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1 **Ectomycorrhizas with *Paxillus involutus* enhance cadmium uptake and tolerance**

2 **in *Populus* × *canescens***

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Running head: Ectomycorrhizas enhance poplar Cd tolerance

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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 nonmycorrhizal controls were exposed to 0 or 50 µM $CdSO_4$. EMs displayed higher net Cd^{2+} influx than nonmycorrhizal roots. Net Cd^{2+} influx was coupled with net H^+ efflux and inactivation of plasma membrane (PM) H^+-ATPases reduced Cd^{2+} uptake of EMs less than of nonmycorrhizal roots. Consistent with higher Cd^{2+} uptake in EMs, in most cases, transcript levels of genes involved in Cd^{2+} uptake, transport and detoxification processes were increased in EMs compared to nonmycorrhizal roots. Higher CO_2 assimilation, improved nutrient and carbohydrate status, and alleviated oxidative stress were found in mycorrhizal compared to nonmycorrhizal poplars despite higher Cd^{2+} accumulation. These results indicate that mycorrhizas increase Cd^{2+} uptake, probably by an enlarged root volume and overexpression of genes involved in Cd^{2+} uptake and transport, and concurrently enhance *P.* × *canescens* Cd tolerance by increased detoxification, improved nutrient and carbohydrate status and defense preparedness.

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Keywords: Poplar; Mycorrhiza; Ion flux; Plasma membrane proton-ATPases; Gene expression; Oxidative stress; Nutrients; Carbohydrates

Introduction

Cadmium (Cd) concentrations in the environment are steadily rising due to rapid expansion of industrial activities and the fact that it is nondegradable (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

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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, e.g., *P. tremula* (Kieffer *et al.* 2009) and *P.* × *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 *P.* × *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.* 2012a). In contrast, it is known that *P. 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

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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, Cd^{2+} fluxes are strictly controlled in roots (Li *et al.* 2012b; Sun *et al.* 2013). Higher Cd^{2+} influxes have been observed in roots of a Cd hyperaccumulator compared with a nonhyperaccumulator ecotypes of *Sedum alfredii* (Sun *et al.* 2013). Net Cd^{2+} influx in the root of *Suaeda salsa*, a halophytic plant, is inhibited by Ca^{2+} channel and thiol blockers, respectively, suggesting that Cd^{2+} 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.* 2012b). In cucumber (*Cucumis sativus* L.) root cells, a plasma membrane (PM) Cd^{2+}/H^+ antiporter system may be responsible for cytosolic Cd^{2+} efflux (Migocka & Klobus 2007; Migocka *et al.* 2011), indicating that Cd^{2+} transport in root cells may be coupled with PM H^+-ATPases which provide the proton motive force for Cd^{2+} movement across the plasma membrane. In woody plants, a strong net Cd^{2+} influx is detected in root apical region of nonmycorrhizal *P.* × *canescens*, but the coupling remains unclear between Cd^{2+} fluxes and PM H^+-ATPases (He *et al.* 2011). Since EMs are known to mediate nutritional ion transport to their host plants (Plassard *et al.* 2002; Gobert & Plassard 2007; Plassard & Dell 2010), it is most likely that Cd^{2+} 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 Cd^{2+} transport in root cells, little information is available on the transcriptional regulation of genes encoding PM

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H^+-ATPases in plants exposed to Cd. In *Arabidopsis thaliana*, PM H^+-ATPases are encoded by 11 genes, denoted as *AHA1* to *AHA11* (**A**rabidopsis **H**$^+$-**A**TPase) (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 *P. 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 *ZIP2* (**Z**RT-**I**RT-like **P**rotein 2), *NRAMP1.1* (**N**atural **R**esistance **A**ssociated **M**acrophage **P**rotein 1.1), *PCS* (Phytochelatin synthase), *ABCC1* (**A**TP-**B**inding **C**assette transporter **C1**), *MTP1* (**M**etal **T**olerance **P**rotein 1), *ATM3* (**A**TP-binding cassette **T**ransporter in **M**itochondria) and *HMA4* (**H**eavy **M**etal **A**TPase 4) 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 *Populus* × *canescens* (syn. *P. tremula* × *P. alba*) in combination with or without *Paxillus involutus* (strain MAJ) to establish mycorrhizal or nonmycorrhizal plants, which were then exposed to 0 or 50 μM $CdSO_4$. The aim of this study is to address the following questions: (i) Do EMs modulate Cd^{2+} fluxes and coupling between Cd^{2+} fluxes and PM H^+-ATPases in poplar roots? (ii) Do EMs mediate transcriptional regulation of key genes involved in Cd^{2+} fluxes in roots? and

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1 (iii) Do EMs affect Cd accumulation and modify physiological responses of host
2 plants to Cd exposure? A study addressing these questions will provide new insights
3 into the physiological and molecular mechanisms underlying Cd tolerance of
4 ectomycorrhizal woody plants with potential for phytoremediation.
5

6 **Materials and Methods**

7 *Cultivation of fungus, plants, inoculation and Cd exposure*

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

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

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1 used for inoculation with *P. involutus* (strain MAJ) and the other served as
2 non-inoculated control.

3 Mycelia of *P. involutus* grown for 4 weeks in liquid culture were used for
4 inoculation. The upper clear supernatant of the culture medium was discarded. Slurry
5 containing mycelia of *P. involutus* was homogenized, and 15 ml was used to inoculate
6 the rooting medium of each plant assigned for inoculation. For inoculated and
7 non-inoculated poplar plants, 50 milliliters sterile LN nutrient solution (300 μM
8 NH_4NO_3, after (Matzner *et al.* 1982)) in the morning and 50 milliliters sterile distilled
9 water in the evening were used to irrigate each plant avoiding runoff. Poplar plants
10 were grown in the climate chamber for 14 weeks before Cd treatment and during this
11 period some plants were harvested to examine the progress of ectomycorrhizal
12 colonization (≥60% ectomycorrhizal root tips). Subsequently, half of the plants (18
13 plants) of each group (i.e. non-inoculated controls (N) and mycorrhizal plants (M))
14 were irrigated either with LN nutrient solution containing additionally either no (NC,
15 MC) or 50 μM $CdSO_4$ (Cd) once a day (NCd, MCd). Plants were grown for 40 days
16 after the Cd treatment.

17

18 *Gas exchange measurement and harvest*

19 Before harvest, 6 plants from each treatment were randomly selected for gas
20 exchange measurements. Subsequently, three mature leaves (leaf plastochron index =
21 7–9) of each plant were selected for gas exchange measurements. Net photosynthetic
22 rate (***A***), stomatal conductance ($\boldsymbol{g_s}$) and transpiration rate (***E***) were determined using a

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portable photosynthesis system (LiCor-6400; LiCor Inc., Lincoln, NE) as described previously (He *et al.* 2011).

After photosynthesis measurements, 12 plants from each experimental condition were harvested and the other 6 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 6 biological replicates per condition. The samples were stored at −80°C for further analysis. Subsamples of fresh nonmycorrhizal 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 the fresh-to-dry mass ratio and the fresh weight of each part. To analyze root characteristics, subsamples of roots (ca. 500 mg fresh weight) were scanned and analyzed using a WinRHIZO Root Analyzer System (WinRHIZO 2012b, 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

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according to biomass of root system and the ratio of biomass of scanned roots to total biomass of root system.

To examine nonmycorrhizal 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 nonmycorrhizal and mycorrhizal root tips were photographed under a light microscope (Eclipse E200, Nikon, Tokyo, Japan) with a CCD (DS-Fi1, Nikon) connected to a computer.

For scanning electron microscopy (SEM), root samples were prepared according to the proposed method by Pitre, 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 nonmycorrhizal and mycorrhizal roots of *P.* × *canescens* exposed to 0 or 50 μM $CdSO_4$, fine roots (diameter < 2 mm) were selected from 6 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., 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

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manufactured and silanized with tributylchlorosilane and the tip was backfilled with a commercially available ion-selective cocktail (Cadmium Ionophore I, 20909, Sigma-Aldrich, Louis, MO 63103, 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 nonmycorrhizal 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 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 seconds. Acquisition of root images was performed with Mageflux software (version 1.0) attached to the NMT system.

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1 At the position from the root tip where the maximal net Cd^{2+} uptake was found,
2 net Cd^{2+} and H^+ fluxes associated with plasma membrane (PM) H^+-ATPases were
3 investigated. The fine root was equilibrated in the measuring solution as described
4 above and subsequently transferred to fresh measuring solution to simultaneously
5 record net Cd^{2+} and H^+ fluxes for 5 min using Cd^{2+}- and H^+-selective microelectrodes.
6 Subsequently, the root was transferred to a Petri dish containing 0.5 mM
7 orthovanadate, which is a specific inhibitor for PM H^+-ATPases acting well in poplar
8 (Luo *et al.* 2013) and incubated for 25 min. Then the root was equilibrated and used to
9 record net Cd^{2+} and H^+ fluxes as mentioned above.
10
11 *Analysis of transcript levels of genes involved in Cd transport and detoxification*
12 The RNA extraction and quantitative RT-PCR were carried out based on the
13 method of Chang, Puryear & Cairney (1993) with minor modification (Li *et al.*
14 2012c). In brief, total RNA of root powder (ca. 500 mg) was isolated and purified
15 with a plant RNA extraction kit (R6827, Omega Bio-Tek, GA, USA). Aliquots of 1 μg
16 total RNA were used for first strand cDNA synthesis in a total volume of 20 μl,
17 containing 0.5 μg oligo d(T)18–primer and 200 U RevertAid Moloney murine
18 leukemia virus reverse transcriptase (DRR037A, Takara, Dalian, China) according to
19 the manufacturer's instruction. Quantitative PCR was performed using 10 μl 2×
20 SYBR Green Premix Ex Taq II (DRR820A, Takara, Dalian, China), 0.5 μl cDNA, and
21 0.2 μM primer which had been designed specifically for each plant gene (Table S1)
22 and tested in a CFX96 Real Time system (CFX96, Bio-Rad, Hercules, CA, USA). The

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18S rRNA was used as a reference gene (Table S1). To ensure the specificity, PCR products were sequenced and aligned with homologues in other model plants (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, Japan) after pressure extraction in HNO_3 (Heinrichs *et al.* 1986). Total carbon (C) and nitrogen (N) in roots and leaves were analyzed by a C/N analyzer (Elemental Analyzer EA1108; Carlo Erba Strumentazione). Other mineral elements in roots and leaves were determined by an inductively coupled plasma-atomic emission spectrometer (Spectroflame; Spectro Analytical Instruments) after Heinrichs *et al.* (1986).

