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Cadmium interferes with maintenance of auxin homeostasis in *Arabidopsis* seedlings

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ABSTRACT

Auxin and its homeostasis play key roles in many aspects of plant growth and development. Cadmium (Cd) is a phytotoxic heavy metal and its inhibitory effects on plant growth and development have been extensively studied. However, the underlying molecular mechanism of the effects of Cd stress on auxin homeostasis is still unclear. In the present study, we found that the root elongation, shoot weight, hypocotyl length and chlorophyll content in wild-type (WT) *Arabidopsis* seedlings were significantly reduced after exposure to Cd stress. However, the lateral root (LR) formation was markedly promoted by Cd stress. The level and distribution of auxin were both greatly altered in primary root tips and cotyledons of Cd-treated plants. The results also showed that after Cd treatment, the IAA content was significantly decreased, which was accompanied by increases in the activity of the IAA oxidase and alteration in the expression of several putative auxin biosynthetic and catabolic genes. Application of the auxin transport inhibitor, 1-naphthylphthalamic acid (NPA) and 1-naphthoxyacetic acid (1-NOA), reversed the effects of Cd on LR formation. Additionally, there was less promotion of LR formation by Cd treatment in *aux1-7* and *pin2* mutants than that in the WT. Meanwhile, Cd stress also altered the expression of *PINs* and *AUX1* in *Arabidopsis* roots, implying that the auxin transport pathway is required for Cd-modulated LR development. Taken together, these findings suggest that Cd stress disturbs auxin homeostasis through affecting auxin level, distribution, metabolism, and transport in *Arabidopsis* seedling.

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Introduction

Heavy metals, as products of human activities (mainly industrial processes and the use of phosphate fertilizers in agriculture), cause serious environmental pollution. Cd, a nonessential element, is one of the most aggressive and persistent heavy metal elements in natural environments. Compared with other heavy metals, Cd has a higher tendency to accumulate in edible parts of crops which subsequently enter into the food chain and lead to the biomagnification and high threats to humans and animals (Wagner, 1993; Arao et al., 2003; Ishikawa et al., 2009). Cd is also very toxic to most plants even at low concentrations. It is absorbed rapidly by roots and is loaded into the xylem for transport into plant aerial parts, causing a general growth inhibition and numerous physiological and metabolic disturbances (Sanità di Toppi and Gabbrielli, 1999; Benavides et al., 2005; Ishikawa et al., 2009). Many studies have shown that the characteristic general symptoms of Cd toxicity are root and leaf growth reduction, cell death, leaf roll and chlorosis, alterations in respiration, photosynthesis and gas exchange, competition with the uptake, transport and use of nutrients and water, disturbance in plant antioxidant defenses, generation of oxidative stress and lipid peroxidation, damage of the cell membrane, inhibition of enzyme activities, disruption of cell transport processes, and disturbance of cellular redox control (Sanità di Toppi and Gabbrielli, 1999; Benavides et al., 2005; Romero-Puertas et al., 2007; Rodriguez-Serrano et al., 2009; Sharma and Dietz, 2009). The mechanisms of specific responses to Cd stress in plants are less clear.

Phytohormone auxin affects several processes throughout the life cycle of plants, including pattern formation during embryogenesis, LR formation, vascular patterning and tropism, and at the cellular level, cell division, expansion, and differentiation (Woodward and Bartel, 2005). Optimal plant growth requires tight control of IAA level, which is accomplished by diverse mechanisms that include IAA biosynthesis, transport among tissues, cycling between active and inactive forms of IAA, and signal perception

Abbreviations: Cd, cadmium; ELISA, enzyme-linked immunosorbent assay; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; IPA, indole-3-pyruvic acid; LR, later root; LRP, later root primordium; MS, Murashige and Skoog; 1-NOA, 1-naphthoxyacetic acid; NIT, nitrilases; NPA, 1-naphthylphthalamic acid; PIN, PIN-FORMED; ROS, reaction oxygen species; TAA, aminotransferase; Trp, tryptophan; WT, wild-type.

