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Exogenous Abscisic Acid Alleviates Cadmium Toxicity by Restricting Cd^{2+} Influx in *Populus euphratica* Cells

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Abstract Abscisic acid (ABA), a widely known phytohormone involved in the plant response to abiotic stress, plays a vital role in mitigating Cd^{2+} toxicity in herbaceous species. However, the role of ABA in ameliorating Cd^{2+} toxicity in woody species is largely unknown. In the present study, we investigated ABA restriction on Cd^{2+} uptake and the relevance to Cd^{2+} stress alleviation in Cd^{2+}-hypersensitive *Populus euphratica*. ABA (5 μM) markedly improved cell viability and growth but reduced membrane permeability in $CdCl_2$ (100 μM)-stressed *P. euphratica* cells. Moreover, ABA significantly increased the activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX), contributing to the scavenging of Cd^{2+}-elicited H_2O_2 within *P. euphratica* cells during the period of $CdCl_2$ exposure (100 μM, 24–72 h). ABA alleviation of Cd^{2+} toxicity was mainly the result of ABA restriction of Cd^{2+} uptake under Cd^{2+} stress. Steady-state and transient flux recordings showed that ABA inhibited Cd^{2+} entry into Cd^{2+}-shocked (100 μM, 30 min) and short-term-stressed *P. euphratica* cells (100 μM, 24–72 h). Non-invasive micro-test technique data showed that H_2O_2 (3 mM) stimulated the Cd^{2+}-elicited Cd^{2+} influx but that the plasma membrane (PM) Ca^{2+} channel inhibitor $LaCl_3$ blocked it, suggesting that the Cd^{2+} influx was through PM Ca^{2+}-permeable channels. These results suggested that ABA up-regulated antioxidant enzyme activity in Cd^{2+}-stressed *P. euphratica* and that these enzymes scavenged the Cd^{2+}-elicited H_2O_2 within cells. The entry of Cd^{2+} through the H_2O_2-mediated Ca^{2+}-permeable channels was subsequently restricted; thus, Cd^{2+} buildup and toxicity were reduced in the Cd^{2+}-hypersensitive species, *P. euphratica*.

Keywords ABA · Antioxidant enzyme · H_2O_2 · NMT · Cd^{2+} influx · *Populus euphratica*

Introduction

Cadmium (Cd) is one of the most toxic heavy metals for herbaceous (DalCorso and others 2008) and woody plants (Elobeid and others 2012; Polle and others 2013). It exerts adverse effects on various physiological processes such as photosynthesis, respiration, nitrogen metabolism, and nutrient uptake, leading to growth retardation and even plant death (di Toppi and Gabbrielli 1999; DalCorso and others 2008). Cd^{2+} is suggested to enter plant cells through non-selective cation channels and Fe^{2+}, Ca^{2+}, and Zn^{2+} transporters/channels such as iron-regulated transporter (IRT) 1, zinc-regulated transporter/IRT-like protein, and natural resistance-associated macrophage protein family transporter (Clemens 2006; Lux and others 2011; Zhu and others 2012; Sasaki and others 2012). Excessive Cd^{2+} in

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the cytoplasm usually promotes a burst of reactive oxygen species (ROS), for example, superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Gallego and others 2012; Chmielowska-Bak and others 2014). ROS accumulation can lead to oxidative stress within the cells, including harmfully changing protein structures, destroying phospholipids, and eventually causing membrane damage and enzyme inactivation (Gallego and others 2012). In contrast, initially produced ROS also can be signaling molecules that regulate a large network in the cellular response to cadmium toxicity (Sandalio and others 2009; Rodríguez-Serrano and others 2009). We previously found that H_2O_2 activates plasma membrane (PM) Ca^{2+} channels, thus enhancing entry of Cd^{2+} into the cytosol (Sun and others 2013).

Abscisic acid (ABA) is a crucial phytohormone that regulates plant growth and development, including seed dormancy, stomatal movement, and lateral root formation (Finkelstein and others 2002). It also plays important roles in plant adaptations to various environmental stresses, including drought, salinity, and freezing (Zhu 2002; Thompson and others 2007; Guo and others 2012; Yang and others 2014). Recently, increasing numbers of reports have confirmed the physiological roles of ABA in heavy metal tolerance in herbaceous plants. For example, exogenous ABA significantly enhances cadmium tolerance by reducing transpiration rates in rice seedlings (Hsu and Kao 2003, 2005). In *Atractylodes macrocephala*, the application of ABA increases antioxidant enzyme activity and decreases Pb^{2+} content in shoot and root tissues, alleviating Pb^{2+}-induced oxidative damage (Wang and others 2013a). Moreover, pretreatment with ABA markedly attenuates the inhibitory effect of Cd^{2+} on adventitious rooting in mung bean seedlings (Li and others 2014). However, the underlying mechanisms responsible for the altered Cd^{2+} toxicity remain largely unknown in woody plants.

As a salt-tolerant tree, *Populus euphratica* is usually used as a model species to explore mechanisms involved in plant responses to salinity (Chen and others 2001, 2002a, 2002b, 2003; Sun and others 2009, 2010a; Chen and Polle 2010; Han and others 2013; Ma and others 2013; Chen and others 2014; Polle and Chen 2015). However, *P. euphratica* is sensitive to Cd^{2+} stress, mainly because of a failure to activate early protective responses upon Cd^{2+} exposure (Polle and others 2013). Therefore, *P. euphratica* is considered ideal for investigating the effects of exogenous chemicals on Cd^{2+} detoxification in woody plants (Sun and others 2013). The present work aims to examine the possible role of exogenous ABA in the alleviation of cellular Cd^{2+} toxicity in *P. euphratica*. In this study, the effects of ABA on antioxidant enzyme activity, H_2O_2 accumulation, and Cd^{2+} uptake were investigated in Cd^{2+}-stressed *P. euphratica* cells. In addition, we used a non-invasive ion flux technique to measure the cellular fluxes of Cd^{2+} in *P. euphratica*. The main objective was to examine ABA-induced alternations in ion fluxes in this poplar under cadmium stress.

