Ectomycorrhizas with *Paxillus involutus* enhance cadmium uptake and tolerance in *Populus × canescens*

Yonglu Ma¹*, Jiali He²*, Chaofeng Ma², Jie Luo³, Hong Li³, Tongxian Liu³, Andrea Polle⁴, Changhui Peng⁵ & Zhi-Bin Luo¹,²

¹College of Forestry and State Key Laboratory of Crop Stress Biology for Arid Areas, ²College of Life Sciences, ³Key Laboratory of Applied Entomology, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China, ⁴Department of Forest Botany and Tree Physiology, Büsgen-Institute, Georg-August University, Büsgenweg 2, 37077 Göttingen, Germany and ⁵Department of Biology Science, Institute of Environment Sciences, University of Quebec at Montreal, Quebec, Canada H3C 3P8

**ABSTRACT**

Ectomycorrhizas (EMs), which are symbiotic organs formed between tree roots and certain fungi, can mediate cadmium (Cd) tolerance of host plants, but the underlying physiological and molecular mechanisms are not fully understood. To investigate EMs mediated Cd tolerance in woody plants, *Populus × canescens* was inoculated with *Paxillus involutus* (strain MAJ) to establish mycorrhizal roots. Mycorrhizal poplars and non-mycorrhizal controls were exposed to 0 or 50 μM CdSO₄. EMs displayed higher net Cd²⁺ influx than non-mycorrhizal roots. Net Cd²⁺ influx was coupled with net H⁺ efflux and inactivation of plasma membrane (PM) H⁺-ATPases reduced Cd²⁺ uptake of EMs less than of non-mycorrhizal roots. Consistent with higher Cd²⁺ uptake in EMs, in most cases, transcript levels of genes involved in Cd²⁺ uptake, transport and detoxification processes were increased in EMs compared to non-mycorrhizal roots. Higher CO₂ assimilation, improved nutrient and carbohydrate status, and alleviated oxidative stress were found in mycorrhizal compared to non-mycorrhizal poplars despite higher Cd²⁺ accumulation. The results indicate that mycorrhizas increase Cd²⁺ uptake, probably by an enlarged root volume and overexpression of genes involved in Cd²⁺ uptake and transport, and concurrently enhance *Po. × canescens* Cd tolerance by increased detoxification, improved nutrient and carbohydrate status and defence preparedness.

**Key-words**: carbohydrates; gene expression; ion flux; mycorrhiza; nutrients; oxidative stress; plasma membrane proton-ATPases; poplar.

**INTRODUCTION**

Cadmium (Cd) concentrations in the environment are steadily rising due to rapid expansion of industrial activities and the fact that it is non-degradable (Clemens et al. 2013). Cd is a highly toxic heavy metal for most organisms. Cd in the soil can be taken up and accumulated in plants and eventually enter the human body through the food chain (Kaplan, Ince & Yaman 2011). Cd enrichment in the environment and its toxicity poses a serious threat to human health (Nawrot et al. 2006). Thus, it is critical to remediate Cd pollution. Phytoremediation is a biological technology using plants to absorb Cd from the soil and allocate it to harvestable parts (Kramer 2010).

Fast-growing woody plants such as *Populus* species have been proposed for phytoremediation due to their large biomass, deep root systems, intermediate Cd concentrations in aerial parts and short rotation coppicing properties (Schutzendubel et al. 2002; Unterbrunner et al. 2007; Zhao & McGrath 2009; Di Lonardo et al. 2011; He et al. 2011, 2013). Poplar roots can form associations with both arbuscular and ectomycorrhizal fungi, but in plantations ectomycorrhizal fungi are predominant (Danielsen et al. 2012). Ectomycorrhizal fungi establish symbiotic organs, ectomycorrhizas (EMs), between root tips and fungal hyphae. In EMs, the fungi provide water and mineral nutrients to the plants and are rewarded with photosynthates by their hosts (Martin & Nehls 2009). Accumulating evidence suggests that EMs can modify physiological and molecular responses of hosts to abiotic and biotic stresses including Cd stress (Schutzendubel & Polle 2002; Bellion et al. 2006; Krznaric et al. 2009; Luo et al. 2009a,b; 2011; Huang et al. 2012; Mrnka et al. 2012; Sousa et al. 2012). EMs can result in elevated, decreased or unchanged Cd concentrations of host plants depending on host species and fungal isolates (Sell et al. 2005; Baum et al. 2006; Krpata et al. 2009; Mrnka et al. 2012; Sousa et al. 2012). Therefore, choosing appropriate hosts and fungal partners is essential for remediation of Cd polluted soils.

In addition to fast growth, leaf and bark tissues of some *Populus* species, for example *Po. tremula* (Kieffer et al. 2009) and *Po. × canescens* (He et al. 2011), can accumulate more than 100 μg Cd g⁻¹ DW, which is the threshold commonly defined for hyperaccumulation (Milner & Kochian 2008). These poplar species may be ideal candidates for phytoremediation of Cd polluted soil if high Cd accumulation is maintained and tolerated in aerial parts, when the trees are colonized by ectomycorrhizal fungi.

The ectomycorrhizal fungus *Paxillus involutus* (strain MAJ) can colonize roots of *Po. × canescens* to form abundant EMs which can attenuate toxicity of salt stress in roots via
transcriptomic priming (Luo et al. 2009a) and mediate enhanced salt tolerance of the plants (Luo et al. 2011). The improved performance of EM plants is partly due to decreased salt uptake (Langenfeld-Heyser et al. 2007; Li et al. 2012b). In contrast, it is known that Pa. involutus can accumulate large amounts of Cd in its vacuoles (Ott et al. 2002). Whether this strong accumulation in the fungus also affects the transfer to its host plant and how EMs modulate long-term physiological and molecular responses to Cd exposure remain unknown.