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through a family of auxin receptors (Vanneste and Friml, 2009). Auxin is mainly synthesized in young aerial tissues and roots, particularly in the meristematic primary root tips (Normanly, 2010). The prevalent form of auxin, IAA, is synthesized from indole precursors via the four main accepted branch routes of tryptophan (Trp)-dependent synthesis or through Trp-independent pathways (Normanly, 2010). Plants can transfer IAA from source tissues to the root and shoot tips and other sink tissues, which require the asymmetric cellular localization of auxin influx and efflux components of the AUX1/LAX, ABCB/PGP/MDR, and PIN-FORMED (PIN) family (Vanneste and Friml, 2009). Once auxin arrives at destination, it binds to a pocket of the TIR1 auxin receptor, an F-box component of the E3 ubiquitin ligase complex SCF^{TIR1} (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Auxin binding strengthens the interaction between TIR1 and the Aux/IAA protein targets for ubiquitination (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). The degradation of Aux/IAA transcriptional repressors by the 26S proteasome then leads to auxin-dependent gene transcription and auxin responses.

Auxin homeostasis results from multiple dynamic and continuous adjustments of regulatory mechanisms and aims at maintaining a fairly stable internal equilibrium for effective plant growth and development. Auxin homeostasis is often disturbed by different stress factors such as salinity, osmolarity, drought, cold, wounds, metals stress, and mechanical damage (Potters et al., 2007, 2009; Tognetti et al., 2010), resulting in alteration of growth and development in plants. However, plants employ significant development plasticity to respond to environmental changes. The stress-induced morphogenetic responses are considered to be part of a general acclimation strategy that prevents or attenuates the deleterious effects of environmental stresses. The alterations in auxin homeostasis induced by environmental stresses can be partly responsible for morphological responses (Pasternak et al., 2005; Potters et al., 2007, 2009; Tognetti et al., 2010). It has been reported that wounding and pathogen attack can affect auxin biosynthesis through Trp-dependent or independent IAA biosynthesis pathways (Ljung et al., 2002). Peto et al. (2011) found that excess Cu^{2+} can affect auxin levels in primary root apices and cotyledons of *Arabidopsis*. Auxin is degraded by oxidizing IAA via elevation of IAA oxidase in tobacco and duckweed species under UV stress (Jansen et al., 2001) or in pea leaves under copper treatment (Chaoui and Ferjani, 2005). The changes in levels of auxin conjugation were also found under salinity, osmotic, drought, cold, and heat stress (Junghans et al., 2006; Park et al., 2007; Tognetti et al., 2010). Environmental stresses can also impact the auxin homeostasis via auxin redistribution and transport, which involves *PIN* gene expression and polar location of auxin carrier proteins. Early studies showed that oxidative stress caused by alloxan reduced the levels of PIN3 and PIN1 genes (Pasternak et al., 2005). In *Arabidopsis*, the expression of auxin efflux carrier was suppressed by salt stress at transcriptional and post-transcriptional levels, which led to changes of the root system architecture (Sun et al., 2008; Wang et al., 2009). Moreover, aluminum-caused inhibition of *Arabidopsis* root growth was shown to result from the altered auxin distribution in roots via effects on the expression of PIN genes (Sun et al., 2010). Li et al. (2011) demonstrated that shoot-supplied ammonium affects root architecture in *Arabidopsis* by interfering with AUX1-dependent auxin transport from shoot to root. Other evidence indicated that auxin homeostasis is changed in *Arabidopsis* roots under cold stress, and this is associated with impacts on intracelluar trafficking of PIN2 and PIN3 proteins, leading to inhibition of the auxin transport (Shibasaki et al., 2009). However, there are fewer reports about how heavy metals, especially Cd, affect auxin homeostasis.

