *P. alba* (TG) with enhanced cadmium (Cd) accumulation and tolerance in comparison with wild-type (WT) plants. Net fluxes of Cd^{2+}, H^+ and Ca^{2+} in WT (a) and TG (b), and the molecular mechanisms of Cd^{2+} transport and detoxification (c). The highest net Cd^{2+} influxes were found under the conditions of pH 5.5 and/or 0.1 mM Ca^{2+}. TG displayed higher net Cd^{2+} influxes and net H^+ effluxes than WT. Inhibition of plasma membrane (PM) H^+-ATPases or Ca^{2+} channels led to drastic decreases in Cd^{2+} uptake or even net Cd^{2+} effluxes. More Cd^{2+} was translocated from roots to the aerial parts in TG than in WT. HA2.1, AHA10.1 and VHA1.1, PM H^+-ATPases 2.1, 10.1 and 1.1; ZIP2 and ZIP6.2, zinc/iron regulated transporter related proteins 2 and 6.2; NRAMP1.3, natural resistance associated macrophage protein 1.3; PCS, phytochelatin synthetase family protein; MTP1, metal tolerance protein 1; ABCC1, ATP-binding cassette transporter 1; ATM3, ABC transporter of the mitochondria 3; HMA4, P-type heavy metal ATPase 4; PCR2, plant cadmium resistance protein 2; CML24, calmodulin-like protein 24; CAM6.1, calmodulin 6.1; CCH1.1, calcium channel 1.1; GSH, glutathione; GSSG, oxidized glutathione; ROS, reactive oxygen species; NOS, nitric oxide synthetase; CAT, catalase; NSCC, nonselective cation channel; LMW Cd-PC, low molecular weight Cd-PC complex; HMW Cd-PC, high molecular weight Cd-PC complex.

compared with wild-type plants agreed closely with the lower H_2O_2 concentrations in the roots of the transgenics, thereby suggesting that the elevated GSH levels in the roots of transgenics could scavenge excess H_2O_2. The signalling function of GSH is related to changes in the ratio of GSH relative to GSSG in plant cells (Seth *et al.*, 2012). Lower GSH to GSSG ratios often appear in stress conditions. In agreement, lower GSH to GSSG ratios were detected in the bark and leaf tissues of transgenic poplars exposed to Cd^{2+} compared with the controls without Cd^{2+}, but these changes were not detected in the wild-type plants. These results suggest that GSH may interact with different signalling pathways in the wild-type and transgenic poplars in response to Cd^{2+} exposure.

GSH is considered to mediate the transcriptional regulation of genes involved in metal uptake and detoxification (Ball *et al.*, 2004; Seth *et al.*, 2012), but little experimental evidence is available to support this hypothesis. In the present study, the elevated GSH levels detected in the roots of transgenic poplars may have helped to increase the transcript levels of several genes involved in Cd^{2+} uptake and detoxification, such as *PCS*, *ZIP6.2*, *NRAMP1.3* and *PCR2*, which were already highly expressed in the control condition without Cd^{2+} exposure. However, further studies are needed to elucidate the mechanisms that underlie the GSH-mediated transcriptional regulation of genes involved in Cd^{2+} uptake and detoxification.

As summarized in Fig. 11, the wild-type and transgenic poplars exhibited the highest net Cd^{2+} influx into the roots at pH 5.5 and 0.1 mM Ca^{2+}, and higher Cd^{2+} uptake rates were found in the transgenics. A greater reduction in the net Cd^{2+} influx was found in the roots of the transgenics compared with the wild-type plants after inactivation of PM H^+-ATPases or Ca^{2+} channels using specific inhibitors. In agreement, the transcript levels of several genes involved in Cd^{2+} transport and detoxification, such as *PCS*, *ZIP6.2*, *NRAMP1.3* and *PCR2*, were increased more in the roots of the transgenics than in the wild-type plants after Cd^{2+} exposure. The transgenic poplars accumulated more Cd in their aerial parts and they exhibited lower decreases in *A* and biomass, as well as higher concentrations of soluble sugars and starch compared with the wild-type plants in response to Cd^{2+} exposure. Moreover, the transgenic plants had lower concentrations of $O_2^{\cdot -}$ and H_2O_2; higher concentrations of total thiols, GSH and GSSG in the roots and/or leaves; elevated concentrations of soluble phenolics and free proline; and greater foliar GR activity compared with the wild-type plants. These data indicate that the transgenic poplars may have been more tolerant of 100 μM Cd^{2+} than the wild-type plants, probably because of the enhanced transcript levels of genes involved in Cd^{2+} transport and detoxification due to mediation by enhanced GSH synthesis.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Root tip and net Cd^{2+} fluxes.

Fig. S2 Alignments of genes.

Fig. S3 Confirmation of the transferred gene.

Fig. S4 Cd concentrations in root, wood, bark and leaf tissues.

Fig. S5 Total soluble sugars and starch in root, wood, bark and leaf tissues.

Fig. S6 $O_2^{\cdot -}$, H_2O_2 and malonaldehyde (MDA) in root, wood, bark and leaf tissues.

Fig. S7 Soluble phenolics, free proline and ascorbate.

Fig. S8 Activities of GPX, CAT, APX and GR.

Table S1 Primers used for qRT-PCR

Table S2 Photosynthesis

Table S3 Biomass, fine root activity and root architecture

Table S4 Concentrations of Ca, Zn and Fe

Table S5 Principal components analysis (PCA)

Methods S1 Methods for gas exchange measurement, harvesting, determination of root characteristics and the short-term Cd^{2+} flux experiment.

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