The root is the first checkpoint for Cd entry into plants and plays a fundamental role in Cd transport and accumulation in plants. In herbaceous species that cannot form EMs, Cd²⁺ fluxes are strictly controlled in roots (Li et al. 2012c; Sun et al. 2013). Higher Cd²⁺ influxes have been observed in roots of a Cd hyperaccumulator compared with a non-hyperaccumulator ecotypes of Sedum alfredii (Sun et al. 2013). Net Cd²⁺ influx in the root of Suæda salsa, a halophytic plant, is inhibited by Ca²⁺ channel and thiol blockers, respectively, suggesting that Cd²⁺ may enter root cells via channels/transporters of essential nutrient ions and that Cd transport in roots may be associated with thiol compounds (Li et al. 2012c). In cucumber (Cucumis sativus L.) root cells, a plasma membrane (PM) Cd²⁺/H⁺ antiporter system may be responsible for cytosolic Cd²⁺ efflux (Migocka & Klobus 2007; Migocka et al. 2011), indicating that Cd²⁺ transport in root cells may be coupled with PM H⁺-ATPases which provide the proton motive force for Cd²⁺ movement across the plasma membrane. In woody plants, a strong net Cd²⁺ influx is detected in root apical region of non-mycorrhizal Po. × canescens, but the coupling remains unclear between Cd²⁺ fluxes and PM H⁺-ATPases (He et al. 2011). Since EMs are known to mediate nutritional ion transport to their host plants (Plüssard et al. 2002; Gobert & Plassard 2007; Plassard & Del1 2010), it is most likely that Cd²⁺ flux and its coupling with PM H⁺-ATPases in plants can be modulated by EMs. However, this hypothesis has not yet been experimentally tested.

Although PM H⁺-ATPases may play a role in Cd²⁺ transport in root cells, little information is available on the transcriptional regulation of genes encoding PM H⁺-ATPases in plants exposed to Cd. In Arabidopsis thaliana, PM H⁺-ATPases are encoded by 11 genes, denoted as AHA11 to AHA11 (Arabidopsis H⁺-ATPase) (Baxter et al. 2003; Haruta & Sussman 2012). Great progress has been made in elucidating the function and regulation of these genes in Arabidopsis, although their involvement in Cd transport is unclear (Morth et al. 2011). In the genome of Po. trichocarpa, 12 genes encoding putative PM H⁺-ATPases have been reported (Tuskan et al. 2006; Beritognolo et al. 2007), but little information is currently available on the function and regulation of these poplar genes (Beritognolo et al. 2007). Previous studies have revealed that ZRT-IRT-like protein 2 (ZIP2), natural resistance associated macrophage protein 1.1 (NRAMP1.1), phytochelatin synthase (PCS), ATP-binding cassette transporter C1 (ABCC1), metal tolerance protein 1 (MTP1), ATP-binding cassette transporter in mitochondria (ATM3) and heavy metal ATPase 4 (HMA4) play pivotal roles in Cd transport and detoxification (Kim et al. 2006; Kramer 2010; Migeon et al. 2010; Mendoza-Cozatl et al. 2011; Lin & Aarts 2012). However, it is unclear whether EMs influence transcriptional regulation of these genes.

In this study, we used Po. × canescens (syn. Po. tremula × Po. alba) in combination with or without Pa. involutus (strain MAJ) to establish mycorrhizal or non-mycorrhizal plants, which were then exposed to 0 or 50 μM CdSO₄. The aim of this study is to address the following questions: (1) Do EMs modulate Cd²⁺ fluxes and coupling between Cd²⁺ fluxes and PM H⁺-ATPases in poplar roots? (2) Do EMs mediate transcriptional regulation of key genes involved in Cd²⁺ fluxes in roots? and (3) Do EMs affect Cd accumulation and modify physiological responses of host plants to Cd exposure? A study addressing these questions will provide new insights into the physiological and molecular mechanisms underlying Cd tolerance of ectomycorrhizal woody plants with potential for phytoremediation.

MATERIALS AND METHODS

Cultivation of fungus, plants, inoculation and Cd exposure

An ectomycorrhizal fungus (Pa. involutus, strain MAJ) was grown on 2% modified Melin-Norkrans agar medium and subsequently cultivated in liquid culture as described elsewhere (Luo et al. 2009a,b, 2011).

Plantlets of Po. × canescens (syn. Po. tremula × Po. alba) were produced by micropropagation (Leple et al. 1992) and cultivated in a climate chamber (day/night temperature, 25/18 °C; relative air humidity, 50–60%; light per day, 14 h and photosynthetic photon flux, 150 μmol m⁻² s⁻¹). After 5 weeks, rooted plantlets were carefully cleaned to remove agar on root surface in Petri dishes with sterilized water. Subsequently, plants were transferred to 10-L plastic pots filled with rooting medium. The rooting medium consisted of sand and soil (1.5 parts: 1 part, v/v) and was autoclaved (LDZH-200KBS, Truelab Laboratory Instrument Co. Ltd, Shanghai, China) at 120 °C for 20 min before filling the pots. Afterwards, plants in pots were grown for 4 weeks in a climate chamber with similar conditions as above. Then plants with similar height and growth performance were selected and divided into two groups (each group with 40 plants) for further treatments. One group of plants was used for inoculation with Pa. involutus (strain MAJ) and the other served as non-inoculated control.

Mycelia of Pa. involutus grown for 4 weeks in liquid culture were used for inoculation. The upper clear supernatant of the culture medium was discarded. Slurry containing mycelia of Pa. involutus was homogenized, and 15 mL was used to inoculate the rooting medium of each plant assigned for inoculation. For inoculated and non-inoculated poplar plants, 50 mL sterile low nitrogen (LN) nutrient solution [300 μM NH₄NO₃, after (Matzen et al. 1982)] in the morning and 50 mL sterile distilled water in the evening were used to irrigate each plant avoiding runoff. Poplar plants were grown in the climate chamber for 14 weeks before Cd treatment and during this period, some plants were harvested to

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Gas exchange measurement and harvest

Before harvest, six plants from each treatment were randomly selected for gas exchange measurements. Subsequently, three mature leaves (leaf plastochron index = 7–9) of each plant were selected for gas exchange measurements. Net photosynthetic rate (A), stomatal conductance (g_s) and transpiration rate (E) were determined using a portable photosynthesis system (LiCor-6400; LiCor Inc., Lincoln, NE, USA) as described previously (He et al. 2011).