Previously, it was found that Cd stress induced elevation of IAA oxidase activity in *Arabidopsis* and pea leaves, which mediates the endogenous level of IAA by acting on auxin catabolism through oxidative decarboxylation (Chaoui and Ferjani, 2005; Xu et al., 2010a). In addition, exogenous NO can alleviate auxin degradation by decreasing the IAA oxidase activity (Xu et al., 2010b). Recently, it was discovered that Cd stress can interfere with the metabolism of auxin, which triggered increases in the activity of GH3 enzymes in poplars (Elobeid et al., 2012). However, despite the increasing knowledge of the auxin physiology response to Cd stress, several key points remain unclear. The aim of this study is to investigate the detailed changes of auxin homeostasis, including auxin levels, distribution, metabolism, and transport in *Arabidopsis* seedling under Cd stress, and to further clarify the relationship between auxin homeostasis and stress-induced morphogenesis responses when exposed to Cd stress.

Materials and methods

Plant materials and culture conditions

This study was carried out on *Arabidopsis thaliana* including WT ecotypes Columbia (Col-0) and the transgenic lines *DR5::GUS* (Ulmasov et al., 1997), and the mutants *aux1-7* (Pickett et al., 1990), *pin2* (Muller et al., 1998). In order to investigate auxin changes, *DR5::GUS Arabidopsis* reporter line, which is generally recognized as an indirect indicator of level or distribution of auxin (Benková et al., 2003; Xu et al., 2010b; Peto et al., 2011), is a useful tool. Seeds were surface sterilized with 70% ethanol for 30 s and 15% sodium hypochlorite for 15 min, and washed five times with sterilized water before sowing on solid 1/2 Murashige and Skoog (MS) medium (pH 5.7) containing 3% (w/v) sucrose, 1% (w/v) agar. After stratification on 1/2 MS medium for 2–4 days at 4 °C, seeds were placed to germinate under controlled growth conditions: 21 °C temperature, 55–60% relative humidity, 180 μmol photon m^{-2} s^{-1} light intensity, and 16/8 h photoperiod. Unless otherwise stated, Cd treatment was performed as follows: for transfer experiments, Col-0, the transgenic lines *DR5::GUS* and mutants *aux1-7* and *pin2* plants were grown for 5 days and then transferred to 1/2 MS agar medium containing $CdSO_4$ (single concentration of Cd was at 50 μM, except in concentration dependence experiments, where concentrations were 0 μM, 12.5 μM, 25 μM, 50 μM, and 75 μM) for indicated times. For the combination treatments, 5-day-old *Arabidopsis* seedlings were treated with 50 μM Cd in the presence or absence of NPA (10 μM), or 1-NOA (10 μM) for another 5 days. At least 20 seedlings were used for each treatment.

Morphology measurements

The length of primary root was recorded before and after 5 days of Cd treatment by Nikon digital camera D60 and analyzed by NIH Image software (Image J, version 1.43). The number of LR (longer than 0.5 mm in length) was counted. The density of LR was determined as the total LR number divided by the primary root length. Lateral root primordia (LRP) were classified and counted according to their stage of development by using the methods and nomenclature described in Malamy and Benfey (1997). The emerged but shorter than 0.5 mm LR is also called LRP, and the density of LRP was determined by counting the LRPs per seedlings. Hypocotyl length of seedlings after 5 days of Cd treatments was measured under a microscope at 5× magnification. For each treatment, at least 20 seedlings were used for morphology measurements. These experiments were repeated three times.

Histochemical analyses

Histochemical assay of the GUS activity was performed as described by Jefferson et al. (1987) with minor modifications. *DR5::GUS* seedlings were collected after different treatments and

then incubated in GUS-staining buffer containing 1 mM X-Gluc, 100 mM sodium phosphate (pH 7.5), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA and 0.1% Triton X-100 for 12 h at 37 °C in the dark. Stained seedlings were cleared by the method of Malamy and Benfey (1997). Briefly, the stained seedlings were transferred to small Petri dishes containing 0.24 N HCl in 20% methanol and incubated on a 57 °C heat block for 15 min. This solution was replaced with another solution containing 7% NaOH, 7% hydroxylamine–HCl in 60% ethanol for 15 min at room temperature. Seedlings were then rehydrated for 5 min in 40%, 20%, and 10% ethanol solutions, respectively, and infiltrated for 15 min in a solution containing 5% ethanol and 25% glycerol. Individual seedlings were mounted in 50% glycerol on microscopic glass slides and were photographed under the Leica CME compound microscope. At least 20 seedlings for each treatment were analyzed and the experiments were repeated three times.