Materials and Methods

Plant Material and Treatments

P. euphratica callus cells were induced from shoots as described previously (Sun and others 2010a, 2010b, 2013). The cells were grown on a Murashige and Skoog (MS) solid medium (pH 5.7), supplemented with 2.5 % sucrose, 0.25 mg L^{-1} 6-BA (6-benzylaminopurine), and 0.50 mg L^{-1} NAA (1-Naphthaleneacetic acid). Cultures were maintained at 25 °C in the dark and sub-cultured every 3 weeks. Dose tests of $CdCl_2$ and ABA effects on cell growth were examined in this study. *P. euphratica* cells were treated with different concentrations of $CdCl_2$ (0, 25, 50, 100, and 200 μM) supplemented with or without ABA (5 μM; Note: ABA was able to reduce the effects of Cd^{2+} on viability and membrane permeability (MP) at 5 μM, Table 1). Cell growth was reduced by increasing Cd^{2+} concentrations in the medium after 3 weeks of culture (Supplementary Fig. S1). ABA could alleviate Cd^{2+} inhibition of cell growth and the ABA effect was more pronounced when the medium was supplemented with 100 μM $CdCl_2$, as compared to the low (25 and 50 μM) or high doses (200 μM) of $CdCl_2$ (Supplementary Fig. S1). Thus 100 μM $CdCl_2$ was used for the following experiments in this study.

Dose tests of ABA on cell viability and MP were examined in Cd^{2+}-stressed cells. After 15 days of transformation onto a fresh solid MS medium, cell cultures were incubated in a liquid MS medium (LMS) for a 6-h equilibration and then treated with or without $CdCl_2$ (100 μM in LMS) in the absence or presence of ABA (0.5, 5, and 20 μM). Control cells were treated without the addition of $CdCl_2$ or ABA. Cell samples were harvested at 72 h to examine cell viability and MP.

To examine the time course of H_2O_2, antioxidant enzymes (SOD, CAT, and APX) and Cd^{2+} fluxes, *P. euphratica* cultures were exposed to $CdCl_2$ (0 or 100 μM in LMS) supplemented with or without ABA (5 μM). Cells were sampled at 0, 24, 48, and 72 h, and used to measure H_2O_2, antioxidant enzymes activity, and Cd^{2+} fluxes.

Determination of Cell Viability

Cell viability was determined with the fluorescent dye fluorescein diacetate as described by Sun and others (2010b, 2012a, 2012b). Briefly, cell samples were

Table 1 Effect of exogenous ABA on cell viability and membrane permeability (MP) in *P. euphratica* cells under Cd^{2+} stress

Treatment	ABA (0 μM)	ABA (0.5 μM)	ABA (5 μM)	ABA (20 μM)
Cell viability (%)				
$-Cd^{2+}$	97 ± 4 a^A	95 ± 3 a^A	93 ± 3 a^A	87 ± 8 a^A
$+Cd^{2+}$	24 ± 2 b^B	33 ± 2 b^B	50 ± 3 a^B	21 ± 3 b^B
MP (%)				
$-Cd^{2+}$	29 ± 4 b^B	32 ± 1 b^B	31 ± 2 b^B	31 ± 4 b^B
$+Cd^{2+}$	64 ± 1 a^A	62 ± 2 a^A	43 ± 4 b^A	64 ± 6 a^A

Cells were treated with ($+Cd^{2+}$) or without ($-Cd^{2+}$) 100 μM $CdCl_2$ for 72 h in the presence or absence of ABA (0, 0.5, 5, and 20 μM). Each value (±SE) is the mean of four to five independent experiments. For cell viability measurement, at least 500 cells were examined in each experiment. Values followed by different letters in the same row (a, b; Kruskal–Wallis non-parametric test) or the same column (A, B; Mann–Whitney *U* test) are significant differences at $P < 0.01$

harvested and stained with 20 μg mL^{-1} fluorescein diacetate (Sigma-Aldrich, St. Louis, MO, USA) for 5 min in the dark at room temperature. Then the fluorescence of living cells was visualized under a Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) with excitation at 488 nm and emission at 515–530 nm. Cell viability was examined by measuring five randomly selected fields on each slide, and for each field at least 100 cells were analyzed.

Membrane Permeability Measurement

MP was determined in terms of relative conductivity according to Wang and others (2007, 2013b) and Sun and others (2010b). The relative change in the conductivity is due to the release of soluble solutes from the disrupted membranes. In brief, callus cells (0.2 g) were cultured in redistilled water at 25 °C for 2 h, and then the conductivity (C1) was measured. Thereafter, cells were heated at 95 °C for 1 h to determine the total conductivity (C2). The electrical conductivities, C1 and C2, were measured with an electrical conductivity meter (DDSJ-318, LeiCi Co., Shanghai, China).

The MP was calculated as follows:

$$\text{MP}\ (\%) = \text{C1}/\text{C2} \times 100$$

Enzyme Activity Assays

Callus samples (0.2 g) were ground to a fine powder and homogenized with 2 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 % polyvinylpyrrolidone. The homogenate was centrifuged at 13,000 rpm for 20 min at 4 °C, and the supernatant was used for the enzyme assay. Protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo, USA). In the case of APX measurement, 1 mM ascorbic acid was added to the extraction buffer. The total activities of CAT and APX were determined as described previously (Sun and others 2010b, 2013). The activity of SOD was measured using an SOD assay kit A001-3 (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The results of this enzymatic assay were given in units of SOD activity per milligram of protein (U mg^{-1} protein) (Wang and others 2007, 2008), where 1 U of SOD was defined as the amount of enzyme producing 50 % inhibition of a colorimetric reaction between superoxide anion and a water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2*H*-tetrazolium, monosodium salt).

Detection of Cellular H_2O_2

H_2O_2 content was detected using the H_2O_2-sensitive fluorescent probe H_2DCF-DA (2′,7′-Dichlorodihydrofluorescein diacetate, Molecular Probes, Eugene, OR, USA). Callus cells were incubated in 50 μM H_2DCF-DA for 5 min in the dark. Then the cells were fixed on poly-L-lysine-pretreated cover slips and washed 3–4 times with LMS solution. The specific fluorescence of H_2O_2 produced in cells was examined with a Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) with excitation at 488 nm and emission at 500–530 nm. Three-dimensional reconstructed images (maximum) of cells were used to calculate the relative fluorescence intensity using Image-Pro Plus version 6.0 software.

