Exogenous abscisic acid alleviates zinc uptake and accumulation in *Populus × canescens* exposed to excess zinc

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Abstract

A greenhouse experiment was conducted to study whether exogenous abscisic acid (ABA) mediates the responses of poplars to excess zinc (Zn). *Populus × canescens* seedlings were treated with either basal or excess Zn levels and either 0 or 10 µM ABA. Excess Zn led to reduced photosynthetic rates, increased Zn accumulation, induced foliar ABA and salicylic acid (SA), decreased foliar gibberellin (GA₃) and auxin (IAA), elevated root H₂O₂ levels, and increased root ratios of glutathione (GSH) to GSSG and foliar ratios of ascorbate (ASC) to dehydroascorbate (DHA) in poplars. While exogenous ABA decreased foliar Zn concentrations with 7-day treatments, it increased levels of endogenous ABA, GA₃ and SA in roots, and resulted in highly increased foliar ASC accumulation and ratios of ASC to DHA. The transcript levels of several genes involved in Zn uptake and detoxification, such as yellow stripe-like family protein 2 (YSL2) and plant cadmium resistance protein 2 (PCR2), were enhanced in poplar roots by excess Zn but repressed by exogenous ABA application. These results suggest that exogenous ABA can decrease Zn concentrations in *P. × canescens* under excess Zn for 7 days, likely by modulating the transcript levels of key genes involved in Zn uptake and detoxification.

Keywords: Heavy metals; Oxidative stress; Phytohormones; Phytoremediation; Transcriptional regulation
Introduction

The heavy metal zinc (Zn) rapidly accumulates in soil and water due to anthropogenic activities (e.g., mining, smelting and fertilization with sewage sludge) that lead to Zn contamination (Hassan & Aarts 2011). Zn is an essential element for plants that can be taken up by roots. Therefore, the utilization of certain plants for remediating Zn-contaminated soil has been suggested (Broadley et al. 2007). Several herbaceous plants have been identified for Zn hyperaccumulation, including Arabidopsis halleri, Noccaea (formerly Thlaspi) caerulescens, and Sedum alfredii, which can accumulate more than 10,000 µg Zn g\(^{-1}\) dry mass in aboveground tissues (Krämer 2010). However, in these plants, the amount of accumulated Zn is limited by slow growth and low biomass. Thus, fast growing woody plants, such as Populus species, which have a large aboveground biomass, a deep root system and the ability to accumulate intermediate metal concentrations, have been proposed for the phytoremediation of Zn-contaminated soil (Langer et al. 2009; Marmiroli et al. 2011; Luo et al. 2014).

Although Zn is a nutrient element for poplars, excess Zn in the soil can be toxic for these plants (Di Baccio et al. 2003, 2005, 2009, 2011). Zn toxicity in plants causes chlorosis, reduced photosynthesis, stunted growth, interference with the uptake of other nutrient elements, outbreak of reactive oxygen species (ROS) and altered antioxidants (Broadley et al. 2007). Excess Zn can also lead to the transcriptional regulation of genes involved in Zn accumulation and detoxification in poplars (Di
In plant cells, zinc-regulated transporter/iron-regulated transporter-related proteins (ZIPs) play an essential role in the uptake of extracellular Zn\(^{2+}\) (Milner et al. 2013). In the genome of *Populus trichocarpa*, the ZIP family contains twenty members (Migeon et al. 2010). Among these ZIPs, ZIP2 (Potri.009G034600), ZIP6.2 (Potri.009G074100), ZIP6.4 (Potri.015G117900) and ZIP7.2 (Potri.010G134300) act as key components in Zn homeostasis in poplars (Migeon et al. 2010; Adams et al. 2012). In herbaceous plants, the genes encoding nicotianamine synthase (NAS) and yellow stripe-like family protein (YSL) play critical roles in Zn detoxification and transport, respectively (DiDonato et al. 2004; Deinlein et al. 2012). Zn\(^{2+}\) can bind to nicotianamine (NA), which is a nonproteinogenic amino acid, to form a Zn-NA chelate, which can be further transported across cellular membranes by YSL proteins (DiDonato et al. 2004). The transcriptional regulation of *NAS* and *YSL* may represent an important mechanism for controlling Zn homeostasis in plants (Curie et al. 2009; Deinlein et al. 2012). In addition, metal tolerance protein 1 (MTP1) is located in tonoplasts to transport Zn\(^{2+}\) into vacuoles (Weber et al. 2013) and both plant cadmium resistance protein 2 (PCR2) and heavy metal ATPase 4 (HMA4) are located in the plasma membrane to pump Zn\(^{2+}\) out of the cytosol for xylem loading. These proteins are important transporters involved in Zn\(^{2+}\) translocation and detoxification in plants (Hanikenne et al. 2008; Song et al. 2010; Adams et al. 2011). Although the transcriptional regulation of these transporters has been investigated in herbaceous plants, little information is available concerning the transcriptional changes of these genes in poplars in response to excess
Abscisic acid (ABA) is a stress phytohormone that often accumulates in plants exposed to abiotic and biotic stresses (Luo et al. 2009, 2011; Wang et al. 2013; Cao et al. 2014). ABA is involved in defense priming in plants (Luo et al. 2009) and can activate antioxidative defense systems that contribute to the alleviation of heavy metal toxicity (Sharma & Kumar 2002; Wang et al. 2013). Moreover, elevated ABA levels in plants can trigger the signaling cascades downstream of phytohormones, such as salicylic acid (SA) and auxin (IAA), which may also mitigate heavy metal-induced toxicity (Stroinski et al. 2010; Noriega et al. 2012; Trinh et al. 2014; Disante et al. 2014). Exogenous ABA application has been reported to reduce transpiration, leading to reduced Zn translocation from the soil to the aerial parts of plants, and to enhance the heavy metal tolerance of plants via defense priming in plants (Hsu & Kao 2003, 2005, 2008; Zhao et al. 2009). In contrast, Zn is involved in the biosynthesis of plant hormones (Broadley et al. 2007). Thus, excess Zn can affect endogenous phytohormone concentrations. Exposure to excess Zn can result in increased ABA concentrations in germinating seeds in chickpea (Cicer arietinum) and in Phaseolus vulgaris (Atici, Agar & Battal 2005; Zengin 2006). Additionally, elevated Zn can increase IAA levels in seedlings of Quercus suber (Disante et al. 2014). Although ABA may mediate heavy metal-induced toxicity, the effects of exogenous ABA on the toxicity of excess Zn and the response of endogenous phytohormones to excess Zn exposure in poplars remain unclear.

In this study, we used seedlings of Populus × canescens (syn. Populus tremula ×
P. alba; clone B1-714) that were exposed to basal or excess Zn levels in combination with exogenous ABA treatments to investigate the effect of exogenous ABA on the physiological and molecular responses of poplars to excess Zn. Because the exposure time of heavy metals may also influence the responses of poplars (He et al. 2011), different exposure times were investigated. The objectives of this study were as follows: (i) to elucidate the physiological and molecular mechanisms by which P. × canescens responds to excess Zn and (ii) to examine whether exogenous ABA addition alleviates Zn accumulation in P. × canescens. For these purposes, photosynthesis, Zn localization and accumulation, phytohormones, transcript levels of several genes involved in Zn uptake and translocation, ROS and antioxidants were analyzed in root, leaf and/or stem (wood + bark) of P. × canescens. Insights into these physiological and molecular responses to excess Zn and/or exogenous ABA will be helpful for the remediation of Zn-contaminated soil via Zn utilization by fast-growing woody plants.