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

Concentrations of the superoxide ($O_2^{\bullet-}$) in samples were determined spectrophotometrically at 530 nm (Dominguez *et al.* 2010). Concentrations of the H_2O_2 were analyzed spectrophotometrically at 410 nm according to the method of Brennan & Frekel (1977) with modification by He *et al.* (2011). The malondialdehyde (MDA)

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1 concentrations in plant materials were analyzed spectrophotometrically at 450, 532 and
2 600 nm as described previously (Lei, Korpelainen & Li 2007).
3
4 *Analysis of enzyme activities and non-enzymatic antioxidants*
5 Soluble proteins in plant materials were extracted and used for quantification
6 (Bradford 1976). The enzyme activities were determined as described by He *et al.*
7 (2011).
8 Concentrations of free proline after Tamas *et al.* (2008), soluble phenolics after
9 Swain & Goldstein (1964) with modification by Luo *et al.* (2008), ascorbate (ASC)
10 after Kampfenkel, Vanmontagu & Inze (1995), reduced glutathione (GSH) after
11 Loggini *et al.* (1999), total thiols (T-SH) after Tamas *et al.* (2008) in plant materials
12 were determined spectrophotometrically.
13
14 *Analysis of total soluble sugars and starch*
15 Concentrations of total soluble sugars and starch in root, wood, bark and leaf
16 tissues were analyzed by the anthrone method of Yemm & Willis (1954) with minor
17 modification (He *et al.* 2013). The standard curve was established by using a serial of
18 diluted solutions of glucose. The final absorbance of total soluble sugars and starch
19 (expressed as glucose equivalent) in samples was determined spectrophotometrically
20 at 620 nm.
21
22 *Analysis of Fourier transform infrared spectroscopy (FTIR)*

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FTIR analysis was performed with dry root and leaf powder of *P.* × *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 6 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 analyzed by three-way-ANOVA. To analyze the effects of vanadate on net fluxes of Cd^{2+} and H^+,

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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 multi-comparisons were corrected by the Tukey-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 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 *P. involutus* formed typical EM structures and non-inoculated plants did not develop any mycorrhizas (Fig. S2). No mycorrhizal fungi in non-inoculated plants and the solely presence of *P. 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 (Table S2). Cd caused growth depression, an effect that was stronger in nonmycorrhizal than in mycorrhizal plants (Table S2), but did not influence the extent of root colonization (65 ± 3% and 61 ± 4% in the absence or presence of Cd,

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respectively) (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 nonmycorrhizal and mycorrhizal poplars. To find out where the maximal Cd^{2+} flux occurs along the nonmycorrhial 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 nonmycorrhizal 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 nonmycorrhizal 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+} influx by 82% or even led to net Cd^{2+} efflux in nonmycorrhizal roots, but only reduced net Cd^{2+} influx by 27% in mycorrhizal roots (Fig. 2a, b). Before vanadate exposure, net H^+ fluxes displayed releases (net effluxes)

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in poplar roots irrespective of mycorrhizal and Cd treatments (Fig. 2c). EMs showed also 3 times stronger H^+ release than nonmycorrhizal 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 Cd^{2+} in EMs and nonmycorrhizal roots led to distinct differences in Cd accumulation in *P.* × *canescens*, which were particularly pronounced in roots (Fig. 3). Cd concentrations in analyzed tissues decreased in the order root > leaf > bark > wood (Fig. 3a-d), the roots of mycorrhizal plant accumulated almost 4 times higher Cd concentrations than those of nonmycorrhizal 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

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encoding PM H^+-ATPases, i.e., *HA2.1* and *AHA10.1*, and seven genes implicated in Cd accumulation were selected for assessment in nonmycorrhizal and mycorrhizal roots of *P.* × *canescens* based on previous studies (Migeon *et al.* 2010; Lin & Aarts 2012). Cd exposure led to an about 2-fold upregulation of *HA2.1* in nonmycorrhizal roots, whereas EMs stimulated transcript level of *HA2.1* even by a factor 2.5 without Cd exposure, and Cd had no further impact under these conditions (Fig. 4a). *AHA10.1* was overexpressed by 3.9- and 3.0- fold in EMs compared to nonmycorrhizal 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, the mRNA levels of *ZIP2* were 4.3- and 5.5-fold, and of *NRAMP1.1* were 4.1- and 4.6-fold higher in EMs than those in nonmycorrhizal roots exposed to 0 or 50 µM Cd, respectively (Fig. 4b). Cd exposure induced transcript levels of *ZIP2* in nonmycorrhizal roots but not in mycorrhizal roots, and transcript levels of *NRAMP1.1* in both nonmycorrhizal and mycorrhizal roots (Fig. 4b). In the cytosol, PCS plays a pivotal role in biosynthesis of phytochelatins binding free Cd^{2+} to form chelates (Cd-PCs) which are subsequently transported into vacuole by tonoplast-localized ABCC1/2 members (Migeon *et al.* 2010; Lin & Aarts 2012). EMs markedly enhanced the transcript levels of *PCS* in roots exposed to 0 or 50 µM Cd, and of *ABCC1* in roots without Cd (Fig. 4c). Cd exposure resulted in overexpression of *PCS* in nonmycorrhizal and mycorrhizal roots, but downregulation of *ABCC1* in mycorrhizal

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roots (Fig. 4c). MTP1 is a tonoplast located transporter for Zn^{2+}/Cd^{2+} entry into vacuoles (Migeon *et al.* 2010; Lin & Aarts 2012) and ATM3 is located at the membrane of mitochondria to export Cd-GSH to cytosol (Kim *et al.* 2006). The transcript level of *MTP1* was 1.3-fold higher in mycorrhizal and Cd-treated roots compared to that in nonmycorrhizal and Cd-treated roots (Fig. 4d). Cd exposure induced *MTP1* transcripts 1.5 times in mycorrhizal roots (Fig. 4d). EMs stimulated *AMT3* transcripts 4-fold in roots without Cd exposure, 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 2-fold compared to nonmycorrhizal roots (Fig. 4e). Cd treatment induced *HMA4* transcripts 2.1- and 2.9-fold in nonmycorrhizal 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 $O_2^{\bullet -}$, H_2O_2, MDA, GPX, CAT, APX, GR, free proline, soluble phenolics, ASC, GSH and T-SH (Figs. 5, 6, S3) and analyzed the data by PCA (Fig. 7, Table S3). The PCA results revealed two main components, which explained 56 (PC1) and 17% (PC2) of the variation, respectively

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1 (Fig. 7, Table S3). PC1 clearly separated the effects of Cd, whereas PC2 separated the
2 mycorrhizal effects on the oxidative stress and antioxidant systems (Fig. 7). The main
3 drivers for PC1 were increases in wood and leaf $O_2^{\bullet-}$, and bark H_2O_2 and decreases in
4 leaf APX, and root and wood GR (Figs. 5, 6 and S3, Table S3). PC2 was mainly
5 determined by root H_2O_2, $O_2^{\bullet-}$ and GSH, and bark GSH (Figs. 5 and 6, Table S3).
6 The detoxification of ROS and functioning of membrane transporters requires
7 energy. Therefore, we also determined the concentrations of soluble carbohydrates
8 and starch in different poplar tissues (Fig. S4). Total soluble sugars were markedly
9 higher in root, bark and leaf tissues of mycorrhizal compared to nonmycorrhizal
10 plants. Unexpectedly, sugars were induced in all analyzed tissues after Cd exposure.
11 Similar to total soluble sugars, starch also displayed induction to mycorrhizas or Cd
12 exposure in poplars. EMs generally improved the nutrient status (Table S4), whereas
13 Cd accumulation led to decreases in Mg in roots and leaves and Ca and Fe in roots
14 (Table S4).
15
16 *FTIR spectra of roots and leaves*
17 FTIR spectra represent a chemical fingerprint of the analyzed tissues, which
18 may reveal novel responses of plants to EMs and/or Cd exposure. Thus, FTIR analysis
19 was performed in roots and leaves of nonmycorrhizal and mycorrhizal *P.* × *canescens*
20 exposed to 0 and 50 μM $CdSO_4$ (Fig. 8). The absorption peaks of the spectra were
21 assigned tentatively to chemical components (Fig. 8a, Table S5) based on previous
22 studies (Luo & Polle 2009; Zhou *et al.* 2011 and references therein). Peaks, where

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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 NH_3^+, 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 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, 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, 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, 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 *P.* × *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

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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.* 2012b; Sun *et al.* 2013) and also in nonmycorrhizal 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 *S. alfredii* (Sun *et al.* 2013). Intriguingly, similar pattern of spatial variation in net Cd^{2+} influx along root tips occurred in both mycorrhizal and nonmycorrhizal poplars (Fig. 1b), although EMs have distinct morphological and anatomical characteristics from those of nonmycorrhizal roots of *P.* × *canescens* (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 experimental evidence that EMs markedly

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increased net Cd^{2+} influx in root apical region of *P.* × *canescens* (Figs. 1b, c and 2a, b). Higher net Cd^{2+} influxes in apical region of mycorrhizal compared to nonmycorrhizal roots of *P.* × *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^{2+} and the hyphal cells may also serve as a sink for Cd because the vacuole of *P. involutus* can accumulate large amounts of Cd (Ott *et al.* 2002). Furthermore, the presence of the Hartig net may facilitate Cd^{2+} uptake because transporters in root cells may be unable to distinguish Cd^{2+} from essential nutritional ions such as Ca^{2+}, Fe^{2+} and Zn^{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^{2+} uptake. Third, overexpressed transcripts of key genes involved in Cd^{2+} transport in mycorrhizal root cells may result in a higher efficiency of Cd^{2+} transport in EMs compared to nonmycorrhizal roots (see below).

PM H^{+}-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^{2+} have yet been identified in plants (Lin & Aarts 2012), it is believed that Cd^{2+} enters root cells via transporters for nutritional ions, e.g., Fe^{2+}

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and Zn^{2+} (Connolly *et al.* 2002; Besson-Bard *et al.* 2009). Thus, PM H^+-ATPases may play a similar role in Cd^{2+} transport across the PM as in Fe^{2+} and Zn^{2+} transport. In nonmycorrhizal and mycorrhizal poplar roots, PM H^+-ATPases are actively involved in mediating Cd^{2+} uptake because vanadate treatment inhibited H^+ release and resulted in marked decreases in Cd^{2+} uptake or even a minor Cd^{2+} release in roots of *P.* × *canescens*. The coupling of Cd^{2+} uptake and H^+ release in nonmycorrhizal and mycorrhizal roots of *P.* × *canescens* before vanadate exposure indicates that some Cd^{2+} may be transported into poplar root cells via a PM antiporter system. Additionally, Cd^{2+} uptake after inhibition of PM H^+-ATPases indicates that other transport systems for Cd^{2+} entry into the cytosol may also exist in poplar root cells. Moreover, higher net Cd^{2+} influx (i.e., less reduction in Cd^{2+} uptake) in EMs compared to nonmycorrhizal roots after vanadate exposure suggests that EMs have mitigated vanadate-induced inhibition of PM H^+-ATPases. This buffering effect in EMs is probably linked with (i) EMs induced transcripts of genes encoding PM H+-ATPases and (ii) the hyphal mantles in EMs because vanadate exposure had no effects on Cd^{2+} uptake of liquid-culture-cultivated hyphae (data not shown). Overall, these results suggest that PM H^+-ATPases play a key role in Cd^{2+} uptake and EMs mediate the function of PM H^+-ATPases on Cd^{2+} absorption in poplar roots.