Visualization of Intracellular Cd^{2+} Levels

Cell cultures were exposed to 0 or 100 μM $CdCl_2$ for 72 h in the absence or presence of 5 μM ABA. A Cd-specific fluorescent dye, Leadmium™ Green AM, was used to detect Cd^{2+} within cells (Sun and others 2013; Han and others 2014). The stock solution of Leadmium™ Green AM was prepared by adding 50 μL dimethyl sulfoxide to

the dye. Then the stock solution was diluted 1:20 with 0.85 % NaCl. Cells were stained with Leadmium™ Green AM for 1 h in the dark and then washed three times with 0.85 % NaCl. The fluorescence of cells was visualized under a Leica SP5 confocal microscope with excitation at 488 nm and emission at 505–530 nm. The relative fluorescence intensity was calculated with Image-Pro Plus version 6.0 software.

Measurement of Cd^{2+} Content

P. euphratica cells were treated with 100 μM $CdCl_2$ for 3 weeks in the absence or presence of 5 μM ABA. Then callus cells were sampled and dried at 60 °C for 48 h. Dry samples (0.1 g) were digested with $HNO_3/HClO_4$ (85/15, v/v), and Cd^{2+} concentration was measured using an inductively coupled plasma optical emission spectrometer (OPTIMA 2000; PerkinElmer, USA).

Cd^{2+} Flux Recordings

Net Cd^{2+} flux was measured non-invasively using the non-invasive micro-test technique (NMT; NMT-YG-100, Younger USA LLC, Amherst, MA01002, USA). The protocols for the preparation of Cd^{2+}-selective electrodes were followed as described by Sun and others (2013) and Han and others (2014) with modifications. Briefly, glass micropipettes (XYPG120-2; Xuyue Sci. and Tech. Co., Ltd.) with an external tip diameter of 2–4 μm were pre-pulled and silanized with tributylchlorosilane. The micropipettes were filled with a backfilling solution (10 mmol/L $Cd(NO_3)_2$ + 0.1 mmol/L KCl) and then front filled with a commercially available ion-selective cocktail (Cadmium Ionophore I, 20909, Sigma-Aldrich, St. Louis, MO, USA). An Ag/AgCl wire electrode holder (XYEH01-1; Xuyue Sci. and Tech. Co., Ltd.) was inserted into the back of the electrode to produce an electrical contact with the electrolyte solution. DRIREF-2 (World Precision Instruments) was used as the reference electrode (CMC-4). The microelectrodes were calibrated in 0, 5, 10, and 50 μM $CdCl_2$ solution prior to the net Cd^{2+} flux measurements. Only electrodes with Nernstian slopes >25 mV/decade were used. The ion flux was calculated using Fick's law of diffusion:

$$J = -D(dc/dx)$$

where J represents the ion flux in the x direction, dc/dx is the ion concentration gradient, and D is the ion diffusion constant in a particular medium. The flux data were acquired with the ASET software, which is part of the NMT system, and calculated using MageFlux developed by the XuYue company (http://xuyue.net/mageflux).

Steady-State Cd^{2+} Flux Recording

P. euphratica cells were exposed to 0 or 100 μM $CdCl_2$ for 0, 24, 48, and 72 h in the absence or presence of 5 μM ABA. Prior to flux measurement, cells were immobilized in the measuring solution (0 or 100 μM $CdCl_2$, 50 μM $CaCl_2$, pH 5.5) and equilibrated for 10 min. Then the steady-state Cd^{2+} flux was recorded for 10 min in each cell.

In addition, the effect of ABA pretreatment on Cd^{2+} flux was examined in this study. *P. euphratica* cells were pretreated with or without 5 μM ABA for 12 h, and then exposed to 0 or 100 μM $CdCl_2$ for 72 h. Then the steady-state Cd^{2+} flux was measured with NMT.

Transient Cd^{2+} Flux Recording

Cells were pretreated with 5 μM ABA, 3 mM H_2O_2, or 5 μM ABA plus 3 mM H_2O_2 for 6 h, and then immobilized in the measuring solution (50 μM $CaCl_2$ supplemented with or without 5 mM $LaCl_3$, pH 5.5). Cd^{2+} fluxes were continuously recorded for 10 min prior to the Cd^{2+} treatment. A stock solution of $CdCl_2$ (100 mM) was slowly added to the measuring solution until the final concentration in the solution reached 100 μM, followed by continuous recording of the Cd^{2+} flux for 30 min. The data measured during the first 2–3 min were discarded because of the diffusion effects of the stock addition.

Data Analysis

Statistical analyses were performed using SPSS version 17.0 software. Unless otherwise stated, differences were considered statistically significant when $P < 0.05$. Non-parametric statistics were used to analyze the percentage data of cell viability and MP between two independent groups (Mann–Whitney U test) or among multiple groups (Kruskal–Wallis test).

Results

ABA Alleviated Cd^{2+} Toxicity in *P. euphratica* Cells

In this study, cell viability and MP (an indicator of lipid peroxidation) were examined to quantify the Cd^{2+}-induced toxicity in *P. euphratica* cells. $CdCl_2$ (100 μM, 72 h) caused a significant decrease in cell viability but markedly increased MP (Table 1). This indicates that Cd^{2+} treatment resulted in a distorted and disrupted membrane, leading to an increased release of intracellular solutes and thus decreased cell viability (Wang and others 2007, 2013b; Sun and others 2010b). Of note, exogenously applied ABA reduced the effects of Cd^{2+} on viability (by 10–29 %) and MP (by 15–82 %), and the effects were more pronounced

at 5 μM compared to the low (0.5 μM) or high dose (20 μM) (Table 1). Treatments with ABA (0.5–20 μM) had no obvious effect on either viability or MP in the absence of Cd^{2+} (Table 1). Furthermore, our results showed that ABA could maintain cell growth under Cd^{2+} stress. As shown in Fig. 1, upon 3 weeks of 100 μM $CdCl_2$ exposure, *P. euphratica* cells treated with ABA (5 μM) exhibited 83 % higher fresh weight than non-ABA-treated cells. Collectively, these results indicated that ABA could alleviate Cd^{2+} toxicity in *P. euphratica* cells.