Materials and Methods

Plant cultivation and treatments

Populus × canescens (syn. Populus tremula × P. alba; clone B1-714) plantlets were obtained via 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, the rooted plantlets were transferred to 10-liter plastic pots filled with sand and subsequently
cultivated in a climate chamber with conditions similar to those described above. Each plant was slowly irrigated to avoid runoff with 30 mL of distilled water in the morning and 50 mL of Hoagland solution in the evening. After cultivation for 10 weeks in the climate chamber, 72 plants with similar heights (ca. 55 cm) were selected and divided equally into four groups. The plants in each group were provided with Hoagland solution (with basal dose of Zn: 2 μM ZnSO₄) containing additionally either 0 or 10 mM ZnSO₄ and either 0 or 10 μM ABA ((±)-ABA, A1049, Sigma, St. Louis, MO, USA). The treatments lasted for 5 hours, 20 hours and 7 days, and 6 plants from each group were harvested at each time point.

Gas exchange measurement and harvest

Before harvest, the gas exchange of each plant was measured. Three mature leaves (leaf plastochron index = 7-9) were selected from each plant for the determination of the net photosynthetic rate (A), the stomatal conductance (gs) and the transpiration rate (E) using a LiCor-6400 portable photosynthesis system (LiCor Inc., Lincoln, NE, USA), as described by He et al. (2011). The measurements were carried out with a light intensity of 1000 μmol photon m⁻² s⁻¹ from 8:00 to 11:00 h. The air flow through the sample chamber was set at 500 μmol s⁻¹, the CO₂ concentration in the sample chamber was 400 μmol mol⁻¹ and the leaf temperature was 25 ± 0.8 °C.

After the measurement of gas exchange, each plant was harvested via separation of the leaf, bark, wood and root tissues. The roots of each plant were carefully washed with distilled water. The samples were wrapped with tinfoil and immediately frozen in
liquid nitrogen. The frozen samples were subsequently ground into a fine powder in liquid nitrogen using a mortar and a pestle; the samples were stored at -80°C for further analysis. To calculate the fresh-to-dry mass ratio, subsamples of fresh material from each tissue (root, wood, bark and leaf) of each plant were dried at 60°C for 72 h.

Subsamples of fine roots, stems and leaves were also harvested for histochemical analysis.

Analysis of foliar pigments

The concentrations of chlorophylls and carotenoids in the leaves were determined spectrophotometrically, as proposed by Wellburn et al. (1994).

Histochemical analysis of Zn localization

Zn distribution was investigated in the root, stem and leaf tissues of P. × canescens using the histochemical staining method described by Todeschini et al. (2011). In brief, hand sections or intact tissues of fresh samples of fine root, stem and leaf were rinsed in deionized H₂O and exposed to a staining solution (30 mg of diphenylthiocarbazone dissolved in 60 mL of acetone, 20 mL of H₂O and 7 drops of glacial acetic acid) for 90 seconds. After a brief rinse in deionized H₂O, the sections or tissues were immediately observed under an Eclipse E200 light microscope (Nikon, Tokyo, Japan). Well-stained samples with zinc-dithizone precipitates that displayed as red-purple were photographed under the light microscope using a DS-Fi1 CCD camera (Nikon) connected to a computer, as described by Cao et al. (2012).
**Determination of Zn concentrations and amounts**

To determine Zn concentrations, fine powder (ca. 100 mg) from fresh samples was digested in a mixture (7 mL of concentrated HNO₃ and 1 mL of concentrated HClO₄) at 170°C. Subsequently, the Zn concentrations in the extract were determined using a Hitachi 180-80 flame atomic absorbance spectrometer (Hitachi, Japan), as described by He et al. (2013a). The total Zn amount per plant was determined as the sum of the Zn amounts in each tissue, which were calculated by multiplying the Zn concentrations in each tissue by the biomass of that tissue.

**Determination of phytohormone concentrations**

The plant hormones ABA, gibberellin (GA₃), SA and IAA were extracted according to the method described by Walker-Simmons (1987), with minor modifications. Powder from fresh samples (ca. 500 mg) was suspended in 4 mL of 80% (v/v) methanol containing 200 mg L⁻¹ of butylated hydroxytoluene and 500 mg L⁻¹ of citric acid monohydrate on ice. The mixture was subsequently shaken overnight at 4 °C before centrifugation for 15 min at 10,000× g and 4 °C. The supernatant was collected. The precipitate was extracted twice for one hour, and the supernatants were combined. Then, the supernatants were dried under N₂ and resuspended in 800 μL of 80% methanol. The phytohormone concentrations in the extracts were analyzed using an LC-20AT high performance liquid chromatography system (Shimadzu, Kinh Do, Japan) and an API 2000™ electrospray tandem mass spectrometer (Allen-Bradley, Japan).
Milwaukee, WI, USA). Two-microliter samples were separated within a Wondasil™ C_{18} column (5 μM, 4.6 × 150 mm) (Shimadzu). ABA ((±)-ABA, A1049), GA_{3} (G7645), SA (S7401) and IAA (I2886) were purchased from Sigma for the preparation of standard curves to quantify hormone concentrations in the samples.

Analysis of transcript levels of genes involved in Zn uptake and translocation

Total RNA extraction and quantitative RT-PCR were performed based on the method described by He et al. (2013b). Briefly, total RNA was isolated from fine powder (ca. 100 mg) from the samples and purified using a plant RNA kit (R6827, Omega Bio-Tek, GA, USA). The purified RNA was used to synthesize cDNA using a PrimeScript RT reagent kit (RR047A, Takara, Dalian, China) based on the manufacturer’s instructions. Quantitative PCR was performed in a 20 μL reaction that contained 10 μL of 2× SYBR Green Premix EX Taq II (DRR820A, Takara), 2 μL of cDNA and 0.2 μL of 20 mM primers, which were designed specifically for each gene (Table S1). Quantitative PCR was performed using an IQ5 real time system (Bio-Rad, Hercules, CA, USA). Tubulin was used as a reference gene (Table S1). To ensure specificity, the PCR products were sequenced and aligned with homologues from other model plants (Fig. S1). The efficiencies of all PCR reactions were between 93 and 113% (Table S1). PCR was performed in triplicate and in combination with a dilution series of the reference gene.

Analysis of superoxide (O_{2}^{•−}) and H_{2}O_{2}

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Superoxide concentrations were determined spectrophotometrically at 530 nm, according to the method described by Ma et al. (2014).

The concentrations of H$_2$O$_2$ in the samples were determined spectrophotometrically at 410 nm, as described by Brennan & Frenkel (1977).