Determination of the shoot fresh weight and the chlorophyll content

Five-day-old plants of approximately uniform size were selected and transferred to 1/2 MS media containing the indicated Cd concentrations. After 5 days of treatment, fifty plants were harvested, and the fresh weight of the shoots was measured immediately. The chlorophyll was extracted and measured according to the procedures of Porra et al. (1989). A 0.2 g sample of fresh leaves was ground to powder in liquid nitrogen and then placed in 5 mL acetone (80%) and incubated at 4 °C in the dark until the leaf powder was colorless. Absorbance was measured at 647 and 664 nm after centrifugation at 10,000 rpm for 10 min, and total chlorophyll content was calculated. At least three biological replicates were analyzed for each treatment.

Determination of the IAA oxidase activity

The IAA oxidase activity was determined according to Xu et al. (2010b). In order to clearly observe the alteration of IAA oxidase activity under Cd stress, 5-day-old WT seedlings were subjected to different concentrations of Cd (12.5, 25, 50 and 75 μM) for 72 h or to 50 μM Cd for different time (12, 24, 48 and 72 h). Then, fresh seedlings (0.5 g) were harvested and ground in 5 mL of cold phosphate buffer (0.06 M, pH 6.1) and centrifuged for 20 min at 4 000 g and 4 °C. The supernatant was collected for the IAA oxidase activity assay, which was carried out in 6 mL reaction mixtures containing 4 mL of phosphate buffer (0.06 M, pH 6.1), 0.5 mL of the supernatants, 0.3 mL of 1 mM $MnCl_2$, 0.6 mL of 1 mM DCP, and 0.6 mL of 4 mM IAA. The enzyme activity was expressed as the percentage of the IAA activity destroyed (where 100% IAA is the control reaction that had no enzyme fraction added). This was calculated from the absorbance of Salkowski's reagent at 530 nm by using a UV-vis Spectrophotometer (UV-5300 PC, Shanghai Metash Instruments Co., Ltd). Six replicates for each treatment were carried out and the measurements were repeated at least three times.

Extraction and determination of endogenous IAA

5-day-old WT seedlings were exposed to 50 μM Cd for 48 h and 72 h, and then were harvested and used to analysis of IAA content. The methods for extraction and purification of endogenous IAA were slightly modified from those described previously by Yang et al. (2001). The frozen samples (1 g) were powdered and extracted for 12 h with 4 mL cold 80% (v/v) aqueous methanol containing 1% (w/v) PVP and 1 mM BHT as an antioxidant at 4 °C, centrifuged for 15 min with 4000 rpm, and the supernatant was collected. The residues were extracted again with 1 mL extraction buffer under 4 °C for 1 h, centrifuged, and the supernatants were combined. The extracts were then passed through a C18 plastic column to remove the pigment and dried in N_2. The residue fractions were dissolved in 1 mL phosphate-buffered saline solution (0.01 M, pH 7.4) containing 0.1% (v/v) Tween-20 and 0.1% (w/v) gelatin for analysis by an enzyme-linked immunosorbent assay (ELISA). DG022 immunoanalyser (Perlong, China) was used to measure the absorbance of 490 nm to determine the content of IAA. Five replicates were carried out for each treatment. All the measurements were repeated three times.

Quantitative reverse transcription PCR analysis

After indicated times of 50 μM Cd treatment, the roots and leaves of WT plants were harvested separately, snap frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA, USA). Before PCR analysis, total RNA was pretreated with RNase-free DNase (Promega, USA) to remove any contaminating genomic DNA. First-strand cDNA was synthesized with 2 μg of total RNA using PrimeScript RT reagent Kit (TaKaRa, Dalian, China). PCR reactions were performed using the CFX96 Real Time System (Bio-Rad, CA, USA) with SYBR Premix Ex Taq II Kit (TaKaRa, Dalian, China), according to the procedure described by the manufacture. Based on primer efficiency, relative expression was calculated in Bio-Rad CFX manager (version 1.0) after normalization to *ACTIN2*. Triplicates for each PCR reaction were performed for each gene and the experiments were repeated for three times. The specific primers for each gene were listed in Supplemental Table S1.