ABA Increased Antioxidant Enzyme Activity and Reduced H_2O_2 Accumulation under Cd^{2+} Stress

To examine the effect of exogenous ABA on antioxidant enzymes, we measured CAT, SOD, and APX activities in *P. euphratica* cells after 0, 24, 48, and 72 h of Cd^{2+} stress.

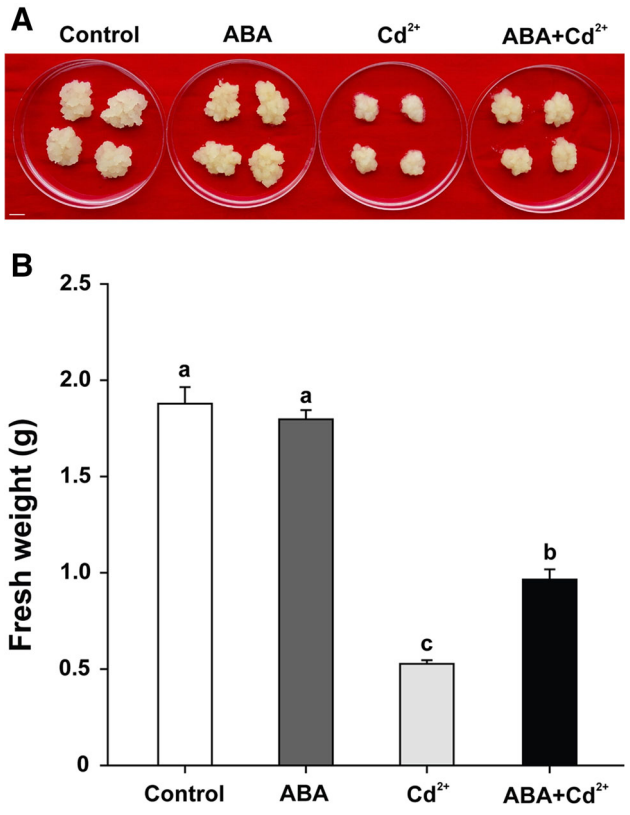

Fig. 1 Effect of exogenous ABA on cell growth of *P. euphratica* under Cd^{2+} stress. Cells were treated with or without $CdCl_2$ (100 μM) for 3 weeks in the presence or absence of 5 μM ABA. **a** Representative images showing cell performance. **b** Fresh weight of cells. Each *column* is the mean of four to five biologically independent samples, and *bars* represent the standard error of the mean. *Columns* labeled with different *letters* (a, b, c) indicate significantly differences at $P < 0.05$. *Scale bar* 1 cm

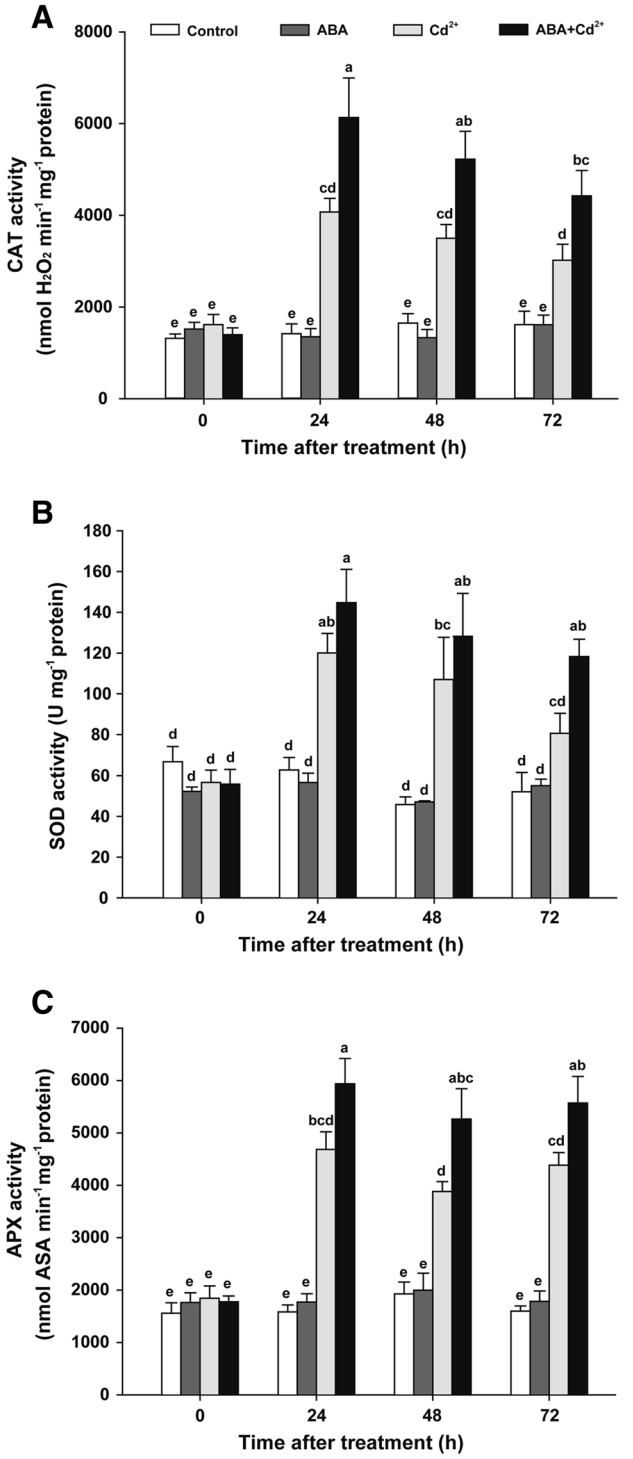

Fig. 2 Effect of ABA on activity of antioxidant enzymes in *P. euphratica* cells under Cd^{2+} stress. **a** CAT; **b** SOD; **c** APX. Cells were subjected to 100 μM $CdCl_2$ for 0, 24, 48, and 72 h in the presence or absence of 5 μM ABA. Each *column* is the mean of three to four biologically independent samples, and *bars* represent the standard error of the mean. *Columns* labeled with different *letters* (a, b, c, d, e) denote significantly differences at $P < 0.05$

There was no significant difference in antioxidant activities when ABA and Cd^{2+} treatments were initiated (0 h, Fig. 2a–c). $CdCl_2$ (100 μM) stress accelerated the activities of the three enzymes at 24, 48, and 72 h (Fig. 2a–c). It was notable that ABA increased the activity of CAT and APX in Cd^{2+}-stressed *P. euphratica* cells at the three time points (24, 48, and 72 h) (Fig. 2a, c). ABA + Cd^{2+} treatment resulted in significantly higher SOD activity than Cd^{2+} treatment at 72 h, although their activities were similar at 24 and 48 h (Fig. 2b). Compared with control cells, ABA alone had no significant effect on the activities of the three antioxidant enzymes in the absence of Cd^{2+} stress (Fig. 2a–c).