**Analysis of non-enzymatic and enzymatic antioxidants**

The concentrations of reduced (GSH) and oxidized glutathione (GSSG) in the samples were analyzed as described by Chen et al. (2011), with minor modifications (Ma et al. 2014). The analysis was based on the 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB)-glutathione reductase (GR) recycling procedure. Fine powder from fresh samples (ca. 100 mg) was extracted on ice in 0.7 mL of 0.5% 5-sulfosalicylic acid and centrifuged for 10 min at 10,000× g and 4 °C. Total glutathione (GSH + GSSG) levels were determined by mixing 0.2 mL of the supernatant with 0.05 mL of H$_2$O, 0.5 mL of 0.5 M sodium phosphate buffer (pH 7.5) containing 2.5 mM EDTA, 0.1 mL of 0.25 mM NADPH, 0.1 mL of 6 mM DTNB and 1 U of GR. To determine GSSG levels, 0.05 mL of 10% 2-vinylpyridine was added to the mixture instead of H$_2$O to remove GSH. The absorbances of total glutathione and GSSG were recorded spectrophotometrically at 412 nm. Finally, the ratio of GSH to GSSG was calculated based on the obtained concentrations of GSH and GSSG.

Total thiols (T-SH) were analyzed according to the method described by Tamás et al. (2008), with minor modifications (Ma et al. 2014).

The concentrations of ascorbate (ASC) and dehydroascorbate (DHA) in the
samples were analyzed according to the protocol described by Chen et al. (2011).

Free proline concentrations in the samples were determined spectrophotometrically at 518 nm, according to the method described by He et al. (2013a). The standard curve was established using a series of diluted solutions of L-proline (Amresco Inc., Solon, OH, USA).

Soluble proteins were extracted and used for enzyme activity assays, as described previously by He et al. (2011). The enzyme activities of superoxide dismutase (SOD, U g\(^{-1}\) protein), catalase (CAT, nkat g\(^{-1}\) protein), peroxidase (POD, nkat g\(^{-1}\) protein), ascorbate peroxidase (APX, nkat g\(^{-1}\) protein) and glutathione reductase (GR, nkat g\(^{-1}\) protein) were determined spectrophotometrically.

Statistical analysis

Statistical tests were performed using Statgraphics (STN, St. Louis, MO, USA). The data were tested for normality prior to the statistical analysis. For experimental variables, three-way ANOVA was applied, with ZnSO\(_4\) (Zn), ABA and time as the three principal factors. Differences between the means were considered significant when the \(P\)-value of the ANOVA F-test was less than 0.05. When interactions were significant, a posteriori comparison of means was done with Tukey method. Significance level of Tukey test was less than 0.05. The letters obtained from multi-comparisons represented the differences between all comparison levels. To reduce the chance on type I errors, all \(P\)-values less than 0.05 of these multi-comparisons were corrected by Tukey method. The data obtained from
quantitative PCR were analyzed using REST (Pfaffl, Horgan & Dempfle 2002). Cluster analysis of gene expression was performed using heatmap.2 in the ‘gplots’ package in R (http://www.r-project.org/), as described by Luo et al. (2013). For principal component analysis (PCA), the data were standardized and subsequently computed using prcomp() in R, as suggested by Li et al. (2012).

Results

Photosynthesis and foliar pigments

The CO₂ assimilation rate (\(A\)) of \(P. \times canescens\) was significantly reduced by excess Zn exposure (Table 1). Similarly, the stomatal conductance (\(g_s\)) and the transpiration rate (\(E\)) were reduced in \(P. \times canescens\) exposed to excess Zn. Depending on treatment time, the concentrations of chlorophyll \(a\) and the total concentrations of chlorophyll \(a\) and \(b\) were reduced by excess Zn. The concentrations of foliar carotenoids in \(P. \times canescens\) were also reduced by excess Zn. Photosynthesis of \(P. \times canescens\) was inhibited by exogenous ABA addition (Table 1). However, \(g_s\) and \(E\) reduction caused by excess Zn were alleviated by exogenous ABA addition for 5 h and 7 d. The concentrations of chlorophyll \(a\), chlorophyll \(b\) and their sums were unaffected by exogenous ABA addition, but concentrations of these pigments were significantly affected by the treatment time (Table 1). The repressive effects of excess Zn and/or exogenous ABA addition on \(A\) and other photosynthesis-related parameters were increasingly obvious with increases in treatment time (Table 1).
Zn localization and concentrations

Zn-dithizone complexes were undetectable in *P. × canescens* treated with excess Zn for either 5 or 20 hours. However, Zn-dithizone precipitates were observed in *P. × canescens* exposed to excess Zn for 7 days (Fig. 1). In the roots, elevated Zn exposure led to Zn accumulation in epidermal cells of *P. × canescens* (Fig. 1a1-a4). In the stems, high Zn levels resulted in Zn enrichment in the phloem, particularly in collenchyma cells, cortical cells and phloem fibers (Fig. 1b1-b5). In the leaves, excess Zn led to Zn accumulation at the base of the trichomes and in the upper epidermis, particularly in the vessel cell walls of the leaf vein, the chloroplasts of palisade cells near the epidermis and spongy cells (Fig. 1c1-c7). Zn localization exhibited a similar pattern in *P. × canescens* exposed to 10 mM ZnSO₄ and 10 μM ABA for 7 days, but the intensity of Zn staining under this condition was lower than that under 10 mM ZnSO₄ without ABA addition (Fig. 1).

Consistent with the Zn localization data, Zn concentrations were the highest in *P. × canescens* exposed to excess Zn for 7 days compared to plants treated for 5 or 20 hours, irrespective of exogenous ABA treatments (Fig. 2). Elevated Zn exposure resulted in increased Zn concentrations in the root, wood, bark and leaf tissues of *P. × canescens*. Exogenous ABA addition reduced Zn concentrations in *P. × canescens* after treatment for 7 days, irrespective of the supply of basal or excess Zn. For instance, exogenous ABA addition decreased Zn concentrations by 56 % in bark with 7-day treatments in comparison to the counterpart under excess Zn without ABA.
addition. Under basal Zn supply conditions, Zn concentration in poplar leaves was reduced by 16% due to exogenous ABA addition for 7 days. Excess Zn caused a 1.7- to 9.2-fold increase in the total amount of Zn per plant in comparison with plants undergoing normal Zn treatment. Exogenous ABA addition resulted in a 9-31% reduction in the total amount of Zn per plant.

**Phytohormone concentrations**

Because elevated ABA may prime defenses against heavy metal stress in plants, ABA concentrations were quantified in the roots and leaves of *P. × canescens* (Fig. 3). In the roots, excess Zn caused lower ABA concentrations, irrespective of ABA treatment and the time of exposure, but the opposite pattern was observed in the leaves. Endogenous ABA concentrations were higher in the roots and leaves of *P. × canescens* plants treated with exogenous ABA than in those of plants without ABA addition, regardless of Zn treatment and the time of exposure. In the roots, endogenous ABA concentrations gradually decreased with increases in treatment time; however, this pattern was not observed in the leaves.

In addition to ABA, the concentrations of GA\textsubscript{3}, SA and IAA, which often respond to abiotic stresses, were also determined in the roots and leaves of *P. × canescens* (Fig. 3). GA\textsubscript{3} concentrations were reduced in the roots and leaves of *P. × canescens* exposed to excess Zn, irrespective of ABA treatment and time of exposure. In the roots, exogenous ABA addition increased GA\textsubscript{3} concentrations in the absence of excess Zn and after treatment times of 5 or 20 hours. However, reduced GA\textsubscript{3}
concentrations were observed after 7 days of ABA treatment, regardless of Zn treatment. GA$_3$ concentrations gradually decreased in the roots and leaves with increases in treatment time, particularly in the presence of excess Zn and exogenous ABA addition.