The fact that EMs induced transcripts of genes encoding PM H^+-ATPases in *P.* × *canescens* corresponds well to the roles of PM H^+-ATPases in Cd^{2+} uptake of plants. Overexpression of *HA2.1* and *AHA10.1* in EMs compared to nonmycorrhizal roots of *P.* × *canescens* may lead to higher activities of PM H^+-ATPases and thus, higher H^+

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release, driving higher Cd^{2+} absorption rates in EMs. Although the roles of PM H^+-ATPases have been documented in uptake of NH_4^+ and NO_3^- (Luo *et al.* 2013), cellular Na^+ 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^+-ATPases. Down-regulated transcripts of genes coding PM H^+-ATPases have been reported for leaves of *P. alba* exposed to salinity (Beritognolo *et al.* 2007). Activities of PM H^+-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^+-ATPases genes, several other genes including *ZIP2*, *NRAMP1.1*, *PCS*, *ABCC1*, *MTP1*, *ATM3* and *HMA4* play fundamental roles in Cd^{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^{2+} uptake in EMs compared with nonmycorrhizal roots, in most cases, EMs stimulated transcript levels of *ZIP2*, *NRAMP1.1*, *PCS*, *ABCC1*, *MTP1*, *ATM3* and *HMA4*, suggesting that EMs may enhance Cd^{2+} uptake via overexpression of genes involved in Cd^{2+} transport and detoxification which can result in a higher Cd^{2+} uptake efficiency.

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

Our study shows that higher Cd^{2+} influx at the root tips of mycorrhizal plants

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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; Krpata *et al.* 2008, 2009; Sousa *et al.* 2012). Induction of Cd accumulation by EMs in these plants has been mainly ascribed to (i) fungal exudates, e.g., oxalic acid, that enhance Cd bioavailability in the rhizosphere, (ii) modified Cd^{2+} mobility in the root apoplast due to the Hartig net, and (iii) 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^+-ATPases-driven Cd^{2+} uptake is a major factor for increased Cd accumulation in mycorrhizal plants. Cd^{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 nonmycorrhizal plants. Higher soluble sugars and starch which can be immediately hydrolyzed to soluble sugars if needed in mycorrhizal *P.* × *canescens* may further function as compatible solutes against Cd stress (Kieffer *et al.* 2009; Keunen *et al.* 2013). Furthermore, stimulated ASC and GSH by EMs in *P.* × *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 nonmycorrhizal 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

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mycorrhizal plants (Elobeid *et al.* 2012). Taken together, our data indicate that EMs can enhance host nutrient and carbohydrate status and defense preparedness, probably leading to higher Cd tolerance in *P.* × *canescens*.

As summarized in Fig. 9, *P.* × *canescens* inoculated with *P. involutus* established EMs with hyphal mantle and Hartig net, leading to increased root volume compared with nonmycorrhizal roots. EMs displayed higher net Cd^{2+} influx than nonmycorrhizal roots. Net Cd^{2+} influx was coupled with net H^+ efflux. Inactivation of PM H^+-ATPases resulted in less reduction in Cd^{2+} uptake in mycorrhizal than nonmycorrhizal roots. In line with higher Cd^{2+} uptake rates in EMs, higher Cd accumulation occurred in mycorrhizal plants. In most cases, transcript levels of *HA2.1*, *AHA10.1*, *ZIP2*, *NRAMP1.1*, *PCS*, *ABCC1*, *MTP1*, *ATM3* and *HMA4* were induced in EMs. Enhanced CO_2 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 Cd^{2+} uptake, probably by an enlarged root volume and overexpressed transcripts of genes involved in Cd^{2+} uptake and transport, and concurrently enhance *P.* × *canescens* Cd tolerance by increased detoxification, improved nutrient and carbohydrate status and defense preparedness.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used for qRT-PCR.

Table S2. Characteristics of photosynthesis, biomass and roots.

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

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

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

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

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

6 **Figure S2.** Micrographs of nonmycorrhizal and mycorrhizal roots.

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

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

9

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20

21 **Conflict of Interest**

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

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References

Alber A., Ehlting B., Ehlting J., Hawkins B.J. & Rennenberg H. (2012) Net NH_4^+ and NO_3^- flux, and expression of NH_4^+ and NO_3^- transporters in roots of *Picea glauca*. *Trees-Structure and Function*, **26**, 1403-1411.

Arend M., Weisenseel M.H., Brummer M., Osswald W. & Fromm J.H. (2002) Seasonal changes of plasma membrane H^+-ATPase and endogenous ion current during cambial growth in poplar plants. *Plant Physiology*, **129**, 1651-1663.

Baum C., Hrynkiewicz K., Leinweber P. & Meissner R. (2006) Heavy-metal mobilization and uptake by mycorrhizal and nonmycorrhizal willows (*Salix × dasyclados*). *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde*, **169**, 516-522.

Baxter I., Tchieu J., Sussman M.R., Boutry M., Palmgren M.G., Gribskov M., Harper J.F. & Axelsen K.B. (2003) Genomic comparison of P-type ATPase ion pumps in Arabidopsis and rice. *Plant Physiology*, **132**, 618-628.

Bellion M., Courbot M., Jacob C., Blaudez D. & Chalot M. (2006) Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. *FEMS Microbiology Letters*, **254**, 173-181.

Beniwal R.S., Langenfeld-Heyser R. & Polle A. (2010) Ectomycorrhiza and hydrogel protect hybrid poplar from water deficit and unravel plastic responses of xylem anatomy. *Environmental and Experimental Botany*, **69**, 189-197.

Beritognolo I., Piazzai M., Benucci S., Kuzminsky E., Sabatti M., Mugnozza G.S. & Muleo R. (2007) Functional characterisation of three Italian *Populus alba* L. genotypes under salinity stress. *Trees-Structure and Function*, **21**, 465-477.

Besson-Bard A., Gravot A., Richaud P., Auroy P., Duc C., Gaymard F., Taconnat L., Renou J.P., Pugin A. & Wendehenne D. (2009) Nitric oxide contributes to cadmium toxicity in Arabidopsis by promoting cadmium accumulation in roots and by up-regulating genes related to iron uptake. *Plant Physiology*, **149**, 1302-1315.

Bonfante P. & Genre A. (2010) Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. *Nature Communications*, **1**.

Bradford M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of

This article is protected by copyright. All rights reserved.

proteins utilizing the principle of protein-dye binding *Anal Biochem*, **72**, 248-254.

Brennan T. & Frekel C. (1977) Involvement of hydrogen peroxide in the regulation of senescence in pear *Plant Physiol*, **59**, 411-416.

Chang S.J., Puryear J. & Cairney J. (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter*, **11**, 113-116.

Clemens S., Aarts M., Thomine S. & Verbruggen N. (2013) Plant science: the key to preventing slow cadmium poisoning. *Trends in Plant Science* (In press).

Colpaert J.V., Wevers J.H.L., Krznaric E. & Adriaensen K. (2011) How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. *Annals of Forest Science*, **68**, 17-24.

Connolly E.L., Fett J.P. & Guerinot M.L. (2002) Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. *Plant Cell*, **14**, 1347-1357.

Danielsen L., Thurmer A., Meinicke P., Buee M., Morin E., Martin F., Pilate G., Daniel R., Polle A. & Reich M. (2012) Fungal soil communities in a young transgenic poplar plantation form a rich reservoir for fungal root communities. *Ecology and Evolution*, **2**, 1935-1948.

Di Lonardo S., Capuana M., Arnetoli M., Gabbrielli R. & Gonnelli C. (2011) Exploring the metal phytoremediation potential of three *Populus alba* L. clones using an in vitro screening. *Environmental Science and Pollution Research*, **18**, 82-90.

Dominguez D.M., Garcia F.C., Raya A.C. & Santiago R.T. (2010) Cadmium-induced oxidative stress and the response of the antioxidative defense system in *Spartina densiflora*. *Physiologia Plantarum*, **139**, 289-302.

Elobeid M., Gobel C., Feussner I. & Polle A. (2012) Cadmium interferes with auxin physiology and lignification in poplar. *Journal of Experimental Botany*, **63**, 1413-1421.

Enstone D.E., Peterson C.A. & Hallgren S.W. (2001) Anatomy of seedling tap roots of loblolly pine (*Pinus taeda* L.). *Trees-Structure and Function*, **15**, 98-111.

Fang Y.Y., Babourina O., Rengel Z., Yang X.E. & Pu P.M. (2007) Spatial distribution of ammonium and nitrate fluxes along roots of wetland plants. *Plant Science*, **173**, 240-246.

Farrell R.E., McArthur D.F.E. & Van Rees K.C.J. (2005) Net Cd^{2+} flux at the root surface of durum wheat (*Triticum turgidum* L. var. durum) cultivars in relation to cultivar differences in Cd

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accumulation. *Canadian Journal of Plant Science*, **85**, 103-107.

Flavel R.J., Guppy C.N., Tighe M., Watt M., McNeill A. & Young I.M. (2012) Non-destructive quantification of cereal roots in soil using high-resolution X-ray tomography. *Journal of Experimental Botany*, **63**, 2503-2511.

Gardes M. & Bruns T.D. (1993) ITS Primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rust. *Molecular Ecology*, **2**, 113-118.

Gaudet M., Pietrini F., Beritognolo I., Iori V., Zacchini M., Massacci A., Mugnozza G.S. & Sabatti M. (2011) Intraspecific variation of physiological and molecular response to cadmium stress in *Populus nigra* L. *Tree Physiology*, **31**, 1309-1318.

Gobert A. & Plassard C. (2007) Kinetics of NO_3^- net fluxes in *Pinus pinaster*, *Rhizopogon roseolus* and their ectomycorrhizal association, as affected by the presence of NO_3^- and NH_4^+. *Plant Cell and Environment*, **30**, 1309-1319.

Haruta M. & Sussman M.R. (2012) The effect of a genetically reduced plasma membrane protonmotive force on vegetative growth of Arabidopsis. *Plant Physiology*, **158**, 1158-1171.

He J.L., Ma C.F., Ma Y.L., Li H., Kang J.Q., Liu T.X., Polle A., Peng C.H. & Luo Z.B. (2013) Cadmium tolerance in six poplar species. *Environmental Science and Pollution Research*, **20**, 163-174.

He J.L., Qin J.J., Long L.Y., Ma Y.L., Li H., Li K., Jiang X.N., Liu T.X., Polle A., Liang Z.S. & Luo Z.B. (2011) Net cadmium flux and accumulation reveal tissue-specific oxidative stress and detoxification in *Populus* × *canescens*. *Physiologia Plantarum*, **143**, 50-63.

Heinrichs H., Brumsack H.J., Loftfield N. & Konig N. (1986) Verbessertes druckaufschlussystem fur biologische und anorganische Materialien. *Z Pflanzenernaehr Bodenkd*, **149**, 350-353.

Huang J., Nara K., Lian C.L., Zong K., Peng K.J., Xue S.G. & Shen Z.G. (2012) Ectomycorrhizal fungal communities associated with Masson pine (*Pinus massoniana* Lamb.) in Pb-Zn mine sites of central south China. *Mycorrhiza*, **22**, 589-602.