In addition, a specific H_2O_2 probe, H_2DCF-DA, was used to detect H_2O_2 accumulation in *P. euphratica* cells after 0, 24, 48, and 72 h of Cd^{2+} stress. *P. euphratica* cells exhibited very low H_2O_2 level at the initiation of ABA and Cd^{2+} treatments (0 h, Fig. 3). $CdCl_2$ stress resulted in a marked elevation of H_2O_2 and the H_2O_2 increased with the period of Cd^{2+} exposure (24–72 h, Fig. 3). Of note, ABA application (5 μM) markedly decreased Cd^{2+}-induced H_2O_2 production over the observation time, 24, 48, and 72 h (Fig. 3). These results suggested that ABA could enhance the activity of antioxidant enzymes and decrease the level of H_2O_2 in Cd^{2+}-stressed *P. euphratica* cells.

ABA Reduced Cd^{2+} Accumulation in *P. euphratica* Cells Under Cd^{2+} Stress

In the present study, a Cd^{2+}-sensitive fluorescent probe, Leadmium™ Green, was used to monitor Cd^{2+} accumulation in *P. euphratica* cells after 72 h of Cd^{2+} treatment. $CdCl_2$ stress (100 μM) caused evident Cd^{2+}-specific fluorescence in *P. euphratica* cells, whereas Cd^{2+}-specific fluorescence was nearly undetectable in control cells (Fig. 4). Of note, under Cd^{2+} stress, the ABA-treated *P. euphratica* cells exhibited 33 % less fluorescence intensity compared with the cells treated without ABA (Fig. 4).

The Cd^{2+} content was also determined in *P. euphratica* cells after long-term exposure to 100 μM $CdCl_2$ (3 weeks). The Cd^{2+} concentration in the ABA-treated cells was 16 % less than in cells treated without ABA (Fig. 5). This result was in accord with the measurements with the Leadmium™ Green probe, suggesting that exogenous ABA application could decrease Cd^{2+} accumulation within *P. euphratica* cells under Cd^{2+} stress.

ABA Reduced Cd^{2+} Influx in Cd^{2+}-Stressed *P. euphratica* Cells

To determine whether the reduced accumulation of Cd^{2+} was the result of a reduction in Cd^{2+} uptake in *P.*

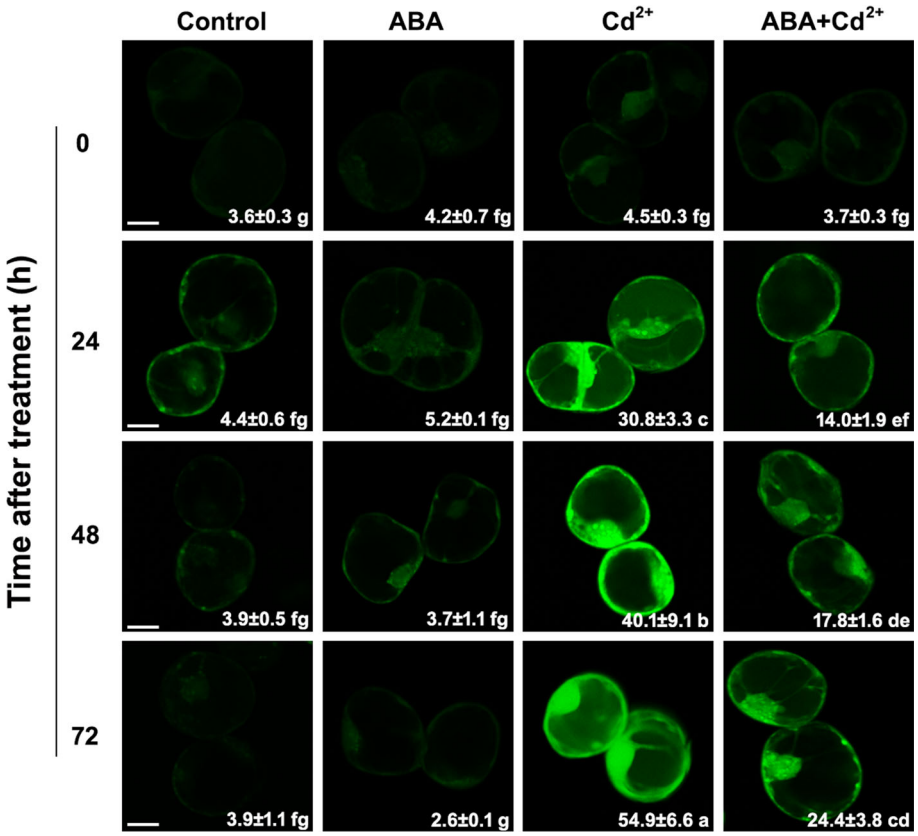

Fig. 3 Effect of exogenous ABA on H_2O_2 accumulation in *P. euphratica* cells under Cd^{2+} stress. Cells were subjected to 100 μM $CdCl_2$ with or without 5 μM ABA for 0, 24, 48, and 72 h and then stained with the H_2O_2-sensitive fluorescent probe H_2DCF-DA. Representative images show H_2O_2 production (green fluorescence) in *P. euphratica* cells. Each value (±standard error) is the mean of three to four biologically independent experiments, and 80–100 individual cells were quantified for each treatment. The mean fluorescence values labeled with different *letters* are significant differences at $P < 0.05$. *Scale bar* 10 μm