In the roots, SA concentrations were increased by excess Zn in the absence of exogenous ABA addition and after 7 days of treatment; however, these values decreased in the presence of elevated Zn and ABA addition, regardless of treatment time (Fig. 3). In the leaves, SA concentrations were elevated upon exposure to excess Zn after 7 days of treatment, irrespective of exogenous ABA addition. In the roots, exogenous ABA addition resulted in higher SA concentrations under normal Zn conditions but lower SA concentrations under excess Zn conditions, irrespective of treatment time. In the leaves, exogenous ABA addition led to lower SA concentrations, regardless of Zn treatment and exposure time. Treatment for 7 days exerted the most pronounced effects on SA concentrations in the roots and leaves of $P. \times$ canescens compared to treatment for 5 or 20 hours.

In the roots, IAA concentrations were increased upon exposure to excess Zn in the absence of ABA addition and after treatment for 5 hours or 7 days; however, these concentrations decreased in the presence of elevated Zn and ABA addition after treatment for 20 hours or 7 days (Fig. 3). In the leaves, IAA concentrations were reduced in response to excess Zn, irrespective of ABA addition and time of treatment (Fig. 3). Exogenous ABA addition resulted in reduced IAA concentrations in the roots and leaves of $P. \times$ canescens, regardless of Zn treatment (Fig. 3).
Transcript levels of genes involved in Zn uptake and translocation

Because altered Zn accumulation was observed in the roots and leaves of *P. × canescens*, the transcriptional regulation of genes involved in Zn uptake and translocation was expected in the presence of excess Zn and/or ABA addition. To study transcriptional responses to excess Zn, nine genes implicated in Zn transport were selected based on our preliminary experiments and previous studies (Migeon et al., 2010; Adams et al. 2012; Lin & Aarks 2012). These genes included the ZIP family members ZIP2, ZIP6.2, ZIP6.4 and ZIP7.2, MTP1, NAS2, YSL2, HMA4 and PCR2, which are homologues of *AtZIP2, AtZIP6, AtIRT1, AtZIP7, AtMTP1, AtNAS2, AtYSL2, AtHMA4* and *AtPCR2* from *Arabidopsis thaliana*, respectively (Table S1). All of these genes were expressed in both roots and leaves of *P. × canescens*, except for *PCR2*, for which no transcripts were detected in the leaves. Among these genes, ZIP6.4, YSL2, HMA4 and PCR2 displayed the most pronounced changes in transcript levels in the roots of *P. × canescens* after excess Zn exposure, ABA addition or altered exposure time (Fig. 4a). Elevated Zn levels led to reduced mRNA levels of ZIP6.4, HMA4 and PCR2 after ABA addition, irrespective of the time of treatment (Fig. 4a). In contrast, excess Zn resulted in overexpressed transcript levels of YSL2, regardless of ABA addition and the time of treatment, and increased mRNA levels of PCR2 in the absence of ABA addition, irrespective of the time of treatment (Fig. 4a). In the leaves of *P. × canescens*, ZIP2, ZIP6.4, ZIP7.2 and YSL2 showed the most pronounced upregulation of mRNA levels after exposure to excess Zn, ABA addition or changes in

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treatment time (Fig. 4b). Specifically, increased transcript levels of ZIP2 were observed after exposure to excess Zn in the absence of ABA addition after 20 hours or 7 days (Fig. 4b). Enhanced mRNA levels of ZIP6.4 were detected in the leaves after exposure to excess Zn, irrespective of ABA addition and the time of treatment (Fig. 4b). Upregulated transcript levels of ZIP7.2 were observed in the leaves after exposure to elevated Zn levels, irrespective of ABA addition and the time of treatment (Fig. 4b). Elevated mRNA levels of YSL2 were detected in the leaves after exposure to excess Zn and ABA addition, irrespective of the time of treatment (Fig. 4b). These results suggest that ABA application resulted in the similar transcriptional regulation pattern of HMA4 in roots and leaves, but in the opposite change trends of ZIP6.4 transcript levels in roots and leaves (Fig. 4).

Oxidative stress and antioxidants

Excess Zn and exogenous ABA addition may result in oxidative stress and an imbalance between ROS and scavengers in P. × canescens. Therefore, oxidants (i.e., O₂⁻ and H₂O₂), non-enzymatic antioxidants (i.e., GSH, GSSG, total thiols, ASC, DHA and free proline) and enzymatic antioxidants (i.e., SOD, CAT, POD, APX and GR) were analyzed in P. × canescens (Figs. 6, 7, S2 and S3). High Zn exposure led to reduced concentrations of O₂⁻ in the root, wood and bark tissues, and reduced concentrations of H₂O₂ in the bark (Fig. 5). Exogenous ABA addition led to reduced concentrations of O₂⁻ in the root and wood tissues and reduced concentrations of H₂O₂ in the bark (Fig. 5). The time of treatment also markedly affected the
concentrations of $O_2^{\bullet-}$ and $H_2O_2$ in $P. \times$ canescens (Fig. 5). For instance, the $O_2^{\bullet-}$ concentration increased in roots treated for 20 hours but decreased in roots treated for 7 days, compared to roots treated for 5 hours with excess Zn in the absence of ABA addition. Similar changes in $O_2^{\bullet-}$ were observed in the leaves after treatment with excess Zn (Fig. 5).

Excess Zn resulted in higher GSH concentrations in the bark but lower GSSG concentrations in the root and wood tissues of $P. \times$ canescens, irrespective of ABA addition and the time of treatment (Fig. 6). Elevated Zn led to higher ratios of GSH to GSSG in the root, wood, bark and leaf tissues of $P. \times$ canescens, regardless of ABA addition and the time of treatment (Fig. 6). Exogenous ABA addition increased GSH concentrations in leaves treated for 20 hours, irrespective of Zn treatment (Fig. 6). In contrast, exogenous ABA addition resulted in reduced GSSG concentrations in the wood and bark tissues of $P. \times$ canescens (Fig. 6). In the roots, exogenous ABA application led to increased ratios of GSH to GSSG (Fig. 6). Effects of treatment time on changes in GSH, GSSG and the ratio of GSH to GSSG were also observed in $P. \times$ canescens and were dependent on excess Zn, ABA addition and the type of tissue (Fig. 6). For instance, the GSH concentration decreased in roots treated for 20 hours but increased in roots treated for 7 days, compared to roots treated for 5 hours with excess Zn in the absence of ABA addition. Excess Zn led to increased ASC concentrations in the bark and leaf tissues and reduced DHA concentrations in $P. \times$ canescens (Fig. 7).

Exogenous ABA addition resulted in lower DHA concentrations in the roots and increased ASC concentrations and ratios of ASC to DHA in leaves treated for 7 days.
Free proline concentrations in the bark of *P. × canescens* were reduced after exposure to excess Zn with ABA addition (Figs. S2). These non-enzymatic antioxidants were affected by the time of treatment, but no clear patterns were observed after exogenous ABA treatments (Figs. 7 and S2). Excess Zn had no effects on total thiols in *P. × canescens* (Fig. S2). Exogenous ABA application led to lower total thiols in poplar bark (Fig. S2). The time of treatment affected total thiols in root, wood and leaf tissues of poplars (Fig. S2).