Kampfenkel K., Vanmontagu M. & Inze D. (1995) Extraction and determination of ascorbate and dehydroascorbate from plant-tissue. *Analytical Biochemistry*, **225**, 165-167.

Kaplan O., Ince M. & Yaman M. (2011) Sequential extraction of cadmium in different soil phases and plant parts from a former industrialized area. *Environmental Chemistry Letters*, **9**, 397-404.

Keunen E., Peshev D., Vangronsveld J., Van den Ende W. & Cuypers A. (2013) Plant sugars are crucial

This article is protected by copyright. All rights reserved.

players in the oxidative challenge during abiotic stress: extending the traditional concept. *Plant, Cell and Environment*, **36**, 1242-1255.

Kieffer P., Planchon S., Oufir M., Ziebel J., Dommes J., Hoffmann L., Hausman J.F. & Renaut J. (2009) Combining proteomics and metabolite analyses to unravel cadmium stress-response in poplar leaves. *Journal of Proteome Research*, **8**, 400-417.

Kim D.Y., Bovet L., Kushnir S., Noh E.W., Martinoia E. & Lee Y. (2006) AtATM3 is involved in heavy metal resistance in Arabidopsis. *Plant Physiology*, **140**, 922-932.

Kramer U. (2010) Metal hyperaccumulation in plants. In: *Annual Review of Plant Biology, Vol 61* (eds S. Merchant, W.R. Briggs, & D. Ort), pp. 517-534. Annual Reviews, Palo Alto.

Krpata D., Fitz W., Peintner U., Langer I. & Schweiger P. (2009) Bioconcentration of zinc and cadmium in ectomycorrhizal fungi and associated aspen trees as affected by level of pollution. *Environmental Pollution*, **157**, 280-286.

Krpata D., Peintner U., Langer I., Fitz W.J. & Schweiger P. (2008) Ectomycorrhizal communities associated with *Populus tremula* growing on a heavy metal contaminated site. *Mycological Research*, **112**, 1069-1079.

Krznaric E., Verbruggen N., Wevers J.H.L., Carleer R., Vangronsveld J. & Colpaert J.V. (2009) Cd-tolerant *Suillus luteus*: A fungal insurance for pines exposed to Cd. *Environmental Pollution*, **157**, 1581-1588.

Langenfeld-Heyser R., Gao J., Ducic T., Tachd P., Lu C.F., Fritz E., Gafur A. & Polle A. (2007) *Paxillus involutus* mycorrhiza attenuate NaCl-stress responses in the salt-sensitive hybrid poplar *Populus* × *canescens*. *Mycorrhiza*, **17**, 121-131.

Langer I., Santner J., Krpata D., Fitz W.J., Wenzel W.W. & Schweiger P.F. (2012) Ectomycorrhizal impact on Zn accumulation of *Populus tremula* L. grown in metalliferous soil with increasing levels of Zn concentration. *Plant and Soil*, **355**, 283-297.

Lei Y.B., Korpelainen H. & Li C.Y. (2007) Physiological and biochemical responses to high Mn concentrations in two contrasting *Populus cathayana* populations. *Chemosphere*, **68**, 686-694.

Leple J.C., Brasileiro A.C.M., Michel M.F., Delmotte F. & Jouanin L. (1992) Transgenic poplars - expression of chimeric genes using 4 different constructs. *Plant Cell Reports*, **11**, 137-141.

Li H., Li M.C., Luo J., Cao X., Qu L., Gai Y., Jiang X., Liu T., Bai H., Janz D., Polle A., Peng C.H. & Luo Z.B. (2012) N-fertilization has different effects on the growth, carbon and nitrogen

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1 physiology, and wood properties of slow- and fast-growing Populus species. *Journal of*
2 *Experimental Botany*, **63**, 6173-6185.
3 Li J., Bao S.Q., Zhang Y.H., Ma X.J., Mishra-Knyrim M., Sun J., Sa G., Shen X., Polle A. & Chen S.L.
4 (2012) *Paxillus involutus* strains MAJ and NAU mediate K^+/Na^+ homeostasis in
5 ectomycorrhizal *Populus × canescens* under sodium chloride stress. *Plant Physiology*, **159**,
6 1771-1786.
7 Li L.Z., Liu X.L., Peijnenburg W., Zhao J.M., Chen X.B., Yu J.B. & Wu H.F. (2011) Pathways of
8 cadmium fluxes in the root of the halophyte *Suaeda salsa*. *Ecotoxicology and Environmental*
9 *Safety*, **75**, 1-7.
10 Li Q., Li B.H., Kronzucker H.J. & Shi W.M. (2010) Root growth inhibition by NH_4^+ in Arabidopsis is
11 mediated by the root tip and is linked to NH_4^+ efflux and GMPase activity. *Plant Cell and*
12 *Environment*, **33**, 1529-1542.
13 Lin Y.F. & Aarts M.G.M. (2012) The molecular mechanism of zinc and cadmium stress response in
14 plants. *Cellular and Molecular Life Sciences*, **69**, 3187-3206.
15 Loggini B., Scartazza A., Brugnoli E. & Navari-Izzo F. (1999) Antioxidative defense system, pigment
16 composition, and photosynthetic efficiency in two wheat cultivars subjected to drought. *Plant*
17 *Physiology*, **119**, 1091-1099.
18 Loth-Pereda V., Orsini E., Courty P.E., Lota F., Kohler A., Diss L., Blaudez D., Chalot M., Nehls U.,
19 Bucher M. & Martin F. (2011) Structure and expression profile of the phosphate Pht1
20 transporter gene family in mycorrhizal *Populus trichocarpa*. *Plant Physiology*, **156**,
21 2141-2154.
22 Luo J., Qin J.J., He F.F., Li H., Liu T.X., Polle A., Peng C.H. & Luo Z.B. (2013) Net fluxes of
23 ammonium and nitrate in association with H^+ fluxes in fine roots of *Populus popularis*. *Planta*
24 *(Berlin)*.
25 Luo Z.B., Calfapietra C., Scarascia-Mugnozza G., Liberloo M. & Polle A. (2008) Carbon-based
26 secondary metabolites and internal nitrogen pools in Populus nigra under Free Air CO_2
27 Enrichment (FACE) and nitrogen fertilisation. *Plant and Soil*, **304**, 45-57.
28 Luo Z.B., Janz D., Jiang X.N., Gobel C., Wildhagen H., Tan Y.P., Rennenberg H., Feussner I. & Polle A.
29 (2009) Upgrading root physiology for stress tolerance by ectomycorrhizas: insights from
30 metabolite and transcriptional profiling into reprogramming for stress anticipation. *Plant*

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Accepted Article

Physiology, **151**, 1902-1917.

Luo Z.B., Li K., Gai Y., Gobel C., Wildhagen H., Jiang X.N., Feussner I., Rennenberg H. & Polle A. (2011) The ectomycorrhizal fungus (*Paxillus involutus*) modulates leaf physiology of poplar towards improved salt tolerance. *Environmental and Experimental Botany*, **72**, 304-311.

Luo Z.B., Li K., Jiang X. & Polle A. (2009) Ectomycorrhizal fungus (*Paxillus involutus*) and hydrogels affect performance of *Populus euphratica* exposed to drought stress. *Annals of Forest Science*, **66**, 106.

Luo Z.B. & Polle A. (2009) Wood composition and energy content in a poplar short rotation plantation on fertilized agricultural land in a future CO_2 atmosphere. *Global Change Biology*, **15**, 38-47.

Ma X.Y., Deng L., Li J.K., Zhou X.Y., Li N.Y., Zhang D.C., Lu Y.J., Wang R.G., Sun J.A., Lu C.F., Zheng X.J., Fritz E., Huttermann A. & Chen S.L. (2010) Effect of NaCl on leaf H^+-ATPase and the relevance to salt tolerance in two contrasting poplar species. *Trees-Structure and Function*, **24**, 597-607.

Martin F. & Nehls U. (2009) Harnessing ectomycorrhizal genomics for ecological insights. *Curr Opin Plant Biol*, **12**, 508-515.

Matzner E., Khanna P.K., Meiwes K.J., Lindheim M., Prenzel J. & Ulrich B. (1982) Elementflusse in waldokosystemen im solling: Datendokumentation. Goettingen Bodenkundliche Berichte, **71,** 1–27.

Mendoza-Cozatl D.G., Jobe T.O., Hauser F. & Schroeder J.I. (2011) Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. *Current Opinion in Plant Biology*, **14**, 554-562.

Migeon A., Blaudez D., Wilkins O., Montanini B., Campbell M.M., Richaud P., Thomine S. & Chalot M. (2010) Genome-wide analysis of plant metal transporters, with an emphasis on poplar. *Cellular and Molecular Life Sciences*, **67**, 3763-3784.

Migocka M. & Klobus G. (2007) The properties of the Mn, Ni and Pb transport operating at plasma membranes of cucumber roots. *Physiologia Plantarum*, **129**, 578-587.

Migocka M., Papierniak A., Kosatka E. & Klobus G. (2011) Comparative study of the active cadmium efflux systems operating at the plasma membrane and tonoplast of cucumber root cells. *Journal of Experimental Botany*, **62**, 4903-4916.

This article is protected by copyright. All rights reserved.

Milner M.J. & Kochian L.V. (2008) Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. *Annals of Botany*, **102**, 3-13.

Morth J.P., Pedersen B.P., Buch-Pedersen M.J., Andersen J.P., Vilsen B., Palmgren M.G. & Nissen P. (2011) A structural overview of the plasma membrane Na^+, K^+-ATPase and H^+-ATPase ion pumps. *Nature Reviews Molecular Cell Biology*, **12**, 60-70.

Mrnka L., Kuchar M., Cieslarova Z., Matejka P., Szakova J., Tlustos P. & Vosatka M. (2012) Effects of endo- and ectomycorrhizal fungi on physiological parameters and heavy metals accumulation of two species from the family Salicaceae. *Water Air and Soil Pollution*, **223**, 399-410.

Nawrot T., Plusquin M., Hogervorst J., Roels H.A., Celis H., Thijs L., Vangronsveld J., Van Hecke E. & Staessen J.A. (2006) Environmental exposure to cadmium and risk of cancer: a prospective population-based study. *Lancet Oncology*, **7**, 119-126.

Nehls U. (2008) Mastering ectomycorrhizal symbiosis: the impact of carbohydrates. In: *Journal of Experimental Botany*, pp. 1097-1108. Oxford Univ Press, Oxford.

Osobova M., Urban V., Jedelsky P.L., Borovicka J., Gryndler M., Ruml T. & Kotrba P. (2011) Three metallothionein isoforms and sequestration of intracellular silver in the hyperaccumulator *Amanita strobiliformis*. *New Phytologist*, **190**, 916-926.

Ott T., Fritz E., Polle A. & Schutzendubel A. (2002) Characterisation of antioxidative systems in the ectomycorrhiza-building basidiomycete *Paxillus involutus* (Bartsch) Fr. and its reaction to cadmium. *FEMS Microbiology Ecology*, **42**, 359-366.

Palmgren M.G. (2001) Plant plasma membrane H^+-ATPases: powerhouses for nutrient uptake. *Annual Review of Plant Physiology and Plant Molecular Biology*, **52**, 817-845.