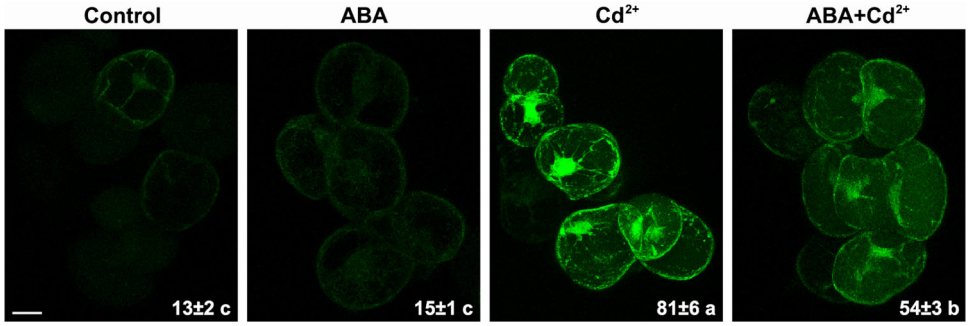

Fig. 4 Effect of exogenous ABA on Cd^{2+} levels within *P. euphratica* cells under Cd^{2+} stress. *P. euphratica* cells were treated with 100 μM $CdCl_2$ for 72 h in the presence or absence of ABA (5 μM) and then incubated with a Cd^{2+}-fluorescent probe (Leadmium™ Green AM) for 1 h. Each value represents the mean of at least 50 individual cells quantified from three to four biologically independent experiments. The mean fluorescence values labeled with different *letters*, a, b, and c, are significant differences at $P < 0.05$. *Scale bar* 10 μm

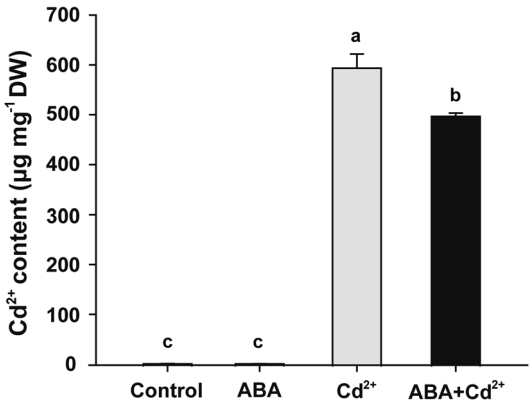

Fig. 5 Effect of exogenous ABA on Cd^{2+} content in *P. euphratica* cells under Cd^{2+} stress. Cells were subjected to 100 μM $CdCl_2$ in the presence or absence of 5 μM ABA for 3 weeks. Each *column* represents the mean of three experiments, and *bars* indicate the standard error of the mean. Different *letters* (a, b, c) denote significantly differences at $P < 0.05$

euphratica cells, we measured the net Cd^{2+} fluxes with NMT. Cd^{2+} flux in *P. euphratica* cells was extremely low or under detection limit before the addition of $CdCl_2$ (100 μM) (0 h; Fig. 6). After exposure to Cd^{2+} for 24–72 h, the steady Cd^{2+} influx was remarkably enhanced (Fig. 6). It was notable that the Cd^{2+} influx increased with the period of Cd^{2+} exposure, reaching 33.7 pmol cm^{-2} s^{-1} at 72 h (Fig. 6). However, application of ABA (5 μM) reduced the Cd^{2+} influx by 56–79 % in $CdCl_2$ (100 μM)-treated cells over the observation periods (24–72 h; Fig. 6).

The effect of ABA pretreatment on steady-state Cd^{2+} flux was examined in this study. *P. euphratica* cells were pretreated with or without 5 μM ABA for 12 h, and then exposed to 0 or 100 μM $CdCl_2$ for another 72 h. ABA pretreatment could significantly inhibit Cd^{2+} uptake in $CdCl_2$-stressed cells (Supplementary Fig. S2). This is similar to the finding when ABA and Cd^{2+} were applied together (Fig. 6). However, the ABA inhibition of Cd^{2+} influx was lower than the concomitant application of Cd^{2+} and ABA (Fig. 6, Supplementary Fig. S2).

The effects of ABA, H_2O_2, and Ca^{2+}-channel inhibitors on transient Cd^{2+} flux were also investigated. *P. euphratica* cells were pretreated with ABA or H_2O_2 for 6 h and then exposed to $CdCl_2$ shock to measure Cd^{2+} flux in the presence or absence of $LaCl_3$ (an inhibitor of Ca^{2+}-permeable channels). ABA pretreatment reduced Cd^{2+} influx by 86 % in $CdCl_2$-stressed cells (Fig. 7). Of note, H_2O_2 pretreatment (3 mM) increased the entry of Cd^{2+} into *P. euphratica* cells (the mean value reached 35.2 pmol cm^{-2} s^{-1}) (Fig. 7); however, ABA significantly reduced H_2O_2-elicited Cd^{2+} influx (Fig. 7). Additionally, in the presence of $LaCl_3$, Cd^{2+} entry was markedly blocked in $CdCl_2$-shocked cells, irrespective of H_2O_2 and ABA pretreatments (Fig. 7).

Discussion

Excessive Cd^{2+} can induce DNA fragmentation, chromatin condensation, and loss of cell viability in woody (Sun and others 2013) and herbaceous species (Iakimova and others 2008; Ma and others 2010), leading to programmed cell death (Iakimova and others 2008; Ma and others 2010; Sun and others 2013). In this study, cellular and subcellular ion analyses revealed that short-term (72 h) or prolonged Cd^{2+} exposure (3 weeks, 100 μM) resulted in evident Cd^{2+} accumulation within *P. euphratica* cells (Figs. 4, 5). This result agrees with those of a previous report (Sun and others 2013). The Cd^{2+} buildup, especially in the cytoplasmic region, led to a significant increase in DCF-dependent fluorescence, indicating an H_2O_2 burst in *P. euphratica* cells (Fig. 3). This Cd^{2+}-elicited H_2O_2 burst has been suggested to contribute to oxidative damage (DalCorso and others 2008) and the occurrence of programmed cell death in *P. euphratica* cells (Sun and others