After photosynthesis measurements, 12 plants from each experimental condition were harvested and the other six plants from each treatment were used for determining net fluxes of Cd^{2+} and H^+ (see below). The root system of each harvested plant was carefully washed and subsequently, the root, wood, bark and leaf tissues of each plant were separated. Fresh weight of each sample was recorded before foil-wrapped samples were frozen in liquid nitrogen. Frozen samples were ground to fine powder in liquid nitrogen. Fresh samples (ca. 80 mg) from each tissue per plant were dried at 60 °C to determine the fresh-to-dry mass ratio. Afterwards, equal amounts of fresh fine powder were combined from the same tissue of two plants for each experimental condition resulting in six biological replicates per condition. The samples were stored at −80 °C for further analysis. Subsamples of fresh non-mycorrhizal and mycorrhizal roots were also harvested for scanning and microscopic analyses.

Determination of biomass and root characteristics

Biomass of roots, wood, bark and leaves of each plant was calculated according to the fresh-to-dry mass ratio and the fresh weight of each part. To analyse root characteristics, subsamples of roots (ca. 500 mg fresh weight) were scanned and analysed using a WinRHIZO Root Analyzer System (WinRHIZO 2012h, Regent Instruments Canada Inc., Montreal, Canada) as described by Flavel et al. (2012). Total root length, root surface area and total root volume of each plant were estimated according to biomass of root system and the ratio of biomass of scanned roots to total biomass of root system.

To examine non-mycorrhizal and mycorrhizal roots, subsamples of harvested roots were observed under a light microscope. The degree of ectomycorrhization was calculated based on the amount of ectomycorrhizal root tips per 100 root tips. Typical non-mycorrhizal and mycorrhizal root tips were photographed under a light microscope (Eclipse E200, Nikon, Tokyo, Japan) with a CCD (DS-Fi1) connected to a computer.

For scanning electron microscopy (SEM), root samples were prepared according to the proposed method by Cooke & Mackay (2007) with minor modification. The SEM observations were made at 13 kV using a scanning electron microscope (JSM-6360LV, Japan Electron Optics Laboratory Co. Ltd, Tokyo, Japan).

Measurements of net fluxes of Cd^{2+} and H^+

To monitor net Cd^{2+} fluxes in non-mycorrhizal and mycorrhizal roots of Po. × canescens exposed to 0 or 50 μM CdSO_4, fine roots (diameter <2 mm) were selected from six plants under each experimental condition. Net Cd^{2+} flux was measured non-invasively by using the non-invasive micro-test technique (the NMT system BIO-IM; Younger Corp., Falmouth, MA, USA) at the company Xuyue Science & Technology Co., Ltd. (Beijing, China). The NMT system and its application in ion flux detection were described in detail (Xu, Sun & Yin 2006; He et al. 2011; Luo et al. 2013). Briefly, the ion-selective microelectrode with an external tip (ca. 2–4 μm in diameter) was manufactured and silanized with tributylchlorosilane and the tip was backfilled with a commercially available ion-selective cocktail (Cadmium Ionophore I, 20909, Sigma-Aldrich, St. Louis, MO, USA). Prior to the flux measurement, the microelectrode was calibrated [for Cd^{2+}: 10 and 100 μM CdSO_4 in addition to other compounds used in the measuring solution (see below); for H^+: pH 6.5 and 5.5 in addition to the compounds used in the measuring solution] and the electrodes were calibrated before the measurement.

To determine the positions along the root tip where the maximal Cd^{2+} flux occurs, a preliminary experiment was carried out with an initial measurement at the root tip followed by 300 μm walk steps. Three fine roots per plant were used for this analysis. The fine roots excised from non-mycorrhizal or mycorrhizal plants exposed to 0 or 50 μM CdSO_4 for 40 days were immediately transferred to a Petri dish containing 10 mL of measuring solution (0.05 mM CdSO_4, 0.05 mM KCl, 0.25 mM NaCl, 0.15 mM 2-(N-Morpholino)ethanesulfonic acid (MES) and 0.1 mM Na_2SO_4, pH 6.0) and equilibrated for 30 min. The equilibrated root was transferred to another Petri dish containing fresh measuring solution and used to record net Cd^{2+} flux for 5 min at each position by a Cd^{2+}-selective microelectrode. Gradients of Cd^{2+} near to the root surface (ca. 2–5 μm) were measured by moving the Cd^{2+}-selective microelectrode between two positions (with a distance of 30 μm) in perpendicular direction to the root surface. The recording rate for Cd^{2+} flux was 10 readings per 64 s. Acquisition of root images was performed with Mageflux software (version 1.0, XuYue Sci. & Tech. Co. Ltd, Beijing, China) attached to the NMT system.

At the position from the root tip where the maximal net Cd^{2+} uptake was found, net Cd^{2+} and H^+ fluxes associated with PM H^+-ATPases were investigated. The fine root was equilibrated in the measuring solution as described above and subsequently transferred to fresh measuring solution to simultaneously record net Cd^{2+} and H^+ fluxes for 5 min using Cd^{2+}- and H^+-selective microelectrodes. Subsequently, the root was transferred to a Petri dish containing 0.5 mM
orthovanadate, which is a specific inhibitor for PM H+-ATPases acting well in poplar (Luo et al. 2013) and incubated for 25 min. Then the root was equilibrated and used to record net Cd2+ and H+ fluxes as mentioned above.

Analysis of transcript levels of genes involved in Cd transport and detoxification

The RNA extraction and quantitative reverse transcription (RT)-PCR were carried out based on the method of Chang, Puryear & Cairney (1993) with minor modification (Li et al. 2012a). In brief, total RNA of root powder (ca. 500 mg) was isolated 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–primer and 200 U RevertAid Moloney murine leukemia virus reverse transcriptase (DRR037A, Takara, Dalian, China) according to the manufacturer’s instruction. 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 which had been designed specifically for each plant gene (Supporting Information Table S1) and tested in a CFX96 Real Time system (CFX96, Bio-Rad, Hercules, CA, USA). The 18S rRNA was used as a reference gene (Supporting Information Table S1). To ensure the specificity, PCR products were sequenced and aligned with homologues in other model plants (Supporting Information Fig. S1). PCR was performed in triplicate together with a dilution series of the reference gene.