Pena R., Simon J., Rennenberg H. & Polle A. (2013) Ectomycorrhiza affect architecture and nitrogen partitioning of beech (*Fagus sylvatica* L.) seedlings under shade and drought. *Environmental and Experimental Botany*, **87**, 207-217.

Pineros M.A., Shaff J.E. & Kochian V. (1998) Development, characterization, and application of a cadmium-selective microelectrode for the measurement of cadmium fluxes in roots of Thlaspi species and wheat. *Plant Physiology*, **116**, 1393-1401.

Pitre F.E., Cooke J.E.K. & Mackay J.J. (2007) Short-term effects of nitrogen availability on wood formation and fibre properties in hybrid poplar. *Trees-Structure and Function*, **21**, 249-259.

Plassard C. & Dell B. (2010) Phosphorus nutrition of mycorrhizal trees. *Tree Physiology*, **30**,

This article is protected by copyright. All rights reserved.

1129-1139.

Plassard C., Guerin-Laguette A., Very A.A., Casarin V. & Thibaud J.B. (2002) Local measurements of nitrate and potassium fluxes along roots of maritime pine. Effects of ectomycorrhizal symbiosis. *Plant Cell and Environment*, **25**, 75-84.

Plaza S., Tearall K.L., Zhao F.J., Buchner P., McGrath S.P. & Hawkesford M.J. (2007) Expression and functional analysis of metal transporter genes in two contrasting ecotypes of the hyperaccumulator *Thlaspi calerulescens*. *Journal of Experimental Botany*, **58**, 1717-1728.

Santi S., Locci G., Pinton R., Cesco S. & Varanini Z. (1995) Plasma-membrane H^+-ATPase in maize roots induced for NO_3^- uptake. *Plant Physiology*, **109**, 1277-1283.

Schutzendubel A., Nikolova P., Rudolf C. & Polle A. (2002) Cadmium and H_2O_2-induced oxidative stress in *Populus* × *canescens* roots. *Plant Physiology and Biochemistry*, **40**, 577-584.

Schutzendubel A. & Polle A. (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *Journal of Experimental Botany*, **53**, 1351-1365.

Sell J., Kayser A., Schulin R. & Brunner I. (2005) Contribution of ectomycorrhizal fungi to cadmium uptake of poplars and willows from a heavily polluted soil. *Plant and Soil*, **277**, 245-253.

Seth C.S., Remans T., Keunen E., Jozefczak M., Gielen H., Opdenakker K., Weyens N., Vangronsveld J. & Cuypers A. (2012) Phytoextraction of toxic metals: a central role for glutathione. *Plant Cell and Environment*, **35**, 334-346.

Sharma S.S. & Dietz K.J. (2009) The relationship between metal toxicity and cellular redox imbalance. *Trends in Plant Science*, **14**, 43-50.

Sorgona A., Cacco G., Di Dio L., Schmidt W., Perry P.J. & Abenavoli M.R. (2010) Spatial and temporal patterns of net nitrate uptake regulation and kinetics along the tap root of *Citrus aurantium*. *Acta Physiologiae Plantarum*, **32**, 683-693.

Sorgona A., Lupini A., Mercati F., Di Dio L., Sunseri F. & Abenavoli M.R. (2011) Nitrate uptake along the maize primary root: an integrated physiological and molecular approach. *Plant Cell and Environment*, **34**, 1127-1140.

Sousa N.R., Ramos M.A., Marques A. & Castro P.M.L. (2012) The effect of ectomycorrhizal fungi forming symbiosis with *Pinus pinaster* seedlings exposed to cadmium. *Science of the Total Environment*, **414**, 63-67.

Sun J., Wang R., Liu Z., Ding Y. & Li T. (2013) Non-invasive microelectrode cadmium flux

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Accepted Article

measurements reveal the spatial characteristics and real-time kinetics of cadmium transport in hyperaccumulator and nonhyperaccumulator ecotypes of *Sedum alfredii*. *Journal of Plant Physiology*.

Swain T. & Goldstein J.L. (1964) The quantitative analysis of phenolic compounds. In: Pridham JB (ed) Methods in polyphenol chemistry. Pergamon Press, Oxford, pp 131–145.

Tamas L., Dudikova J., Durcekova K., Halugkova L., Huttova J., Mistrik I. & Olle M. (2008) Alterations of the gene expression, lipid peroxidation, proline and thiol content along the barley root exposed to cadmium. *Journal of Plant Physiology*, **165**, 1193-1203.

Tuskan G.A., DiFazio S., Jansson S., et al. (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science*, **313**, 1596-1604.

Unterbrunner R., Puschenreiter M., Sommer P., Wieshammer G., Tlustos P., Zupan M. & Wenzel W.W. (2007) Heavy metal accumulation in trees growing on contaminated sites in Central Europe. *Environmental Pollution*, **148**, 107-114.

Wellburn A.R. (1994) The spectral determination of chlorophyll-a and chlorophyll-b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *Journal of Plant Physiology*, **144**, 307-313.

Xu Y., Sun T. & Yin L.P. (2006) Application of non-invasive microsensing system to simultaneously measure both H^+ and O^{2-} fluxes around the pollen tube. *Journal of Integrative Plant Biology*, **48**, 823-831.

Yemm E.W. & Willis A.J. (1954) The estimation of carbohydrates in plant extracts by anthrone. . *Biochem J*, **57**, 508-514.

Zhao F.J. & McGrath S.P. (2009) Biofortification and phytoremediation. *Current Opinion in Plant Biology*, **12**, 373-380.

Zhou G.W., Taylor G. & Polle A. (2011) FTIR-ATR-based prediction and modelling of lignin and energy contents reveals independent intra-specific variation of these traits in bioenergy poplars. *Plant Methods*, **7**.

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Figure Legends

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 nonmycorrhizal (N) or mycorrhizal (M) *P.* × *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 ANOVAs of $CdSO_4$ (Cd), mycorrhizas (M) and position (P) are indicated. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$. The arrow in panel A points to the tip of the electrode. Note: net influxes correspond to positive values and net effluxes indicate negative values, respectively. The measuring solution (pH 6.0) contained 0.05 mM $CdSO_4$, 0.05 mM KCl, 0.25 mM NaCl, 0.15 mM MES and 0.1 mM Na_2SO_4.

Figure 2. Net fluxes of Cd^{2+} (a) and H^+ (c) in 5 minutes, and the mean fluxes of Cd^{2+} (b) and H^+ (d) within the measuring period at 600 µm from the root tips of nonmycorrhizal (N) or mycorrhizal (M) *P.* × *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 ANOVAs of $CdSO_4$ (Cd), mycorrhizas (M) and vanadate (V) are indicated. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$. The measuring solution is the same as indicated in Fig. 1.

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Figure 3. Cd concentrations (a-d) in root, wood, bark and leaf tissues of nonmycorrhizal (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 ANOVAs 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 nonmycorrhizal (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 gene indicate significant difference between the treatments. For each gene, the expression level was set to 1 in nonmycorrhizal (N) *P.* × *canescens* roots exposed to 0 µM $CdSO_4$ (C) and, subsequently, fold changes of transcripts were calculated in roots with other treatments.

Figure 5. $O_2^{\bullet -}$, H_2O_2 and malondialdehyde (MDA) in root, wood, bark and leaf tissues of nonmycorrhizal (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 ANOVAs of $CdSO_4$ (Cd) and mycorrhiza (M) are indicated. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$; ns: not significant.

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1

2 **Figure 6.** Free proline, soluble phenolics, ascorbate (ASC), reduced glutathione (GSH)
3 and total thiols (T-SH) in root, wood, bark and leaf tissues of nonmycorrhizal (N) or
4 mycorrhizal (M) *P.* × *canescens* exposed to 0 (C) or 50 μM $CdSO_4$ (Cd) for 40 days.
5 Bars indicate means ± SE (n = 6). Different letters on the bars for the same tissue
6 indicate significant difference between the treatments. *P*-values of the ANOVAs of
7 $CdSO_4$ (Cd) and mycorrhiza (M) are indicated. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$;
8 ****: $P<0.0001$; ns: not significant.

9

10 **Figure 7.** Principal component analysis (PCA) plots of oxidants and antioxidants in
11 nonmycorrhizal (N) or mycorrhizal (M) *P.* × *canescens* exposed to 0 (C) or 50 μM
12 $CdSO_4$ (Cd) for 40 days. PCA was conducted based on data (both values were
13 averaged in the same tissue with the same treatment) presented in Figs. 5, 6 and S3.

14

15 **Figure 8.** FTIR spectra (a) and principal component analysis (PCA) plot (b) of roots
16 (R) and leaves (L) of nonmycorrihzal (N) or mycorrhizal (M) *P.* × *canescens* exposed
17 to 0 (C) or 50 μM $CdSO_4$ (Cd) for 40 days. The FTIR spectrum is an average
18 spectrum of roots or leaves under each treatment condition (n = 6). PCA was
19 conducted using absorbance data (both values were averaged in the same tissue with
20 the same treatment) of picked peaks in A.

21

22 **Figure 9.** A schematic model for enhanced Cd tolerance in ectomycorrhizal compared

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to nonmycorrhizal *P.* × *canescens*. a: a nonmycorrhizal poplar; b: an ectomycorrhizal poplar; c: a cross section of nonmycorrhizal 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, PCS, 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.