Excess Zn resulted in increased activities of SOD and GR in the root and bark, but these conditions reduced CAT activity in the root and leaf tissues of *P. × canescens* (Fig. S3). Exogenous ABA addition led to higher activities of SOD and GR in the leaf, but this treatment reduced the activities of POD and APX in the root and the activity of CAT in the leaf of *P. × canescens* (Fig. S3). The analyzed enzyme activities were also affected by the treatment time, and in most cases, increases in the time of treatment resulted in more pronounced changes in enzyme activities in *P. × canescens* (Fig. S3). For instance, SOD activities in leaves decreased with the treatment for 20 hours but increased for 7 days, compared to that treated for 5 hours with excess Zn in the absence of ABA addition.

**PCA of physiological responses**

To elucidate the response patterns of *P. × canescens* to excess Zn, exogenous ABA addition and treatment time, PCA was performed using data related to photosynthesis and concentrations of Zn, phytohormones, ROS and antioxidants (Fig. S3).
The response patterns of *P. × canescens* to excess Zn and/or exogenous ABA addition were distinct at different time points. In comparison to treatment for 5 or 20 hours, treatment for 7 days exerted the most pronounced effects on the physiological responses of *P. × canescens* to Zn and/or exogenous ABA treatments. After treatment for 5 hours or 7 days, PC1 separated the effects of Zn treatment and PC2 uncovered variations in the effects of ABA. PC1 and PC2 accounted for 26 and 16% of the observed variation, respectively. Zn concentrations in the roots and leaves, SA concentrations in the leaves and the ratios of GSH to GSSG in the roots were key contributors to PC1. In contrast, the concentrations of ABA in the leaves and IAA in the roots were important factors for PC2. After treatment for 20 hours, PC1 separated the effects of ABA treatment and PC2 revealed variations in Zn levels. These PCA results indicate that treatment for 20 hours induced distinct physiological responses to Zn and/or ABA in comparison to treatment for 5 hours or 7 days. In addition, *P. × canescens* demonstrated different physiological acclimation patterns to excess Zn and exogenous ABA addition, which resulted primarily from distinct effects of excess Zn and exogenous ABA on Zn concentrations in the roots and leaves, SA concentrations in the leaves, and concentrations of endogenous ABA in the leaves and IAA in the roots.

**Discussion**

**Physiological and molecular responses of *P. × canescens* to excess Zn**

Although Zn is an essential nutrient for the growth and development of plants,
excess Zn can be toxic to plants (Broadley et al. 2007). The threshold of Zn toxicity in plants is highly variable among species and even within species. For *Populus* species, concentrations of 0.5-10 mM Zn$^{2+}$ in solution were toxic to plants grown using hydroponics (Di Baccio et al. 2003, 2009; Castiglione et al. 2007; Franchin et al. 2007). In this study, 10 mM Zn$^{2+}$ was applied to seedlings of *P. × canescens* grown in sand culture as an excess Zn treatment. Irrespective of the treatment time, reduced $A$ in *P. × canescens* after exposure to excess Zn in the absence of exogenous ABA demonstrated the toxicity of high concentrations of this transitional metal in these poplars. Moreover, gradual reductions in $A$ were observed with increases in exposure time to excess Zn, suggesting that the severity of Zn toxicity is enhanced with increased exposure time. The toxicity of excess Zn to *P. × canescens* is associated with increasing Zn accumulation in different tissues of poplars with increasing exposure time, as evidenced by that the amount of Zn in poplars elevated with increased exposure time to excess Zn (Fig. 2). Previous studies consistently demonstrated that the inhibition of photosynthesis by excess Zn and Zn accumulation in the roots and leaves of plants, including *Populus* species, are also enhanced with elevated exposure time to excess Zn (Küpper et al. 2002; Di Baccio et al. 2003, 2009; Hermle et al. 2007). The repressive effect of excess Zn on $A$ in poplars is likely due to degeneration of the photosynthetic apparatus (Di Baccio et al. 2009; Todeschini et al. 2011) and the transcriptional downregulation of genes involved in photosynthetic processes in the presence of high Zn levels (Di Baccio et al. 2011). These results indicate that excess Zn can accumulate in poplar plants, causing the inhibition of
photosynthesis and growth.

Phytohormones play key roles in signaling in response to abiotic and biotic stresses, including excess Zn stress (Luo et al. 2009, 2011; Sofo et al. 2013; Cao et al. 2014). ABA, GA$_3$, SA and IAA are crucial components of the phytohormonal signaling network that responds to heavy metals in plants (Vicente & Plasencia 2011; Sofo et al. 2013). In this study, endogenous ABA concentrations decreased in the roots in response to excess Zn with no ABA addition after treatment for 20 hours, but increased in the leaves of $P. \times$ canescens in response to excess Zn in the absence of exogenous ABA addition after treatment for 20 hours or 7 days (Fig. 3). Previous studies demonstrated that higher ABA concentrations are present in several plant species after exposure to elevated Zn levels (Atici et al. 2005; Zengin 2006; Yang et al. 2011). However, no changes in ABA concentration were observed in plants exposed to excess Zn (Sofo et al. 2013). With respect to other heavy metals, excess Mn caused higher ABA concentrations in a Mn-tolerant population of $P. \ cathayana$ than in a Mn-sensitive population (Lei, Korpelainen & Li 2007) and Cd exposure led to increased ABA concentrations in a tolerant cultivar of rice but not in a sensitive cultivar (Hsu & Kao 2003, 2005). These results indicate that endogenous ABA accumulation may contribute to enhanced tolerance to heavy metals in plants.

GA$_3$ can affect plant growth and development via cell elongation and differential transcriptional expression of genes (Peleg & Blumwald 2011). GA$_3$ concentrations are sensitive to changes in environmental conditions. The finding that excess Zn decreased GA$_3$ concentrations in the roots and leaves of $P. \times$ canescens in
the absence of exogenous ABA addition, irrespective of exposure time, is consistent with previously reported changes in GA3 in germinated seeds of chickpea and in the roots of *A. thaliana* after high Zn exposure (Atici *et al.* 2005; Sofo *et al.* 2013). These observations suggest that GA3 is involved in the acclimation of plants to excess Zn.

Although SA is known for protecting plants against biotic stress by inducing local and systemic acquired resistance (Loake & Grant 2007; Luo *et al.* 2009, 2011), little information is available on the changes in endogenous SA concentrations that occur in plants in response to heavy metals (Llugany *et al.* 2013). The induction of endogenous SA in the roots and leaves of *P. × canescens* by excess Zn treatment for 7 days is consistent with findings from previous studies (Fig. 3). For instance, cadmium (Cd) exposure tended to increase foliar SA concentrations in *N. praecox*, partially explaining Cd hyperaccumulation in this species (Llugany *et al.* 2013). Moreover, SA is thought to be involved in signaling pathways that mediate heavy metal-based defense against pathogens in hyperaccumulating plants, although SA concentrations often remain unaltered in these plants after exposure to heavy metals (Freeman *et al.* 2005; Fones *et al.* 2013). Additionally, exogenous SA application often increases the metal tolerance of plants via enhanced antioxidative defense systems against heavy metal-induced oxidative stress (Drazic & Mihailovic 2005; Guo, Liang & Zhu 2009; Noriega *et al.* 2012). These results indicate that SA may act as an important player in the signaling network of plants that is activated in response to heavy metal stress.