Statistical analysis

Values were expressed as means ± SE. For all experiments, the overall data were statistically analyzed in the SPSS version 17.0 (SPSS). One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used for testing differences in growth and root developmental responses. In all cases, the confidence coefficient was set at $p < 0.05$.

Results

Effect of Cd stress on the growth of Arabidopsis seedlings

Firstly, some symptoms of Cd toxicity were evaluated in *Arabidopsis* seedlings with Cd treatment in comparison with controls. As shown in Fig. 1A, a low Cd concentration (12.5 μM) did not significantly decrease the shoot weight, while 25 μM or higher Cd concentrations resulted in marked reduction in this parameter (Fig. 1A) Although treatment with 12.5, 25 or 50 μM Cd did not affect hypocotyl length, after exposure to 75 μM Cd, hypocotyl length decreased to 82% of normal (Fig. 1B). Root growth decreased significantly as Cd concentration increased, and exposure from 12.5 to 75 μM Cd progressively reduced the root growth up to 91% compared to untreated control plants (Fig. 1C). Seedling exposed to 50 or 75 μM Cd also exhibited reduction of chlorophyll content, and chlorophyll content decreased by 30.5% and 42.1%, respectively (Fig. 1D).

Cd stress affects auxin homeostasis

To confirm whether the inhibition of growth in *Arabidopsis* seedlings with Cd treatment is involved in changes in the level or distribution of auixn, we investigated auxin homeostasis changes using the *DR5::GUS* reporter line (Ulmasov et al., 1997). As shown in Fig. 2A, *DR5::GUS* gene expression in root tips and cotyledons of Cd-treated plants slightly decreased after 24 h of treatment compared with that of 0 h. However, a significant reduction of the GUS activity in the area around the quiescent center and cotyledons was observed after exposed to Cd for 48 h, suggesting that auxin levels were decreased in Cd-treated plants (Fig. 2A). The