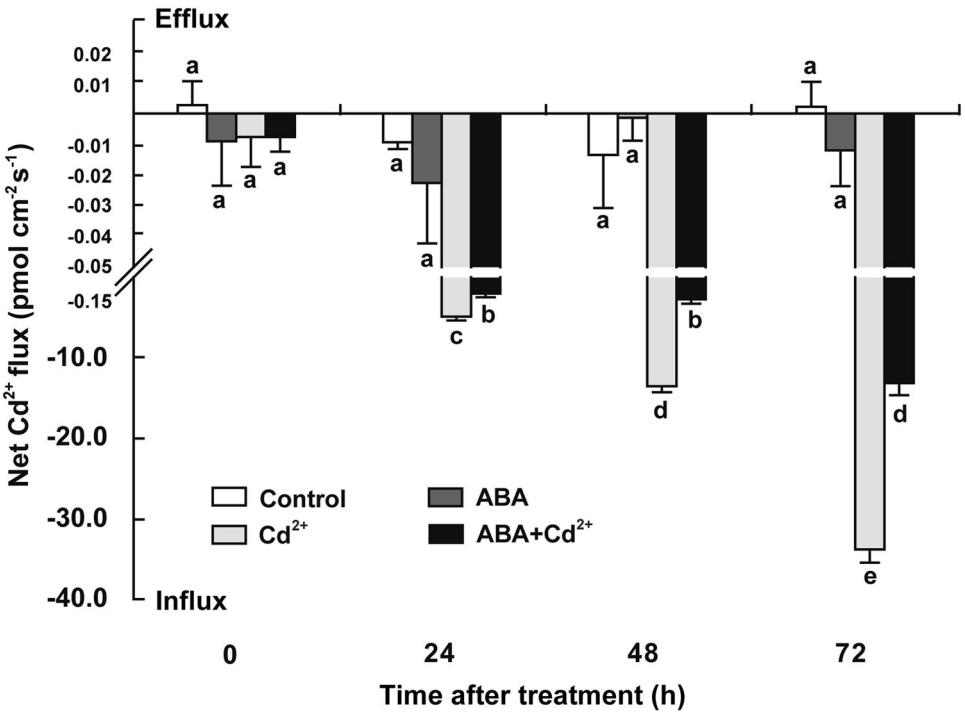

Fig. 6 Effect of exogenous ABA on steady-state Cd^{2+} fluxes in *P. euphratica* cells under Cd^{2+} stress. Cells were treated with 100 μM $CdCl_2$ for 0, 24, 48, and 72 h in the presence or absence of 5 μM ABA. Steady-state Cd^{2+} fluxes were continuously recorded for 10 min for each cell. Each *column* represents the mean of 15 individual cells quantified from three to four biologically independent samples. *Bars* indicate the standard error of the mean. Different *letters* (a, b, c, d, e) denote significantly differences at $P < 0.05$

2013). In the present study, ABA reduced Cd^{2+} suppression of cell growth and viability in *P. euphratica* cells, although the alleviation of Cd^{2+} toxicity was dose dependent (Fig. 1, Table 1, Supplementary Fig. S1). Exogenous ABA application has been reported to reduce Cd^{2+} toxicity in herbaceous plant species, such as rice and mung bean seedlings (Hsu and Kao 2003, 2005; Li and others 2014). Our data showed that ABA alleviation of Cd^{2+} toxicity mainly resulted from the restriction of Cd^{2+} under Cd^{2+} stress (Figs. 4, 5). Similarly, Hsu and Kao (2003) showed that ABA pretreatment reduces Cd^{2+} content in $CdCl_2$-stressed rice seedlings.

The NMT data indicated that the Cd^{2+} restriction in ABA-treated *P. euphratica* cells was the result of a reduced Cd^{2+} influx. Steady-state and transient flux recordings showed a net Cd^{2+} influx in Cd^{2+}-shocked and short-term stressed *P. euphratica* cells (Figs. 6, 7). Moreover, the steady Cd^{2+} influx increased with increasing exposure time to Cd^{2+} stress (24–72 h; Fig. 6). ABA application significantly decreased the Cd^{2+} influx during the observation periods (24–72 h; Fig. 6). Similarly, ABA pretreatment could significantly inhibit Cd^{2+} uptake in $CdCl_2$-stressed cells (Supplementary Fig. S2). However, the ABA inhibition of Cd^{2+} influx was lower than the concomitant application of Cd^{2+} and ABA (Fig. 6, Supplementary Fig. S2). This is likely due to the difference in the duration of ABA treatment, 12 h (ABA pretreatment) versus 72 h (Cd^{2+}+ABA). ABA may not interfere with the uptake of Cd^{2+} as the ABA concentration was 5 μM, which is much lower than the applied Cd^{2+}, 100 μM.

In pharmacological experiments, the Cd^{2+} influx was blocked by the PM Ca^{2+} channel inhibitor $LaCl_3$, indicating that the Cd^{2+}-elicited Cd^{2+} influx was through the PM Ca^{2+} channels (Fig. 7). This finding is in agreement with Sun and others (2013), who suggested that the Cd^{2+} influx into the cytosol is mediated by PM Ca^{2+}-permeable channels (Sun and others 2013). Electrophysiological evidence revealed that Cd^{2+} ions can be transported into cells through Ca^{2+} channels. Using the whole-cell patch-clamp technique, Perfus-Barbeoch and others (2002) have confirmed that Cd^{2+} permeates through the PM calcium channels in *Arabidopsis* guard cells. The permeability of Cd^{2+} through wheat voltage-dependent calcium channels was detected when the PM derived from root cells was incorporated into planar lipid bilayers (White 1998). Of note, H_2O_2 application increased the net Cd^{2+} influx into *P. euphratica* cells (Fig. 7). Moreover, the Cd^{2+} influx increased with the rise of endogenous H_2O_2 accumulation in Cd^{2+}-stressed cells (24–72 h; Figs. 3, 6). Therefore, the Cd^{2+}-induced H_2O_2 production is thought to stimulate the entry of Cd^{2+} into Cd^{2+}-stressed *P. euphratica* cells. In accordance, Sun and others (2013) also showed that H_2O_2 addition enhances an immediate Cd^{2+} influx in *P. euphratica* cells.