IAA can affect a range of physiological processes, such as cell division, expansion and differentiation (Robert & Friml 2009; Popko *et al.* 2010). Stresses
often disturb IAA homeostasis in plants, resulting in changes in growth and development (Hu et al. 2013). Zn is involved in the metabolism of IAA, and excess Zn in plants may impact IAA homeostasis (Broadley et al. 2007; Yamaji et al. 2013). In this study, reductions in IAA concentrations were observed in the roots and leaves of P. × canescens exposed to excess Zn under most ABA treatment conditions and at most time points (Fig. 3). High Zn concentrations caused increased IAA concentrations in the leaves of Quercus suber seedlings (Disante et al. 2014), but no changes in IAA concentrations were observed in the roots and shoots of A. thaliana (Sofo et al. 2013). Changes in IAA concentrations were also reported for plants exposed to other heavy metals. For instance, Cd exposure caused reduced IAA concentrations in Arabidopsis and Populus plants (Elobeid et al. 2012; Hu et al. 2013); these changes are likely due to increased activity of the IAA oxidase and due to the transcriptional regulation of genes involved in IAA biosynthesis and catabolism. These results indicate that changes in IAA concentrations in plants exposed to heavy metals, including excess Zn, are likely associated with heavy metal-induced alterations in IAA metabolism.

Zn is an essential element for plants. Thus, Zn\(^{2+}\) uptake and transport are critical for Zn homeostasis in plants (Milner et al. 2013; Olsen & Palmgren 2014). The differential transcriptional expression of ZIP6.4, YSL2, HMA4 and PCR2 in the roots and ZIP2, ZIP6.4, ZIP7.2 and YSL2 in the leaves of P. × canescens in response to excess Zn (Fig. 4) suggests that these genes play pivotal roles in Zn uptake and transport and help maintain Zn homeostasis in poplars in the presence of excess Zn. In
the roots and shoots of *A. thaliana*, AtZIP2 transcript levels decreased in response to
Zn deficiency (Milner *et al.* 2013); however, transcriptional changes of AtZIP2 remain
to be determined under excess Zn conditions. Based on the EST database, ZIP2 was
highly expressed in the roots of *P. trichocarpa* (Migeon *et al.* 2010). AtIRT1 and
AtZIP7 are the closest homologs to ZIP6.4 and ZIP7.2 from *P. trichocarpa*,
respectively. High expression of IRT1 was suggested to contribute to enhanced Zn
transport in the Zn hyperaccumulator *N. caerulescens* in comparison to the
non-hyperaccumulator *N. arvense* (Lasat *et al.* 2000). The transcript abundance of
AtZIP7 was elevated in the roots and shoots of *Arabidopsis* exposed to Zn deficiency
(Grotz *et al.* 1998). In addition to ZIPs, YSL2, HMA4 and PCR2 are also involved in
Zn translocation in plants. YSL2 transports metal-NA complexes in plants, and
transcript levels of YSL2 decreased in the roots and shoots of *Arabidopsis* exposed to
Fe deficiency or excess Cu (DiDonato *et al.* 2004). Moreover, chelates of Zn-NA
were detected in yeast and in several plant species (Trampczynska *et al.* 2010;
Nishiyama *et al.* 2012; Deinlein *et al.* 2012). Thus, excess Zn-induced YSL2 mRNA
levels in the roots and leaves of *P. × canescens* indicate that YSL2 can play a role in
Zn-NA mobilization in poplars during exposure to excess Zn. HMA4 is implicated in
pumping Zn$^{2+}$ out of the cytosol in poplar cells, and repressed HMA4 transcript levels
have been reported in poplars exposed to excess Zn (Adams *et al.* 2011). Consistently
downregulated HMA4 mRNA levels were detected in the roots and leaves of *P. ×
canescens* in response to excess Zn. AtPCR2 is reported to be a zinc exporter involved
in both zinc extrusion and long-distance zinc transport, but transcript levels of

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AtPCR2 remain unaltered in *Arabidopsis* exposed to excess Zn (Song *et al.* 2010). In this study, *PCR2* transcripts were detected only in the roots of *P. × canescens* and were induced by excess Zn in the absence of exogenous ABA addition (Fig. 4), suggesting that *PCR2* may be involved in Zn transport in poplar roots during exposure to excess Zn. Taken together, these data indicate that the transcriptional regulation of these genes can contribute to Zn uptake, translocation and accumulation in *P. × canescens* in response to excess Zn.

Heavy metals often induce ROS production in plants, resulting in a shift of the balance between ROS and antioxidants (Sharma & Dietz 2009). Although Zn$^{2+}$ is not directly involved in the Fenton and/or Haber-Weiss reactions that lead to ROS overproduction (Sharma & Dietz 2009), excess Zn$^{2+}$ can cause oxidative stress via the displacement of redox-active ions, such as iron ions from binding sites in proteins and other compounds (Arrivault, Senger & Krämer 2006). In the current study, excess Zn induced higher H$_2$O$_2$ concentrations in only the roots of *P. × canescens* and in most cases, the concentrations of O$_2^\bullet^-$ and H$_2$O$_2$ remained unaltered or reduced in these poplars in response to high Zn concentrations in the absence of exogenous ABA addition (Fig. 5). Oxidative stress is often observed in plants, including poplar species, exposed to elevated Zn levels (Di Baccio *et al.* 2003, 2005, 2009; Remans *et al.* 2012).

To eliminate the induced ROS, non-enzymatic and enzymatic antioxidants act as important scavengers (He *et al.* 2011; Luo *et al.* 2014). Specifically, GSH can play a central role in the equilibrium between ROS and antioxidant levels in plant cells under heavy metal stress (Di Baccio *et al.* 2005; Seth *et al.* 2012). In plants, GSH can
function as an antioxidant via the GSH-ASC cycle and/or as a chelator for heavy metals that may be depleted in plant cells in response to excess metals (Di Baccio et al. 2005; Seth et al. 2012). In this study, the increased GSH levels observed in the root and bark tissues and the decreased GSSG concentrations observed in the roots of *P. × canescens* exposed to excess Zn suggest that enhanced GSH biosynthesis likely drives the observed reduction of GSSG in plant cells. This is corroborated by the increased GR activities observed in the roots of *P. × canescens* exposed to excess Zn in the absence of exogenous ABA addition (Fig. S3). Additionally, ASC and DHA are also important scavengers for ROS in poplars (Ma et al. 2014). Elevated concentrations of ASC in bark and leaf tissues and decreased DHA levels in roots and leaves exposed to excess Zn with no ABA addition for 7 days suggest that these molecules play a role in ROS homeostasis in these tissues. Overall, these data indicate that excess Zn causes a limited disturbance of the balance between ROS and antioxidants in *P. × canescens*; this disturbance is likely to be associated with enhanced GSH biosynthesis in the presence of excess Zn.