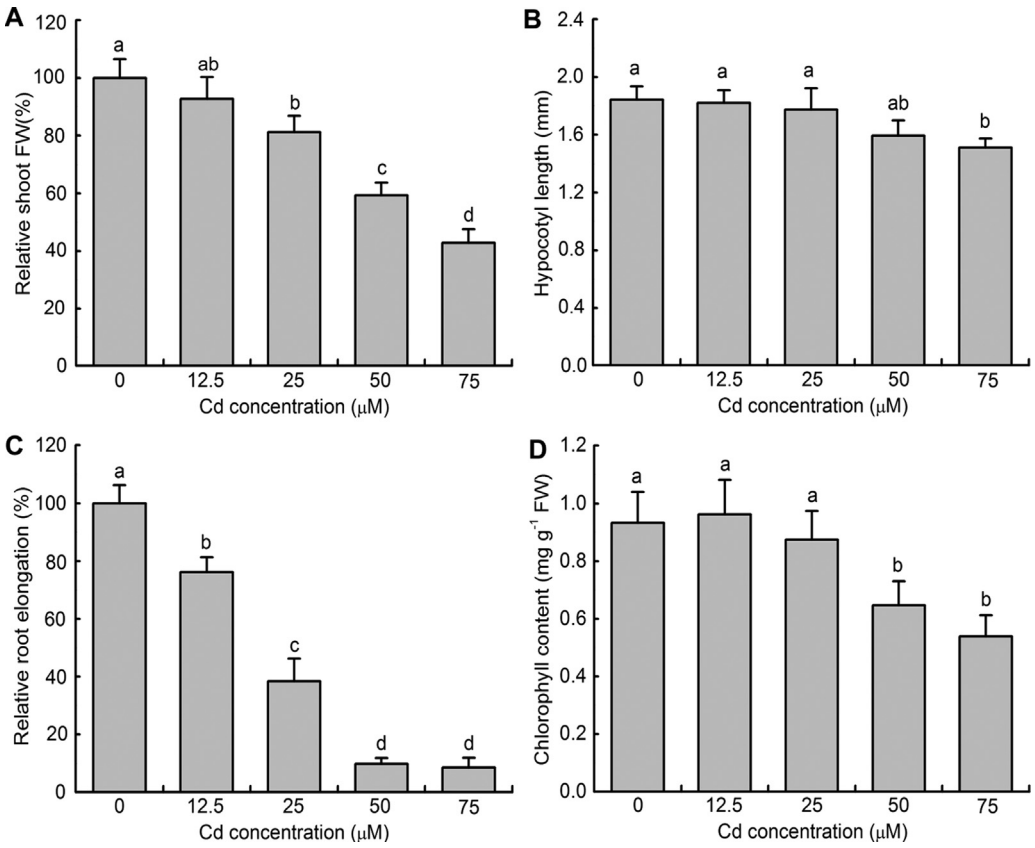

Fig. 1. Inhibitory effects of Cd stress on shoot and root growth in *Arabidopsis* (Col-0). 5-day-old *Arabidopsis* (Col-0) seedlings were transferred to 1/2 MS agar medium supplemented with various concentrations of Cd and grown for additional 5 days. (A) Inhibition of shoot fresh weight ($n = 50$). (B) Hypocotyl length ($n = 20$). (C) Relative primary root elongation ($n = 20$) and (D) the chlorophyll content. Data shown are the means ± SE. Different letters indicate significant differences at $p < 0.05$ (Duncan's multiple range tests).

light and diffused GUS activity was also detected in the root middle zones of Cd-treated seedlings (data not shown), indicating the alteration in auxin redistribution. Our results showed that the reduction of starch granules is consistent with the changes in auxin levels in primary root apices after 24 h and 48 h of Cd treatment (Fig. 2B).

Effects of Cd stress on the IAA content and the activity of IAA oxidase in Arabidopsis seedlings

To further examine the effects of Cd stress on auxin levels, we used ELISA to determine the contents of endogenous IAA in Cd-treated *Arabidopsis* seedlings. Compared to untreated con-

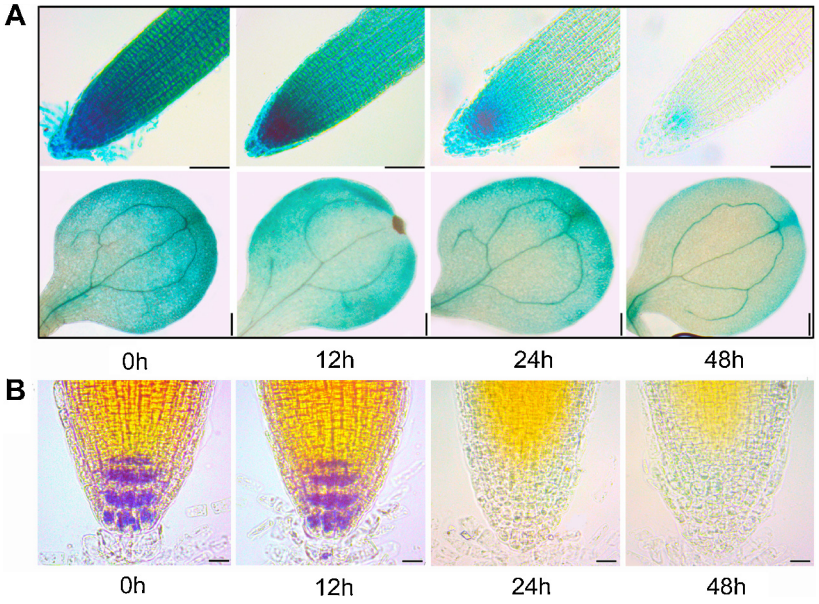

Fig. 2. Effects of Cd stress on the auxin level of *Arabidopsis* seedlings monitored by *DR5::GUS*. (A) GUS activity in primary root apices and cotyledons in 5-day-old *Arabidopsis DR5::GUS* seedlings treated with 50 μM Cd for 0, 12, 24 and 48 h. Scale bars = 10 μm (root tips), 0.5 mm (cotyledons). (B) Amyloplast-containing columella cells in primary root tips of 5-day-old *Arabidopsis* (Col-0) seedlings treated with 50 μM Cd. Scale bar = 10 μm. One representative sample from each treatment ($n = 25$) is shown.