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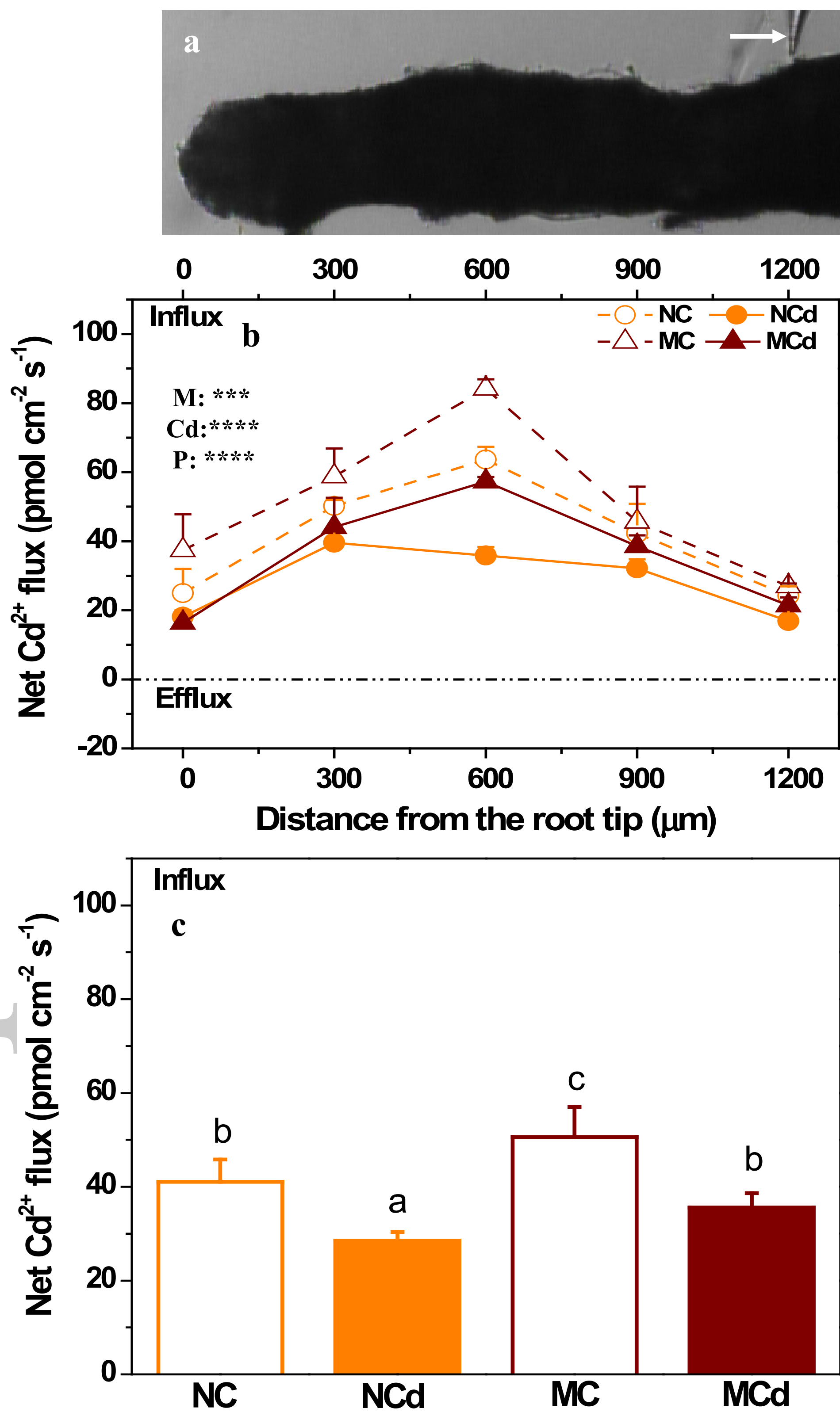

Figure 1

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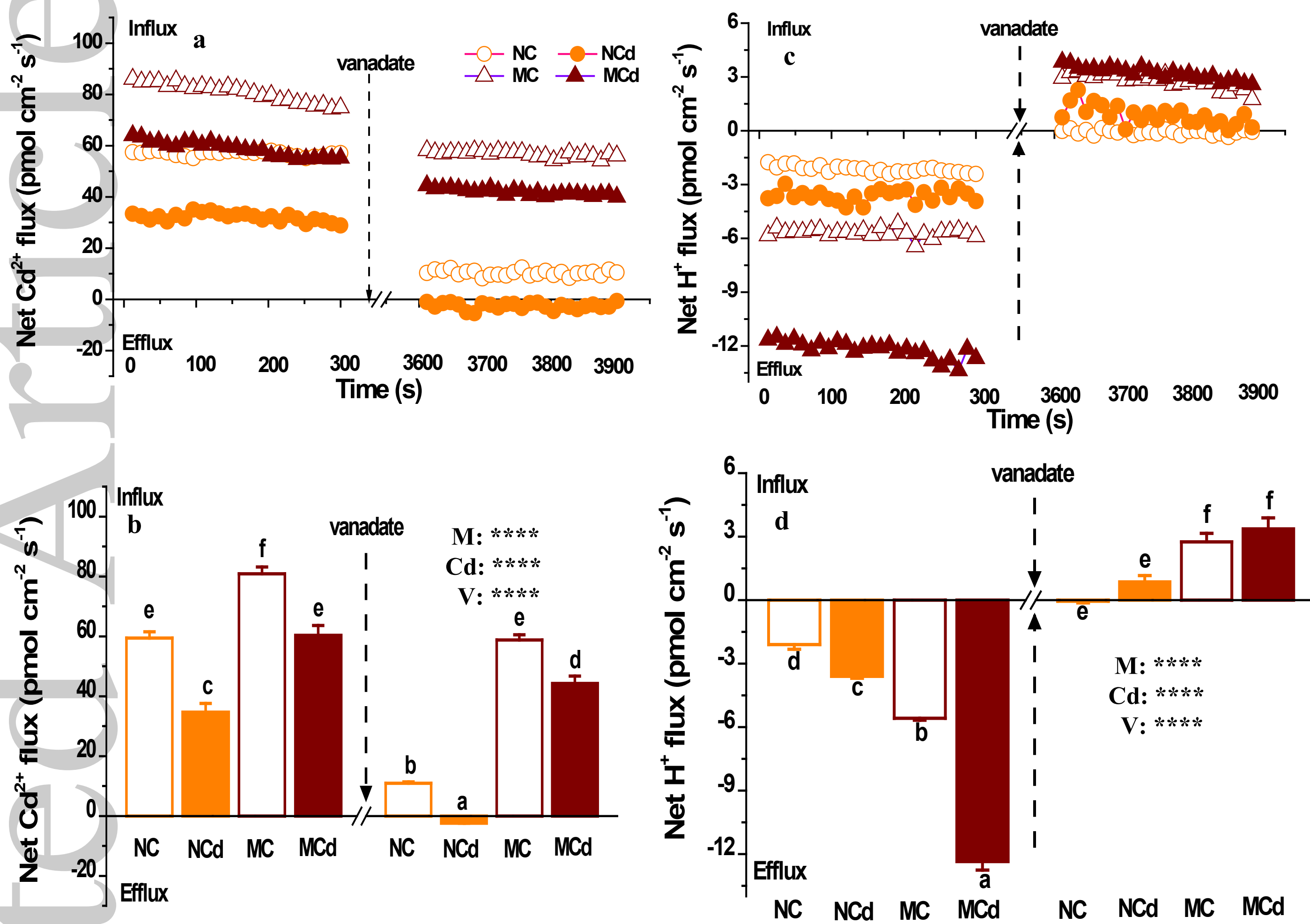

Figure 2

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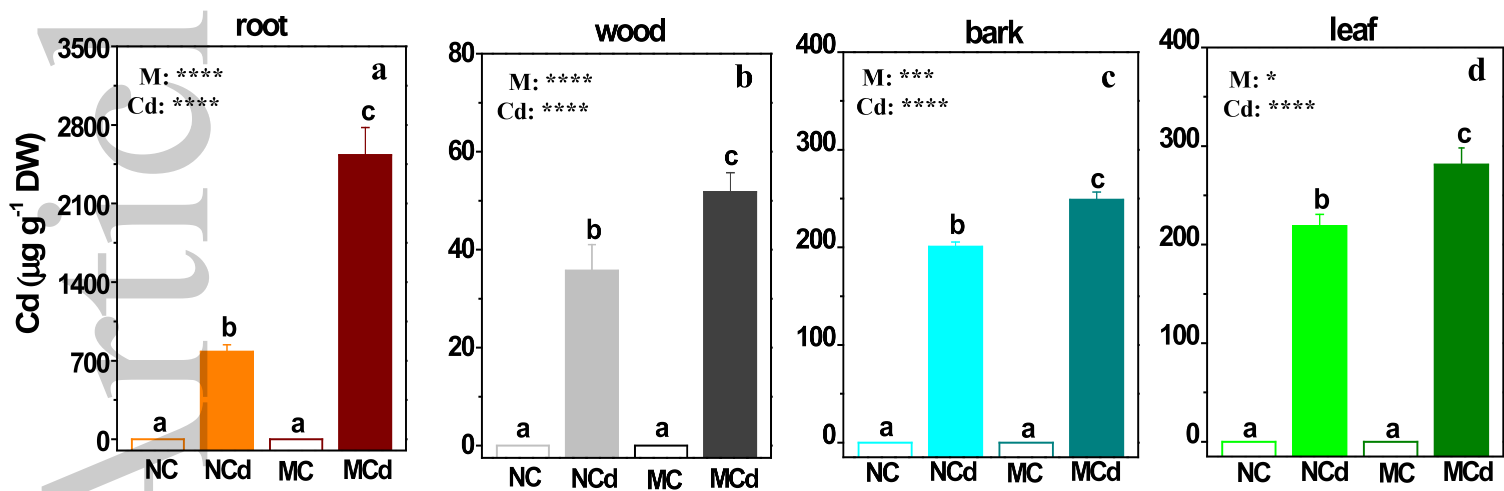

Fig. 3

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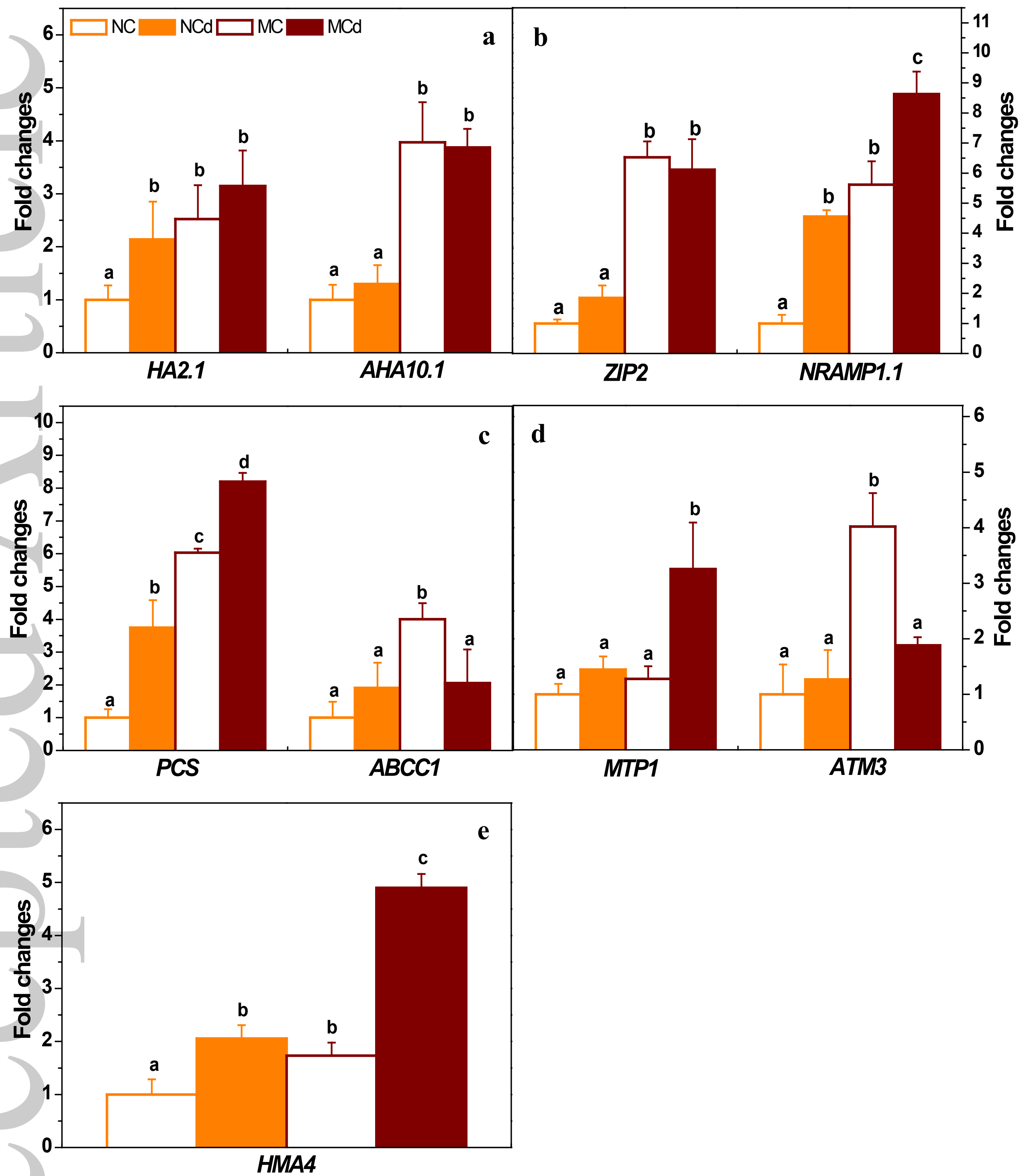