**Effects of exogenous ABA on Zn toxicity in *P. × canescens***

ABA is a stress-inducible phytohormone that regulates several growth and development processes in plants during acclimation to stress. Exogenous ABA application can result in the partial closure of stomata and reduced transpiration rates in plants; these changes may alleviate heavy metal toxicity in plants because heavy metals are transported to the aerial parts of plants via the transpiration stream (Hsu &
Previous studies demonstrated that exogenous ABA reduced the transpiration rates and enhanced the Cd tolerance of rice seedlings (Hsu & Kao 2003, 2005, 2008). The low concentrations of exogenous ABA also alleviated lead (Pb) toxicity in *Atractylodes macrocephala* and rice seedlings (Zhao *et al.* 2009; Wang *et al.* 2013). However, in the current study, transpiration rate in excess-Zn-treated *P. × canescens* with exogenous ABA addition was similar to that in poplars without exogenous ABA application. Thus, the reduced Zn uptake and accumulation in bark and leaf tissues of *P. × canescens* treated with excess Zn for 7 days induced by exogenous ABA addition was probably not due to reduced transpiration rates. The alleviation of heavy metal toxicity in plants by exogenous ABA application is likely ascribed to the following factors: (i) exogenous ABA triggers signaling cascades in plants that prime defenses against heavy metal stress, (ii) exogenous ABA enhances the transcriptional regulation of genes involved in the detoxification of heavy metals and the contents of chelators for heavy metals (Stroinski *et al.* 2010), and (iii) exogenous ABA increases the activities of antioxidative enzymes, leading to reduced heavy metal-induced oxidative stress (Wang *et al.* 2013). Although direct experimental evidence related to the effect of exogenous ABA application on Zn toxicity in plants is lacking, previous studies demonstrated that exogenous ABA can ameliorate Cd/Pb toxicity in plants by mediating changes in endogenous phytohormones. Increases in the concentrations of exogenous ABA applied to rice seedlings caused a gradual elevation of endogenous ABA concentrations in the root and leaf tissues, resulting in enhanced Cd tolerance in plants (Hsu & Kao 2003).
Similarly, Stroinski et al. (2010) found that exogenous ABA addition could increase endogenous ABA concentrations in tuber discs of potato, leading to increased Cd tolerance. Consistent with these studies, increased endogenous ABA concentrations and alterations in concentrations of other phytohormones were detected in the roots and leaves of \( P. \times \) canescens after exogenous ABA addition (Fig. 3). These findings indicate that exogenous ABA application can trigger the signaling network of plants in response to heavy metals via changes in the concentrations of endogenous ABA and other phytohormones. Moreover, exogenous ABA application can result in increased transcript levels of \( PCS1 \), increased enzyme activities of phytochelatin synthase (PCS) and increased phytochelatin (PC) concentrations in potato tuber discs, contributing to the amelioration of Cd-induced toxicity (Stroinski et al. 2010). However, in the current study, reduced transcript levels of genes involved in Zn uptake and transport, such as \( NAS2 \), ZIP6.4, PCR2 and HMA4, were observed in the roots of \( P. \times \) canescens after exogenous ABA addition (Fig. 4). Further, higher concentrations of GSH and T-SH, which can act as chelators for \( \text{Zn}^{2+} \), were detected in the roots and leaves of \( P. \times \) canescens after exogenous ABA addition in the absence of excess Zn treatment. These results indicate that exogenous ABA application can modulate the mRNA levels of genes involved in heavy metal transport and detoxification and enhance the contents of chelators to reduce heavy metal toxicity in plants. Finally, exogenous ABA addition can modulate the activities of antioxidative enzymes in plants to detoxify the excess ROS induced by heavy metals. Exogenous ABA is reported to cause increased activities of antioxidative enzymes, such as SOD, APX, CAT and POD, in plants,
alleviating the oxidative stress induced by heavy metals (Zhao et al. 2009; Wang et al. 2013). In the current study, higher activities of SOD were observed in the roots and leaves of *P. × canescens* after exogenous ABA addition, which may contribute to the scavenging of overproduced ROS after excess Zn exposure.

As summarized in Fig. 9, excess Zn was taken up by the roots and translocated to the aerial parts of *P. × canescens*, leading to reduced A and increased Zn accumulation in poplars. However, Zn concentrations in bark and leaves were decreased upon exogenous ABA addition for 7 days. Endogenous ABA concentrations were lower in the roots but higher in the leaves of *P. × canescens* after excess Zn exposure. These concentrations were elevated in poplars treated with exogenous ABA.

In the roots, the transcript levels of several genes involved in Zn uptake and transport, such as *YSL2* and *PCR2*, were enhanced in *P. × canescens* treated with excess Zn but repressed by exogenous ABA addition. Moreover, increased mRNA levels of *ZIP2*, *ZIP6.4*, *ZIP7.2* and *YSL2* were found in the leaves of *P. × canescens* exposed to excess Zn. H₂O₂ concentrations were increased in the roots of *P. × canescens* after exposure to excess Zn, but this effect was counteracted by exogenous ABA addition. Higher ratios of GSH to GSSG were detected in *P. × canescens* exposed to excess Zn. These results suggest that excess Zn is absorbed and accumulated in *P. × canescens* via the transcriptional regulation of key genes involved in Zn uptake and transport and that signaling cascades are triggered by changes in the concentrations of endogenous phytohormones such as GA₃, SA and IAA. In addition, exogenous ABA application can decrease Zn concentrations in bark and leaf of *P. × canescens* exposed to excess...
Zn for 7 days, likely by modulating the transcript levels of key genes involved in Zn transport, and activating the antioxidative defense system.

Acknowledgements

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Conflict of interest

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

Supporting Information

The following materials are available in the online version of this article:

Table S1. Primers used for qRT-PCR.

Table S2. Principal component analysis (PCA) of physiological parameters.

Fig. S1. Alignments of genes involved in Zn uptake and translocation.

Fig. S2. Concentrations of T-SH and free proline.

Fig. S3. Activities of SOD, CAT, POD, APX and GR.
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Table 1. CO₂ assimilation rate (A, μmol CO₂ m⁻² s⁻¹), stomatal conductance (gs, mol H₂O m⁻² s⁻¹), transpiration rate (E, mmol H₂O m⁻² s⁻¹) and photosynthetic pigments (mg g⁻¹ DW) in leaves of *P. × canescens* exposed to either basal or excess Zn and either 0 (-ABA) or 10 μM ABA (+ABA) for 5, 20 hours (h) or 7 days (d), respectively. Data indicate means ± SE (n = 6). Different letters behind the values in the same column indicate significant difference between the treatments. Chl a: chlorophyll a; Chl b: chlorophyll b; Chl (a + b): chlorophyll a and b; Car: carotenoids. *P*-values of the ANOVAs of ZnSO₄ (Z), ABA (A), Time (T) and their interactions (Z × A: ZA; Z × T: ZT; A × T: AT; and Z × A × T: ZAT) are indicated. *: *P* < 0.05; **: *P* < 0.01; ***: *P* < 0.001; ****: *P* < 0.0001; ns: not significant.

<table>
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<tr>
<th>Zn treatments</th>
<th>ABA treatments</th>
<th>Time</th>
<th>A</th>
<th>gₛ</th>
<th>E</th>
<th>Chl a</th>
<th>Chl b</th>
<th>Chl (a+b)</th>
<th>Car</th>
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<td>0.035 ± 0.000 d</td>
<td>1.03 ± 0.06 e</td>
<td>8.80 ± 0.02 bc</td>
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<td>7 d</td>
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<td>0.034 ± 0.000 d</td>
<td>0.99 ± 0.02 e</td>
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Figure legends

Figure 1. Zn localization revealed by dithizone staining in the root (a1-a7), stem (b1-b9) and leaf (c1-c12) tissues of *P. × canescens* exposed to either basal or excess Zn and either 0 (-ABA) or 10 µM ABA (+ABA) for 7 days. Basal Zn and without ABA (a1, b1, c1 and c2); excess Zn without ABA (a2-a4, b2-b5, c3-c7); excess Zn with ABA (a5-a7, b6-b9, c8-c12). Arrows point to Zn-dithizone precipitates. Root surface (a1, a2, a5); epidermal cells of fine roots (a3, a4, a6, a7); cross sections of stem (b1, b2, b6); collenchyma (col; b3, b7); phloem fiber (pf; b4, b8); cortical cells (cor; b5, b9); leaf trichomes (lt; c1, c3, c8); cross sections of leaf (lc; c2, c4, c9); cross section of leaf vein (lv; c5, c10); palisade cells near epidermis (ep; c6, c11); palisade cell near spongy cells (sp; c7, c12). ph: phloem; ue: upper epidermis; le: lower epidermis; v: vessel; ch: chloroplasts.