Analysis of foliar pigments, Cd and nutrient elements

Chlorophyll and carotenoid concentrations in leaves were determined spectrophotometrically as suggested by Wellburn (1994). Concentrations of Cd, Mg, Ca, Fe and Zn were determined in root, wood, bark and leaf tissues by a flame atomic absorbance spectrometry (Hitachi 180-80, Hitachi Ltd, Tokyo, Japan) after pressure extraction in HNO3 (Heinrichs et al. 1986). Total carbon (C) and nitrogen (N) in roots and leaves were analysed by a C/N analyzer (Elemental Analyzer EA1108; Carlo Erba Strumentazione, Rodano, Milan, Italy). Other mineral elements in roots and leaves were determined by an inductively coupled plasma-atomic emission spectrometer (Spectroflame; Spectro Analytical Instruments, Kleve, Germany) after Heinrichs et al. (1986).

Analysis of O2•−, H2O2 and malondialdehyde (MDA)

Concentrations of the superoxide (O2•−) in samples were determined spectrophotometrically at 530 nm (Dominguez et al. 2010). Concentrations of the H2O2 were analysed spectrophotometrically at 410 nm according to the method of Brennan & Frekel (1977) with modification by He et al. (2011). The MDA concentrations in plant materials were analysed spectrophotometrically at 450, 532 and 600 nm as described previously (Lei, Korpelainen & Li 2007).

Analysis of enzyme activities and non-enzymatic antioxidants

Soluble proteins in plant materials were extracted and used for quantification (Bradford 1976). The enzyme activities were determined as described by He et al. (2011). Concentrations of free proline after Tamas et al. (2008), soluble phenolics after Swain & Goldstein (1964) with modification by Luo et al. (2008), ascorbate (ASC) after Kampfenkel, Vanmontagu & Inze (1995), reduced glutathione (GSH) after Loggini et al. (1999), total thiols (T-SH) after Tamas et al. (2008) in plant materials were determined spectrophotometrically.

Analysis of total soluble sugars and starch

Concentrations of total soluble sugars and starch in root, wood, bark and leaf tissues were analysed by the anthrone method of Yemm & Willis (1954) with minor modification (He et al. 2013). The standard curve was established by using a serial of diluted solutions of glucose. The final absorbance of total soluble sugars and starch (expressed as glucose equivalent) in samples was determined spectrophotometrically at 620 nm.

Analysis of Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed with dry root and leaf powder of Po. × canescens using the FTIR spectrometer Equinox 55 (Bruker Optics, Ettlingen, Germany), equipped with a deuterium triglycine sulfate detector and an attenuated total reflectance (ATR) unit (DuraSamplIR, SensIR Europe, Warrington, UK). The fine powder of roots and leaves was pressed against the diamond crystal of the ATR device. Thirty-two scans were obtained and averaged for each measurement, and six technical replicates (measurements) of each sample were performed and these spectra were averaged. Spectra of roots or leaves from each treatment were averaged and the mean spectra were used for further analysis.

For spectra analysis, the region of 1800–800 cm−1 of the FTIR spectra was baseline-corrected via the Rubberband method and vector-normalized with OPUS software (version 5.5, Bruker Optics, http://www.brukeroptics.com/). Then peaks of the spectra were picked and putatively assigned to chemical moieties as compiled previously (Luo & Polle 2009; Zhou, Taylor & Polle 2011 and references therein). The absorbance data of picked peaks in spectra were used for principal component analysis (PCA).

Statistical analysis

Statistical tests were performed with Statgraphics (STN, St. Louis, MO, USA) or with R. To test significant changes in net
Cd$^{2+}$ flux in roots, the main effects CdSO$_4$ (Cd), mycorrhiza (M) and position along the root tip (P) were analysed by three-way analysis of variance (ANOVA). To analyse the effects of vanadate on net fluxes of Cd$^{2+}$ and H$^+$, three-way ANOVA was performed using CdSO$_4$ (Cd), mycorrhiza (M) and vanadate (V) as main factors. To investigate significant changes of other experimental variables, two-way ANOVAs were applied with CdSO$_4$ (Cd) and mycorrhiza (M) as two main factors. When the interaction of the main factors was significant, a posteriori comparison of means was made. To reduce the chance of type I errors, all P-values of these multicomparisons were corrected by the Tukey-honestly significant difference (HSD) method. Data were tested for normality prior to the statistical analysis. Differences between means were considered significant when the P-value of the ANOVA F-test was less than 0.05. For PCA of reactive oxygen species (ROS) and antioxidants, data were standardized and subsequently computed by the command prcomp() in R (http://www.r-project.org/).

RESULTS

Growth characteristics and net fluxes of Cd$^{2+}$ and H$^+$ in roots

Poplar roots inoculated with Pa. involutus formed typical EM structures and non-inoculated plants did not develop any mycorrhizas (Supporting Information Fig. S2). No mycorrhizal fungi in non-inoculated plants and the solely presence of Pa. involutus in EM roots of poplars were further confirmed using internal transcribed spacer (ITS1F/ITS4B) from Gardes & Bruns (1993) (data not shown). In line with previous studies (Luo et al. 2009a, 2011), EMs resulted in positive effects on photosynthetic performance and growth (Supporting Information Table S2). Cd caused growth depression, an effect that was stronger in non-mycorrhizal than in mycorrhizal plants (Supporting Information Table S2), but did not influence the extent of root colonization (65 ± 3% and 61 ± 4% in the absence or presence of Cd, respectively) (Supporting Information Fig. S2, Table S2). Therefore, EMs must have positive effects on Cd uptake and/or detoxification in poplars. To investigate Cd uptake, Cd$^{2+}$ fluxes were determined at the root tips of non-mycorrhizal and mycorrhizal poplars. To find out where the maximal Cd$^{2+}$ flux occurs along the non-mycorrhizal and mycorrhizal root tip, an initial measurement at the root tip was carried out and followed by 300 μm distance (Fig. 1a). Net Cd$^{2+}$ fluxes showed a large variation along the root tip (Fig. 1b). The maximal Cd$^{2+}$ influxes appeared at 600 μm to the root tip irrespective of EMs and preceding Cd exposure (Fig. 1b). Net Cd$^{2+}$ influxes in mycorrhizal root tips were markedly higher than those in non-mycorrhizal roots (Fig. 1b,c). Moreover, Cd pre-exposure (ca. 40 days with 50 μM CdSO$_4$) resulted in ca. 30% decrease in net Cd$^{2+}$ influxes (Fig. 1b,c).