Figure 4

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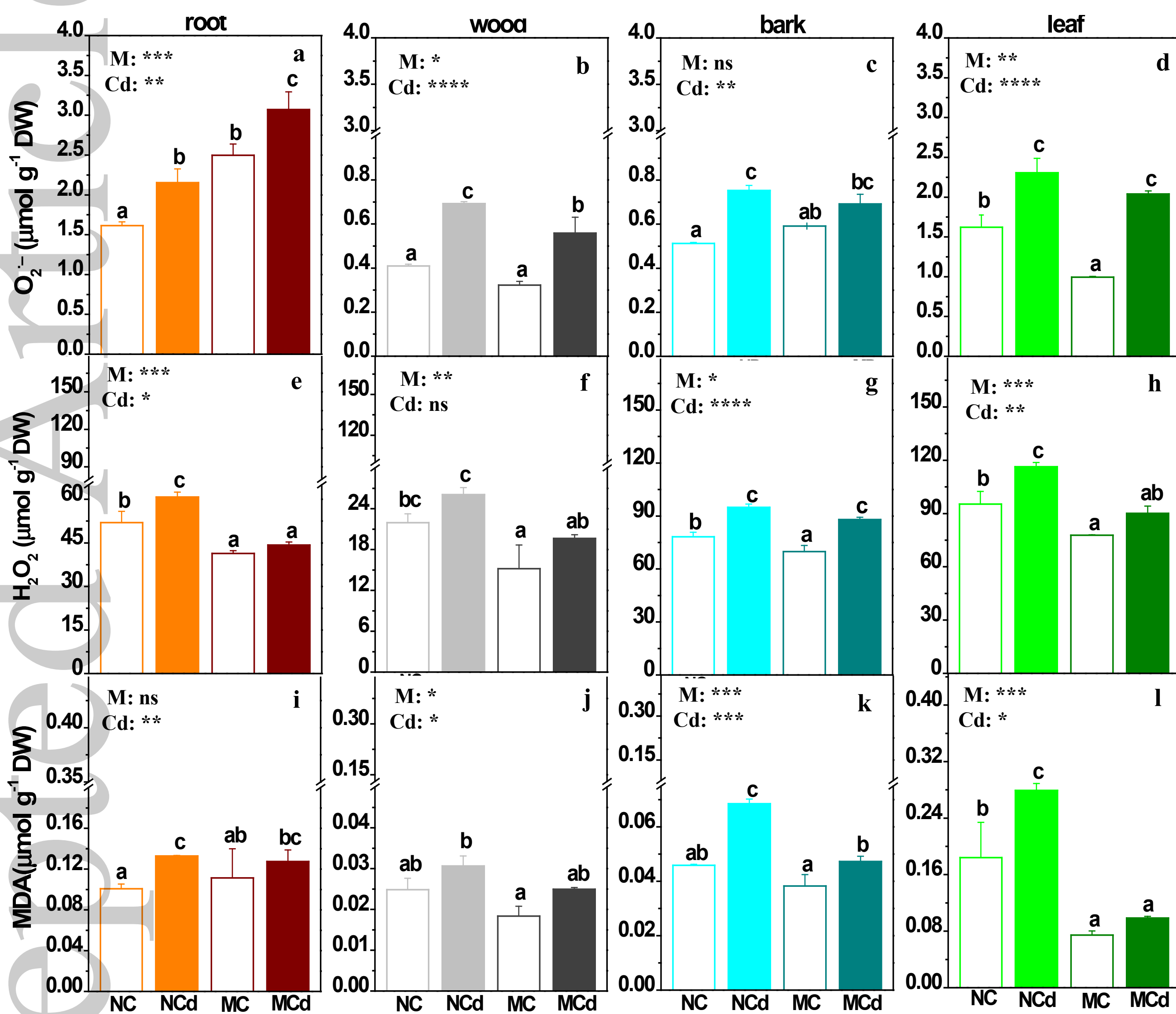

Figure 5

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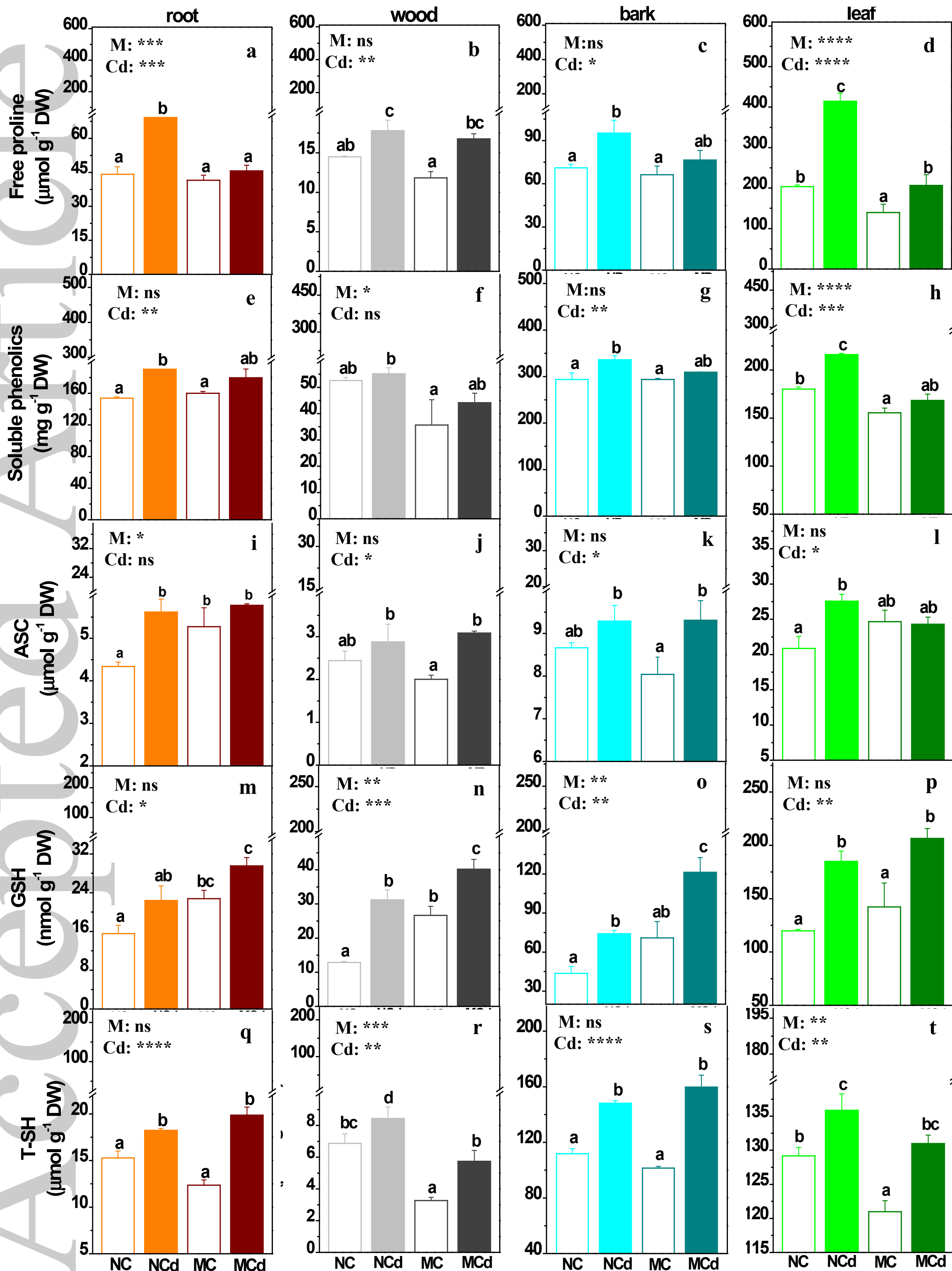

Figure 6

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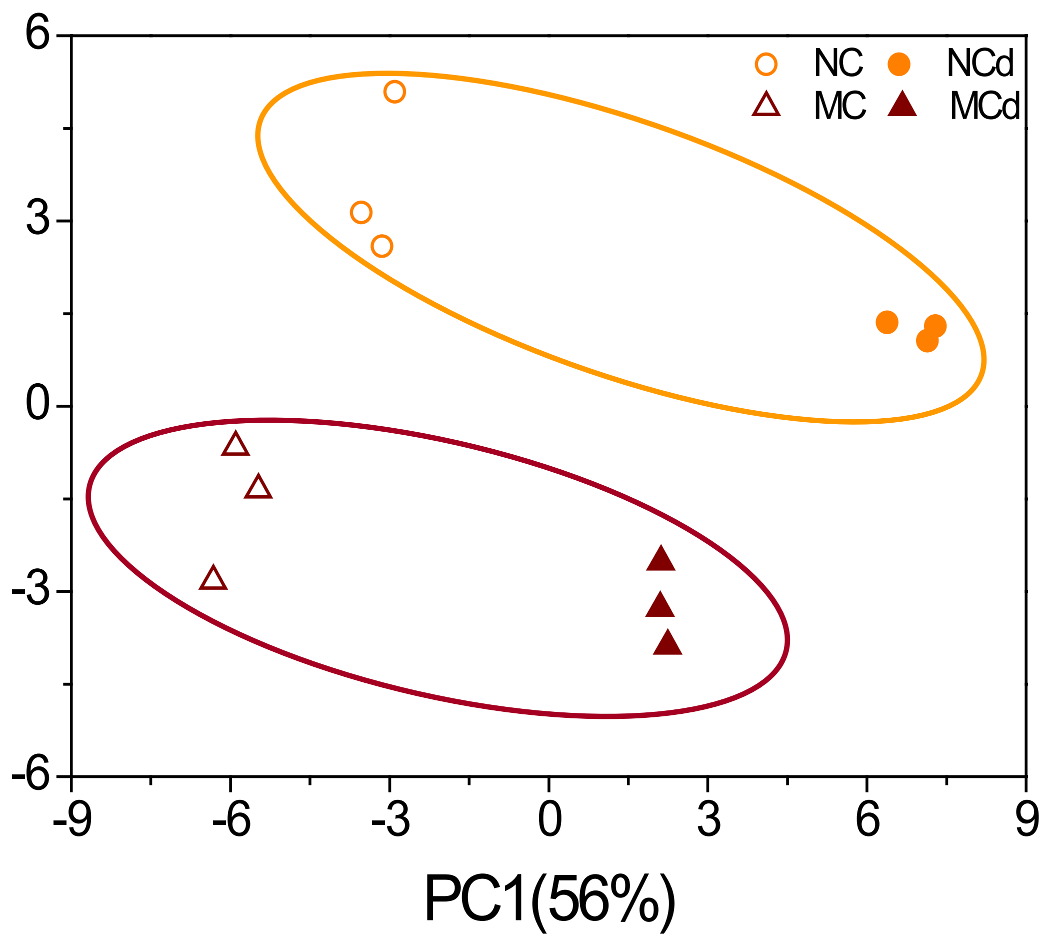