Figure 2. Zn concentrations and total Zn amount per plant of *P. × canescens* exposed to either basal or excess Zn and either 0 (-ABA) or 10 µM ABA (+ABA) for 5 hours (h), 20 hours (h) or 7 days (d). The bars indicate the means ± SE (n = 6). Different letters on the bars for the same tissue indicate significant differences between the treatments. *P*-values for the ANOVA tests of ZnSO₄ (Z), ABA (A), Time (T) and their interactions (Z × A: ZA; Z × T: ZT; A × T: AT; and Z × A × T: ZAT) are indicated. *: *P* < 0.05; **: *P* < 0.01; ***: *P* < 0.001; ****: *P* < 0.0001; ns: not significant.

Figure 3. ABA, GA₃, SA and IAA in the root and leaf tissues of *P. × canescens*.

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exposed to either basal or excess Zn and either 0 (-ABA) or 10 μM ABA (+ABA) for 5 hours (h), 20 hours (h) or 7 days (d). The data indicate the means ± SE (n = 6).

Different letters on the bars for the same tissue indicate significant differences between the treatments. P-values for the ANOVA tests of ZnSO₄ (Z), ABA (A), Time (T) and their interactions (Z × A: ZA; Z × T: ZT; A × T: AT; and Z × A × T: ZAT) are indicated. *: P < 0.05; **: P < 0.01; ***: P < 0.001; ****: P < 0.0001; ns: not significant.

**Figure 4.** Cluster analysis of transcriptional fold-changes of genes involved in Zn uptake and transport in the root (a) and leaf (b) of *P. × canescens* exposed to either basal or excess Zn and either 0 (-ABA) or 10 μM ABA (+ABA) for 5 hours (h), 20 hours (h) or 7 days (d). For each gene, the transcript level in the leaves or roots treated without Zn and ABA for 5 h was defined as 1, and the corresponding transcriptional fold-changes of genes under other conditions were subsequently calculated.

**Figure 5.** O₂⁻ and H₂O₂ in the root, wood, bark and leaf tissues of *P. × canescens* exposed to either basal or excess Zn and either 0 (-ABA) or 10 μM ABA (+ABA) for 5 hours (h), 20 hours (h) or 7 days (d). The data indicate the means ± SE (n = 6). Different letters on the bars for the same tissue indicate significant differences between the treatments. P-values for the ANOVA tests of ZnSO₄ (Z), ABA (A), Time (T) and their interactions (Z × A: ZA; Z × T: ZT; A × T: AT; and Z × A × T: ZAT) are indicated. *: P < 0.05; **: P < 0.01; ***: P < 0.001; ****: P < 0.0001; ns: not significant.
Figure 6. GSH, GSSG and GSH/GSSG in the root, wood, bark and leaf tissues of *P. × canescens* exposed to either basal or excess Zn and either 0 (-ABA) or 10 μM ABA (+ABA) for 5 hours (h), 20 hours (h) or 7 days (d). The data indicate the means ± SE (*n* = 6). Different letters on the bars for the same tissue indicate significant differences between the treatments. *P*-values for the ANOVA tests of ZnSO₄ (Z), ABA (A), Time (T) and their interactions (Z × A: ZA; Z × T: ZT; A × T: AT; and Z × A × T: ZAT) are indicated. *: *P* < 0.05; **: *P* < 0.01; ***: *P* < 0.001; ****: *P* < 0.0001; ns: not significant.

Figure 7. ASC, DHA and ASC/DHA in the root, wood, bark and leaf tissues of *P. × canescens* exposed to either basal or excess Zn and either 0 (-ABA) or 10 μM ABA (+ABA) for 5 hours (h), 20 hours (h) or 7 days (d). The data indicate the means ± SE (*n* = 6). Different letters on the bars for the same tissue indicate significant differences between the treatments. *P*-values for the ANOVA tests of ZnSO₄ (Z), ABA (A), Time (T) and their interactions (Z × A: ZA; Z × T: ZT; A × T: AT; and Z × A × T: ZAT) are indicated. *: *P* < 0.05; **: *P* < 0.01; ***: *P* < 0.001; ****: *P* < 0.0001; ns: not significant.

Figure 8. Principal component analysis (PCA) plots of physiological parameters of *P. × canescens* exposed to either basal or excess Zn and either 0 (-ABA) or 10 μM ABA (+ABA) for 5 hours (h), 20 hours (h) or 7 days (d). The data indicate the means ± SE (*n* = 6). Different letters on the bars for the same tissue indicate significant differences between the treatments. *P*-values for the ANOVA tests of ZnSO₄ (Z), ABA (A), Time (T) and their interactions (Z × A: ZA; Z × T: ZT; A × T: AT; and Z × A × T: ZAT) are indicated. *: *P* < 0.05; **: *P* < 0.01; ***: *P* < 0.001; ****: *P* < 0.0001; ns: not significant.
(+ABA) for 5 hours (h), 20 hours (h) or 7 days (d). Open circle: -Zn-ABA; closed circle: -Zn+ABA; open triangle: +Zn-ABA; closed triangle: +Zn+ABA.

**Figure 9.** A schematic diagram illustrating the effects of ABA on Zn uptake and translocation in *P. × canescens* exposed to excess Zn for 7 days. The left chart depicts *P. × canescens* exposed to excess Zn (a-b), and the right chart represents *P. × canescens* treated with excess Zn and exogenous ABA (c-d). Excess Zn was absorbed by the roots and translocated to the aerial parts of *P. × canescens*, leading to Zn accumulation along the transport pathway, but Zn uptake and accumulation in bark and leaf tissues was decreased by exogenous ABA. Excess Zn caused lower ABA concentrations in the roots and changes in the concentrations of other phytohormones, which may trigger signaling cascades in *P. × canescens* that prime defenses against excess Zn-induced stress. Excess Zn exposure led to increased transcript levels of *YSL2* and *PCR2*, which are involved in Zn uptake and translocation. Excess Zn increased H$_2$O$_2$ concentrations in the roots. Exogenous ABA repressed transcript levels of *ZIP6.2*, *ZIP6.4*, *PCR2* and *HMA4* but enhanced mRNA levels of *YSL2*, inhibiting Zn accumulation and increasing resistance to excess Zn in *P. × canescens*. Moreover, exogenous ABA relieved excess Zn-induced oxidative stress in the roots via increased ratios of GSH to GSSG. CW: cell wall; PM: plasma membrane.
Fig. 1

PCE_12434_F1
Fig. 2

PCE_12434_F2

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Fig. 3
Fig. 4

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Fig. 6
Fig. 7

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Fig. 8

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Fig. 9

PCE_12434_F9
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