To further examine temporal dynamics of net Cd$^{2+}$ fluxes and the coupling between net Cd$^{2+}$ fluxes and PM H$^+$-ATPases, temporal dynamics of net fluxes of Cd$^{2+}$ and H$^+$ were investigated in detail at 600 μm to the root tips where the maximal net Cd$^{2+}$ influxes occurred (Fig. 2). Before vanadate treatment, little fluctuation of net Cd$^{2+}$ fluxes for each mycorrhizal and Cd treatment combination was observed in the tested period (Fig. 2a). Mycorrhizal root tips displayed 36–74% higher net Cd$^{2+}$ influxes compared to non-mycorrhizal roots (Fig. 2b), whereas Cd pre-exposure decreased net Cd$^{2+}$ influxes by 26–42% in comparison with controls (Fig. 2b). Vanadate treatment decreased net Cd$^{2+}$

![Figure 1. Ectomycorrhizal root tip (a), net Cd$^{2+}$ fluxes along root tips (b) and mean of net Cd$^{2+}$ fluxes along root tips (c) in non-mycorrhizal (N) or mycorrhizal (M) Po. × canescens exposed to 0 (C) or 50 μM CdSO$_4$ (Cd) for 40 days. Data indicate means ± SE (n = 6). Different letters on the bars indicate significant difference between the treatments. P-values of the analyses of variance of CdSO$_4$ (Cd), mycorrhizas (M) and position (P) are indicated. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

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influx by 82% or even led to net Cd²⁺ efflux in non-mycorrhizal roots, but only reduced net Cd²⁺ influx by 27% in mycorrhizal roots (Fig. 2a,b). Before vanadate exposure, net H⁺ fluxes displayed releases (net effluxes) in poplar roots irrespective of mycorrhizal and Cd treatments (Fig. 2c). EMs showed also three times stronger H⁺ release than non-mycorrhizal roots (Fig. 2d). Cd pre-exposure resulted in 71–121% higher net H⁺ effluxes in roots before vanadate treatment (Fig. 2d). Vanadate exposure inhibited H⁺ releases from roots and even led to significant net H⁺ influxes (H⁺ uptake) (Fig. 2c,d), indicating that vanadate is an effective inhibitor for PM H⁺-ATPases.

Cd accumulation in roots and leaves

Differential uptake of Cd²⁺ in EMs and non-mycorrhizal roots led to distinct differences in Cd accumulation in *Po. × canescens*, which were particularly pronounced in roots (Fig. 3). Cd concentrations in analysed tissues decreased in the order root > leaf > bark > wood (Fig. 3a–d), the roots of mycorrhizal plant accumulated almost four times higher Cd concentrations than those of non-mycorrhizal plants, whereas the increases in other tissues were moderate (Fig. 3a–d). The present results indicate that Cd concentrations in root, leaf and bark tissues are well above the threshold of 100 μg Cd g⁻¹ DW commonly defined for hyperaccumulation (Milner & Kochian 2008) and that EMs enhance Cd accumulation in both belowground and aerial parts of plants.

**Transcript levels of genes involved in Cd transport and detoxification**

Since increased Cd uptake and accumulation were found in roots and aerial parts of plants, transcriptional regulation of genes involved in Cd transport and detoxification is expected. To study molecular responses to Cd, two important genes encoding PM H⁺-ATPases, that is HA2.1 and AHA10.1, and seven genes implicated in Cd accumulation were selected for assessment in non-mycorrhizal and mycorrhizal roots of *Po. × canescens* based on previous...
Ectomycorrhizas enhance poplar Cd tolerance

AHA10.1 was overexpressed by 3.9- and 3.0-fold in EMs compared to non-mycorrhizal roots under 0 and 50 μM Cd exposure, respectively, but the transcript levels of this gene were unaffected by Cd exposure (Fig. 4a). ZIP2 and NRAMP1.1 are located at the plasma membrane to control Cd$^{2+}$ entry into the cytosol of root cells (Migeon et al. 2010; Lin & Aarts 2012). Consistent with increased Cd$^{2+}$ uptake in mycorrhizal roots, whereas Cd treatment inhibited transcript level of AMT3 in mycorrhizal roots (Fig. 4d). HMA4 is located at the plasma membrane to export Cd$^{2+}$ out of the cytosol so that released Cd$^{2+}$ can be further transported to the central cylinder (Migeon et al. 2010; Lin & Aarts 2012). EMs increased mRNA levels of HMA4 about twofold compared to non-mycorrhizal roots (Fig. 4e). Cd treatment induced HMA4 transcripts 2.1- and 2.9-fold in non-mycorrhizal and mycorrhizal roots, respectively (Fig. 4e). Overall, these data suggest that most Cd uptake and detoxification systems are stimulated by EMs.

**Oxidative stress, detoxification, carbohydrates and nutrients**

Inoculation with mycorrhizal fungi and Cd exposure may cause oxidative stress and a shift of the balance between ROS and scavengers in plants. To investigate how EMs and Cd stress affected the redox balance, we measured O$_2^{-}$, H$_2$O$_2$, MDA, guaiacol peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), free proline, soluble phenolics, ASC, GSH and T-SH (Figs 5, 6 & Supporting Information Table S3) and analysed the data by PCA (Fig. 7, Supporting Information Table S3). The PCA results revealed two main components, which explained 56% (PC1) and 17% (PC2) of the variation, respectively (Fig. 7, Supporting Information Table S3). PC1 clearly separated the effects of Cd, whereas PC2 separated the mycorrhizal effects on the oxidative stress and antioxidant systems (Fig. 7). The main drivers for PC1 were increases in wood and leaf O$_2^{-}$, and bark H$_2$O$_2$; and decreases in leaf APX, and root and wood GR (Fig. 5 & Supporting Information Table S3). PC2 was mainly determined by root H$_2$O$_2$, O$_2^{-}$ and GSH, and bark GSH (Figs 5 & 6, Supporting Information Table S3).