Figure 7

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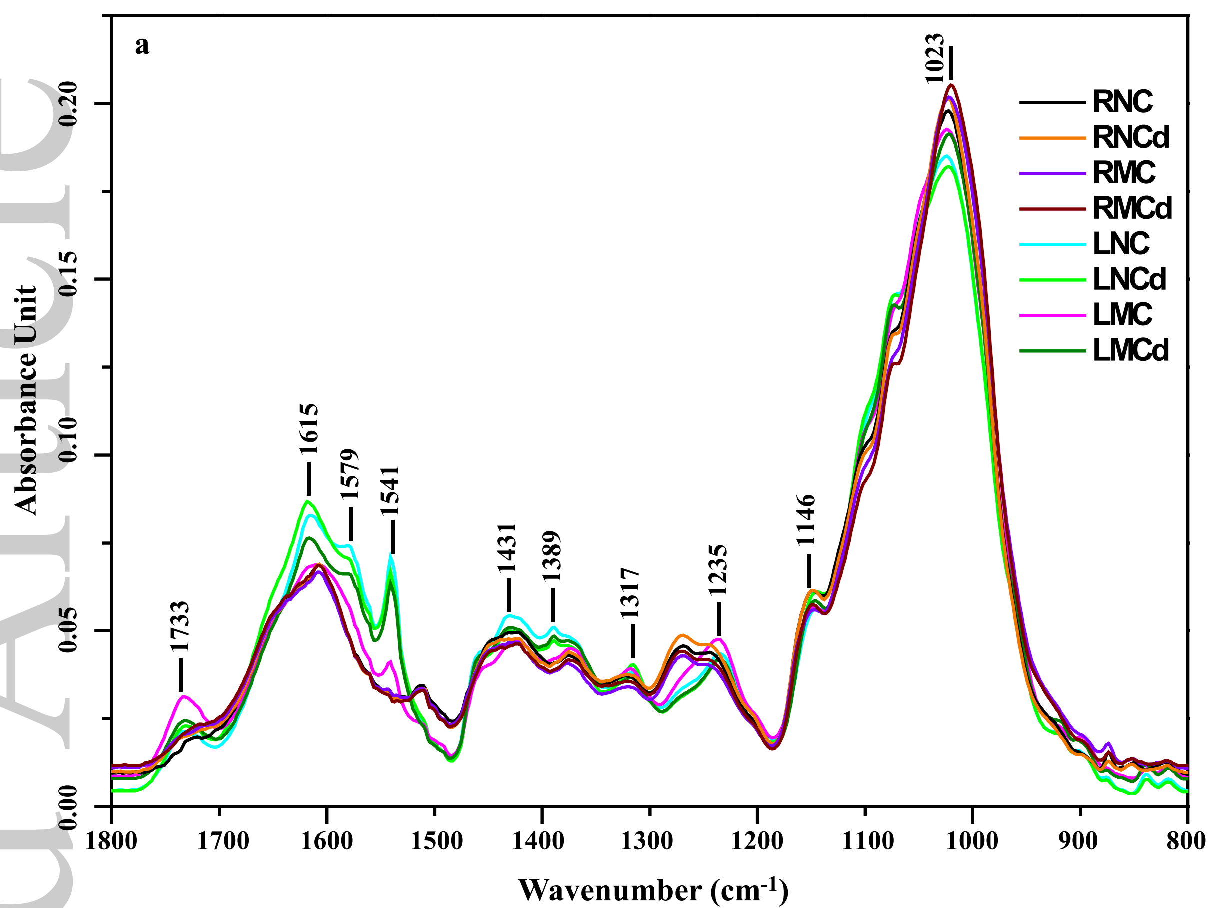

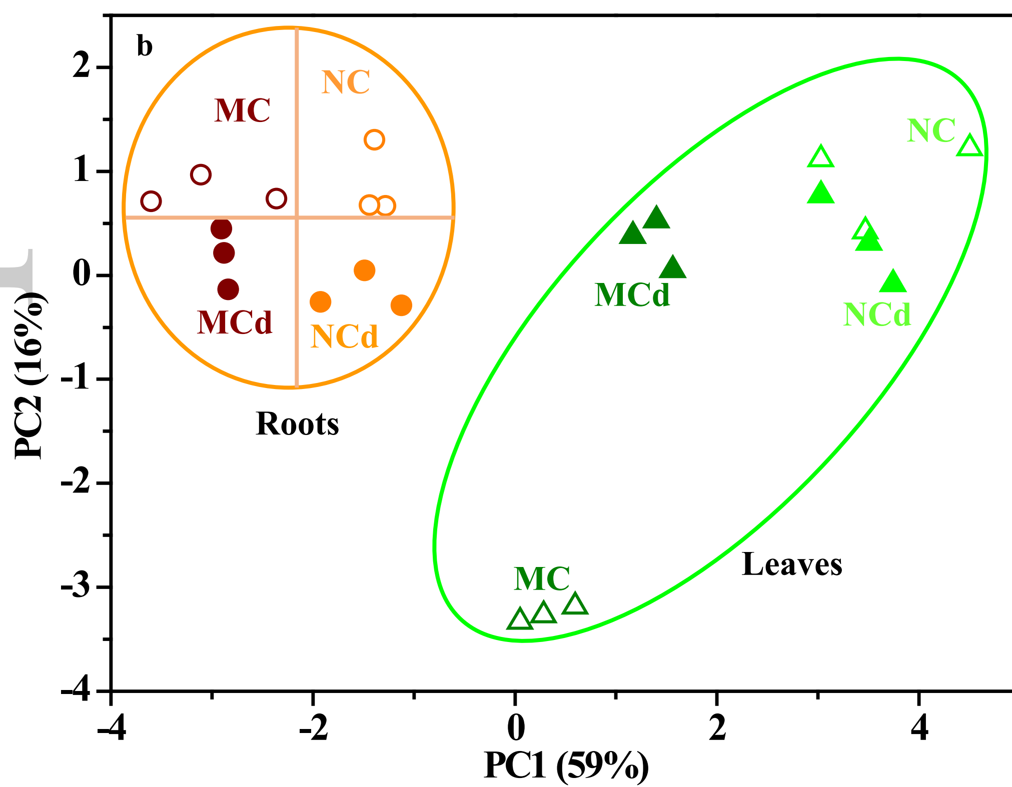

Figure 8

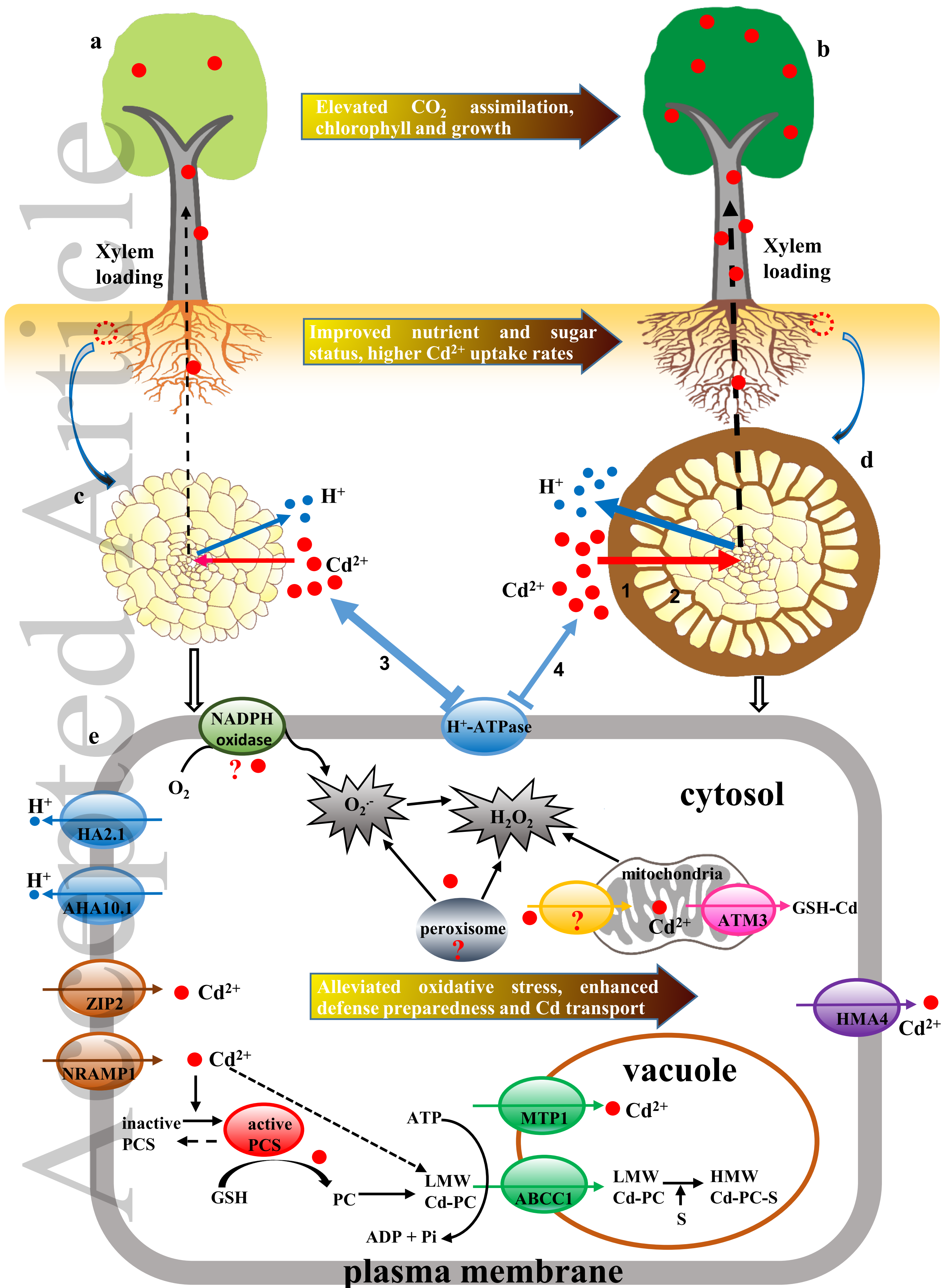

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Figure 9