ABA significantly reduced the influx of Cd^{2+} in *P. euphratica* cells (Fig. 6). This is presumably related to the increased activity of antioxidant enzymes, CAT, SOD, and APX (24–72 h; Figs. 2, 3). The activated antioxidant enzymes benefited *P. euphratica* cells to scavenge the Cd^{2+}-elicited H_2O_2, and thus limited the H_2O_2-stimulated

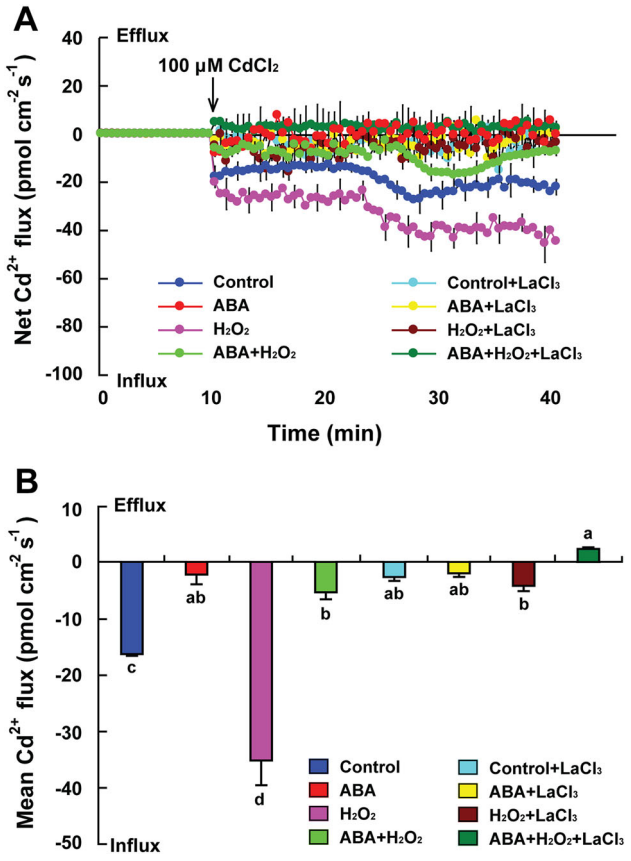

Fig. 7 Effect of ABA, H_2O_2, and PM Ca^{2+} channel inhibitor ($LaCl_3$) on transient Cd^{2+} fluxes in *P. euphratica* cells. **a** Transient Cd^{2+} kinetics. Cells were pretreated with or without ABA (5 μM) in the presence or absence of H_2O_2 (3 mM) for 6 h. Prior to the Cd^{2+} shock, a steady Cd^{2+} flux was continuously recorded for 10 min. Cd^{2+} fluxes in ABA- and/or H_2O_2-pretreated cells were measured in the presence or absence of $LaCl_3$ (an inhibitor of Ca^{2+}-permeable channels; 5 mM). Each *point* is the mean of six individual cells, and *bars* represent the standard error. The mean fluxes of Cd^{2+} after the addition of $CdCl_2$ are shown in (**b**). Different *letters* (a, b, c, d) denote significantly differences at $P < 0.05$

Cd^{2+} influx in a long term of stress. We hypothesize that ABA stimulated the initial generation of ROS, which up-regulated the activities of antioxidant enzymes. It has shown that ABA triggers the increased generation of ROS and activates antioxidant enzymes in water-stressed maize leaves (Jiang and Zhang 2002). Our data showed that enzyme activities of CAT, SOD, and APX increased correspondingly to the rise of H_2O_2 during the period of Cd^{2+} stress (24–72 h; Figs. 2, 3). It is likely that the Cd^{2+}-elicited H_2O_2 up-regulated the activities of antioxidant enzymes. It has shown that H_2O_2 acted as a signaling molecule in the activation of CAT and APX in scot pine roots under Cd^{2+} stress (Schützendübel and others 2001). Collectively, ABA application significantly enhanced these enzyme activities, which contributed to the scavenging of

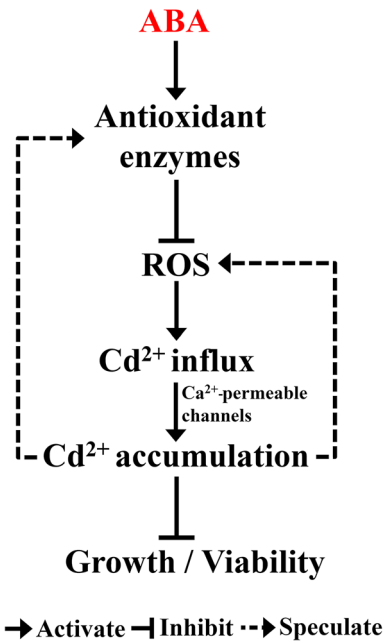

Fig. 8 A schematic model of ABA alleviation of Cd^{2+} toxicity in *P. euphratica* cells. *Solid arrows* indicate a promotion effect, a *bar* indicates a repression effect, and *dotted arrows* indicate an unconfirmed signaling cascade

H_2O_2 under Cd^{2+} stress (Figs. 2, 3). As a result, the H_2O_2-stimulated entry of Cd^{2+} was inhibited by ABA in Cd^{2+}-stressed *P. euphratica* (Figs. 6, 7).

In conclusion, ABA plays a crucial role in alleviating Cd^{2+} toxicity in *P. euphratica* cells under Cd^{2+} stress conditions. We postulate the following model of ABA signaling in Cd^{2+} toxicity alleviation in this poplar (Fig. 8): In Cd^{2+}-stressed *P. euphratica*, ABA up-regulates the activity of antioxidant enzymes, which scavenge the Cd^{2+}-elicited H_2O_2 within cells. As a result, the entry of Cd^{2+} is subsequently reduced because H_2O_2 otherwise would stimulate Cd^{2+} influx through the PM Ca^{2+} channels in *P. euphratica*. The buildup of Cd^{2+} and Cd^{2+}-elicited oxidative damage thus is limited in ABA-treated *P. euphratica*.

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