The detoxification of ROS and functioning of membrane transporters requires energy. Therefore, we also determined the concentrations of soluble carbohydrates and starch in

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**Figure 3.** Cd concentrations (a–d) in root, wood, bark and leaf tissues of non-mycorrhizal (N) or mycorrhizal (M) *P. × canescens* exposed to 0 (C) or 50 μM CdSO$_4$ (Cd) for 40 days. 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 analyses of variances of CdSO$_4$ (Cd) and mycorrhiza (M) are indicated. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns: not significant.

Figure 4. Fold changes of transcripts of genes encoding proteins involved in Cd\(^{2+}\) uptake, transport and detoxification in fine roots of non-mycorrhizal (N) or mycorrhizal (M) *Po. × canescens* exposed to 0 (C) or 50 \(\mu\)M CdSO\(_4\) (Cd) for 40 days. Bars indicate means ± SE \((n = 6)\). Different letters on the bars for the same gene indicate significant difference between the treatments. For each gene, the expression level was set to 1 in non-mycorrhizal (N) *Po. × canescens* roots exposed to 0 \(\mu\)M CdSO\(_4\) (C) and, subsequently, fold changes of transcripts were calculated in roots with other treatments.

different poplar tissues (Supporting Information Fig. S4). Total soluble sugars were markedly higher in root, bark and leaf tissues of mycorrhizal compared to non-mycorrhizal plants. Unexpectedly, sugars were induced in all analysed tissues after Cd exposure. Similar to total soluble sugars, starch also displayed induction to mycorrhizas or Cd exposure in poplars. EMs generally improved the nutrient status (Supporting Information Table S4), whereas Cd accumulation led to decreases in Mg in roots and leaves and Ca and Fe in roots (Supporting Information Table S4).

FTIR spectra of roots and leaves

FTIR spectra represent a chemical fingerprint of the analysed tissues, which may reveal novel responses of plants to EMs and/or Cd exposure. Thus, FTIR analysis was performed in roots and leaves of non-mycorrhizal and mycorrhizal Po. × canescens exposed to 0 and 50 μM CdSO₄ (Fig. 8). The absorption peaks of the spectra were assigned tentatively to chemical components (Fig. 8a, Supporting Information Table S5) based on previous studies (Luo & Polle 2009; Zhou et al. 2011 and references therein). Peaks, where major differences between the spectra were found, are at 1023 cm⁻¹ indicating vibrations of C-O from cellulose and hemicellulose, at 1235 cm⁻¹ for the syringyl ring and C = O stretch in lignin and xylans, at 1317 cm⁻¹ for vibrations of C-H and deformation of N-H from proteins, at 1579 cm⁻¹ for asymmetrical deformation of NH₃⁺, at 1615 cm⁻¹ for aromatic skeletal vibration in lignin and at 1733 cm⁻¹ for vibration of C = O from lignin and esters. Although these spectra might be

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Figure 6. Free proline, soluble phenolics, ascorbate (ASC), reduced glutathione (GSH) and total thiols (T-SH) in root, wood, bark and leaf tissues of non-mycorrhizal (N) or mycorrhizal (M) Po. × canescens exposed to 0 (C) or 50 μM CdSO₄ (Cd) for 40 days. 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 analyses of variance of CdSO₄ (Cd) and mycorrhiza (M) are indicated. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns: not significant.
separated at certain peaks according to EMs and/or Cd exposure, the general pattern was similar (Fig. 8a). To further characterize the spectral difference, PCA was performed using absorbance data at the indicated peaks (Fig. 8b, Supporting Information Table S6). PC1 and PC2 accounted for 59 and 16% of the variation among these spectral peaks, respectively. PC1 separated root and leaf tissues as well as the mycorrhizal effect in roots, and peaks at 1579, 1023 and 1615 cm\(^{-1}\) were the three most important contributors to PC1 (Fig. 8b, Supporting Information Tables S5 and S6). PC2 separated the Cd effect in roots but not in leaves and peaks at 1235 and 1733 cm\(^{-1}\) were the two most important factors to PC2 (Fig. 8b, Supporting Information Tables S5 and S6). These results indicate that EMs and/or Cd exposure cause changes in chemical composition, mainly lignin-related components, in roots and leaves of *Po. × canescens*.

**DISCUSSION**

**EMs have no effects on spatial variation pattern of Cd\(^{2+}\) uptake along root tips, but increase net Cd\(^{2+}\) influxes via distinct ectomycorrhizal structure and differential gene expression**

Spatial variation in uptake of Cd\(^{2+}\) along root apical region may be linked with different anatomical properties along root tip. The root apical region includes root cap, meristematic, elongation and maturation zones, which have distinct functional features leading to different capacities for uptake of nutrient ions (Enstone, Peterson & Hallgren 2001; Fang et al. 2007; Li et al. 2010; Alber et al. 2012; Luo et al. 2013). Spatial variability of net Cd\(^{2+}\) influx along root tips has been documented in herbaceous plants (Pineros, Shaff & Kochian 1998; Farrell, McArthur & Van Rees 2005; Li et al. 2012; Sun et al. 2013) and also in non-mycorrhizal poplars (He et al. 2011). For instance, net Cd\(^{2+}\) influx along wheat (*Triticum aestivum cv Grandin*) roots is greatest in the region of 0.6–1.2 mm from the root tip (Pineros et al. 1998) and the maximal net Cd\(^{2+}\) influx has been detected in the meristematic zone (not in the mature zone and root hairs) of *Se. alfredii* (Sun et al. 2013). Intriguingly, similar pattern of spatial variation in net Cd\(^{2+}\) influx along root tips occurred in both mycorrhizal and non-mycorrhizal poplars (Fig. 1b), although EMs have distinct morphological and anatomical characteristics from those of non-mycorrhizal roots of *Po. × canescens* (Supporting Information Fig. S2). Overall, these results suggest that spatial variation in Cd\(^{2+}\) uptake may be linked with different anatomical properties along the root tip and the root tip plays a fundamental role in Cd\(^{2+}\) uptake.

Previous flux studies indicate that some EMs can increase uptake of nutritional ions such as K\(^{+}\) and NO\(_3\)\(^{-}\) (Plassard et al. 2002; Gobert & Plassard 2007), but it was unknown whether EMs enhance absorption of non-essential and toxic heavy metal ions such as Cd\(^{2+}\). Here, we have provided

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experimental evidence that EMs markedly increased net Cd\textsuperscript{2+} influx in root apical region of Po. × canescens (Figs 1b,c & 2a,b). Higher net Cd\textsuperscript{2+} influxes in apical region of mycorrhizal compared to non-mycorrhizal roots of Po. × canescens can be ascribed to several reasons. First, in an ectomycorrhiza, a fungal mantle covering the root tip and a Hartig net of intercellular hyphae surrounding epidermal cells exist (Nehls 2008; Bonfante & Genre 2010). The hyphae mantle is expected to increase the uptake surface for Cd\textsuperscript{2+} and the hyphal cells may also serve as a sink for Cd because the vacuole of Po. involutus can accumulate large amounts of Cd (Ott et al. 2002). Furthermore, the presence of the Hartig net may facilitate Cd\textsuperscript{2+} uptake because transporters in root cells may be unable to distinguish Cd\textsuperscript{2+} from essential nutritional ions such as Ca\textsuperscript{2+}, Fe\textsuperscript{2+} and Zn\textsuperscript{2+} (Connolly, Fett & Guerinot 2002; Besson-Bard et al. 2009). Second, the fungal partner induces formation of short lateral roots (Nehls 2008; Bonfante & Genre 2010; Loth-Pereda et al. 2011) and enlargement of epidermal cells (Luo et al. 2009a), which increases the number of root tips per root length (Pena et al. 2013) and, thus the critical sites for Cd\textsuperscript{2+} uptake. Third, overexpressed transcripts of key genes involved in Cd\textsuperscript{2+} transport in mycorrhizal root cells may result in a higher efficiency of Cd\textsuperscript{2+} transport in EMs compared to non-mycorrhizal roots (see below).

PM H\textsuperscript+-ATPases extrude protons from the cytosol to the outside at the expense of ATP to create the electrochemical gradients across the PM for driving transport processes such as uptake of nutrient ions (Palmgren 2001; Luo et al. 2013). Since no specific transporters for Cd\textsuperscript{2+} have yet been identified in plants (Lin & Aarts 2012), it is believed that Cd\textsuperscript{2+} enters root cells via transporters for nutritional ions, for example Fe\textsuperscript{2+} and Zn\textsuperscript{2+} (Connolly et al. 2002; Besson-Bard et al. 2009). Thus, PM H\textsuperscript+-ATPases may play a similar role in Cd\textsuperscript{2+} transport across the PM as in Fe\textsuperscript{2+} and Zn\textsuperscript{2+} transport. In non-mycorrhizal and mycorrhizal poplar roots, PM H\textsuperscript+-ATPases are actively involved in mediating Cd\textsuperscript{2+} uptake because vanadate treatment inhibited H\textsuperscript{+} release and resulted in marked decreases in Cd\textsuperscript{2+} uptake or even a minor Cd\textsuperscript{2+} release in roots of Po. × canescens. The coupling of Cd\textsuperscript{2+} uptake and H\textsuperscript{+} release in non-mycorrhizal and mycorrhizal roots of Po. × canescens before vanadate exposure indicates that some Cd\textsuperscript{2+} may be transported into poplar root cells via a PM antiporter system. Additionally, Cd\textsuperscript{2+} uptake after inhibition of PM H\textsuperscript+-ATPases indicates that other transport systems for Cd\textsuperscript{2+} entry into the cytosol may also exist in poplar root cells. Moreover, higher net Cd\textsuperscript{2+} influx (i.e. less reduction in Cd\textsuperscript{2+} uptake) in EMs compared to non-mycorrhizal roots after vanadate exposure suggests that EMs have mitigated vanadate-induced inhibition of PM H\textsuperscript+-ATPases. This buffering effect in EMs is probably linked with (1) EMs induced transcripts of genes encoding PM H\textsuperscript+-ATPases and (2) the hyphal mantles in EMs because vanadate exposure had no effects on Cd\textsuperscript{2+} uptake of liquid-culture-cultivated hyphae (data not shown). Overall, these results suggest that PM H\textsuperscript+-ATPases play a key role in Cd\textsuperscript{2+} uptake and EMs mediate the function of PM H\textsuperscript+-ATPases on Cd\textsuperscript{2+} absorption in poplar roots.

The fact that EMs induced transcripts of genes encoding PM H\textsuperscript+-ATPases in Po. × canescens corresponds well to the roles of PM H\textsuperscript+-ATPases in Cd\textsuperscript{2+} uptake of plants. Overexpression of HA2.1 and AHA10.1 in EMs compared to non-mycorrhizal roots of Po. × canescens may lead to higher activities of PM H\textsuperscript+-ATPases and thus, higher H\textsuperscript{+} release, driving higher Cd\textsuperscript{2+} absorption rates in EMs. Although the roles of PM H\textsuperscript+-ATPases have been documented in uptake of NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{−} (Luo et al. 2013), cellular Na\textsuperscript{+} extrusion (Beritognolo et al. 2007; Ma et al. 2010) and mediating seasonal signals in cambial cells of poplars (Arend et al. 2002), scarce information is available on transcriptional regulation of genes encoding PM H\textsuperscript+-ATPases. Down-regulated transcripts of genes coding PM H\textsuperscript+-ATPases have been reported for leaves of Po. alba exposed to salinity (Beritognolo et al. 2007). Activities of PM H\textsuperscript+-ATPases and transcript levels of the corresponding genes are induced in response to nitrate in roots of maize and citrus plants (Santi et al. 1995; Sorgona et al. 2010, 2011). In addition to PM H\textsuperscript+-ATPases genes, several other genes including ZIP2, NRAMP1.1, PCS, ABC1, MTP1, ATM3 and HMA4 play fundamental roles in Cd\textsuperscript{2+} transport and detoxification in plant roots (Kim et al. 2006; Plaza et al. 2007; Kramer 2010; Migeon et al. 2010; Mendoza-Cozatl et al. 2011; Lin & Aarts 2012). In line with higher Cd\textsuperscript{2+} uptake in EMs compared with non-mycorrhizal roots, in most cases, EMs stimulated transcript levels of ZIP2, NRAMP1.1, PCS, ABC1, MTP1, ATM3 and HMA4, suggesting that EMs may enhance Cd\textsuperscript{2+} uptake via overexpression of genes involved in Cd\textsuperscript{2+} transport and detoxification which can result in a higher Cd\textsuperscript{2+} uptake efficiency.

**EMs improve poplar growth, nutrient and carbohydrate status and defence preparedness, which can lead to enhanced Cd tolerance**

Our study shows that higher Cd\textsuperscript{2+} influx at the root tips of mycorrhizal plants results in higher Cd accumulation of all poplar tissues. Increased Cd accumulation in ectomycorrhizal fungi-colonized plants has been found in previous studies (Sell et al. 2005; Baum et al. 2006; Krapta et al. 2008, 2009; Sousa et al. 2012). Induction of Cd accumulation by EMs in these plants has been mainly ascribed to (1) fungal exudates, for example oxalic acid, that enhance Cd bioavailability in the rhizosphere, (2) modified Cd\textsuperscript{2+} mobility in the root apoplast due to the Hartig net, and (3) changes in hormonal balance or metabolites of host plants (Schutzendubel & Polle 2002; Sell et al. 2005; Colpaert et al. 2011; Osobova et al. 2011; Langer et al. 2012). The present data show that active PM H\textsuperscript+-ATPases-driven Cd\textsuperscript{2+} uptake is a major factor for increased Cd accumulation in mycorrhizal plants. Cd\textsuperscript{2+} uptake as well as detoxification is energy consuming processes. Ectomycorrhizal plants contain higher concentrations of soluble carbohydrates and starch (Luo et al. 2009a,b, 2011; Beniwal, Langenfeld-Heyser & Polle 2010) and therefore may be better prepared to alleviate Cd toxicity than non-mycorrhizal plants. Higher soluble sugars and starch which can be immediately hydrolyzed to soluble sugars if needed in mycorrhizal Po. × canescens may further function as
Figure 9. A schematic model for enhanced Cd tolerance in ectomycorrhizal compared to non-mycorrhizal *P. × canescens*. (a) a non-mycorrhizal poplar; (b) an ectomycorrhizal poplar; (c) a cross section of non-mycorrhizal root and net fluxes of Cd\(^{2+}\) and H\(^{+}\); (d) a cross section of ectomycorrhizal root and net fluxes of Cd\(^{2+}\) and H\(^{+}\); (e) processes of Cd transport, toxicity and detoxification at the cellular level. Hyphal mantle (1); Hartig net (2); Inhibition of PM H\(^{+}\)-ATPases leading to marked decreases (3) and slight reduction (4) in Cd\(^{2+}\) uptake.

HA2.1, AHA10.1, ZIP2, NRAMP1, ABCC1, MTP1, ATM3 and HMA4 are membrane-localized proteins involved in Cd\(^{2+}\) uptake, transport and detoxification in plants. NADPH oxidase is a PM-localized protein contributing to the production of ROS.
compatible solutes against Cd stress (Kieffer et al. 2009; Keunen et al. 2013). Furthermore, stimulated ASC and GSH by EMs in Po. × canescens may play a role in Cd detoxification and enhance Cd tolerance, as documented in other studies (Sharma & Dietz 2009; Gaudet et al. 2011; Seth et al. 2012). Additionally, the higher peak ratios between mycorrhizal and non-mycorrhizal plants at 1733 cm⁻¹ (vibration of C = O from lignin and esters) of FTIR spectra indicate higher lignin concentrations in mycorrhizal roots/leaves, probably contributing to the attenuation of Cd toxicity in mycorrhizal plants (Elobeid et al. 2012). Taken together, our data indicate that EMs can enhance host nutrient and carbohydrate status and defence preparedness, probably leading to higher Cd tolerance in Po. × canescens.

As summarized in Fig. 9, Po. × canescens inoculated with Pa. involutus established EMs with hyphal mantle and Hartig net, leading to increased root volume compared with non-mycorrhizal roots. EMs displayed higher net Cd²⁺ influx than non-mycorrhizal roots. Net Cd²⁺ influx was coupled with net H⁺ efflux. Inactivation of PM H⁺-ATPases resulted in less reduction in Cd²⁺ uptake in mycorrhizal than non-mycorrhizal roots. In line with higher Cd²⁺ uptake rates in EMs, higher Cd accumulation occurred in mycorrhizal plants. In most cases, transcript levels of PCS, ABC1, MP1, ATP3 and HMA4 were induced in EMs. Enhanced CO2 assimilation, nutrient and carbohydrate status, and alleviated oxidative stress as well as stimulated ASC and GSH were found in mycorrhizal poplars. These results indicate that mycorrhizas increase Cd²⁺ uptake, probably by an enlarged root volume and overexpressed transcripts of genes involved in Cd²⁺ uptake and transport, and concurrently enhance Po. × canescens Cd tolerance by increased detoxification, improved nutrient and carbohydrate status and defence preparedness.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest on this work.

REFERENCES


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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** Alignments of examined genes.

**Figure S2.** Micrographs of non-mycorrhizal and mycorrhizal roots.

**Figure S3.** Activities of antioxidant enzymes.

**Figure S4.** Concentrations of total soluble sugars and starch.

**Table S1.** Primers used for qRT-PCR.

**Table S2.** Characteristics of photosynthesis, biomass and roots.

**Table S3.** PCA of oxidants and antioxidants.

**Table S4.** Concentrations of nutrient elements.

**Table S5.** Peak assignments of FTIR spectra.

**Table S6.** PCA of FTIR spectra.