trol plants, Cd treatment for 48 h reduced the endogenous IAA content by 22.8%, and the IAA content was further reduced by 35.7% after 72 h of Cd treatment (Fig. 3A). Since the IAA oxidase is responsible for the degradation of IAA in plants, we test whether cadmium stress altered the activity of IAA oxidase in Cd-treated plants. Our data indicated that the activity of IAA oxidase markedly increased by 108–216% in the presence of 25 μM or higher concentration of Cd (75 μM) (Fig. 3B). In the time-course experiment, after 24 h or longer time of Cd treatment, the activity of IAA oxidase also increased significantly compared to the control (Fig. 3C).

Cd stress affects LR development but is not involved in auxin redistribution in LRP

LRs, which typically constitute the majority of the root systems in plants, contribute greatly to nutrient acquisition from soil as well as to responses to environmental stress. Compared to the control, 12.5 μM Cd treatments did not affect the density of LRs. However, the density of the LR increased significantly after 25 or 50 μM Cd treatments (Fig. 4A). We next investigated the stages of LRP development affected by Cd stress. LRP were classified according to their stage of development as reported by Malamy and Benfey (1997). As indicated in Fig. 4B, the stage distribution of LRP was affected by different concentrations of Cd treatment. In particular, 25 or 50 μM Cd increased the number of emerged LRP (shorter than 0.5 mm) and LRP at late stages (stages IV–VII), but the number of LRP stages I–III, which refer to developing LRP at early stages of development, was reduced markedly compared with the control (Fig. 4B). Unlike the response of LR formation to Cd stress, 25 or 50 μM of Cd treatment markedly inhibited the LR length (Fig. 4C).

In order to further understand whether the Cd stress modulates LR initiation, we investigated the number of *DR5::GUS* sites along the primary roots of the Cd-treated plants. Our results showed that the number of GUS sites increased within the 72 h test period in the control seedlings, but the number of GUS sites started to reduce after 25 or 50 μM Cd treatment for 24 h, implying a former response of LR initiation to Cd stress (Fig. 5A). After 48 h of 25 or 50 μM Cd treatments, approximately 68% and 64% GUS sites disappeared in the primary roots of stress plants, respectively. With a 72 h prolonged period of 25 or 50 μM Cd treatments, the number of GUS sites markedly decreased in Cd-treated seedlings compared with the untreated seedlings (Fig. 5A).

We next examined the distribution or levels of auxin in several stages of LR development in *DR5::GUS* transgenic seedlings under Cd treatments (Fig. 5B). In control seedlings, maximal accumulation of *DR5::GUS* expression was observed at the position of LRP initiation, and the GUS activity remained high at next stages of LR development including the formation of LRP and the emergence of LRP, partially in adjacent cells (Fig. 5B), indicating that maintenance of optimal auxin levels is required for the entire developmental stage of LR. Very intriguingly, compared to the untreated control plants, no significant modifications in the *GUS* gene expression was detected in the entire stage of LR development (stages I–VII and emergence) of Cd-treated seedlings. However, Cd stress markedly decreased the GUS levels in the apex of mature LR (Fig. 5B).

Involvement of auxin transport in root development under Cd stress

It is clear that polar auxin transport plays an important role in root system development. Therefore, we used auxin-transport inhibitor NPA and 1-NOA to examine the role of auxin transport in the response of root architecture to Cd stress. The results indicated that supplementing with 10 μM NPA inhibited primary root

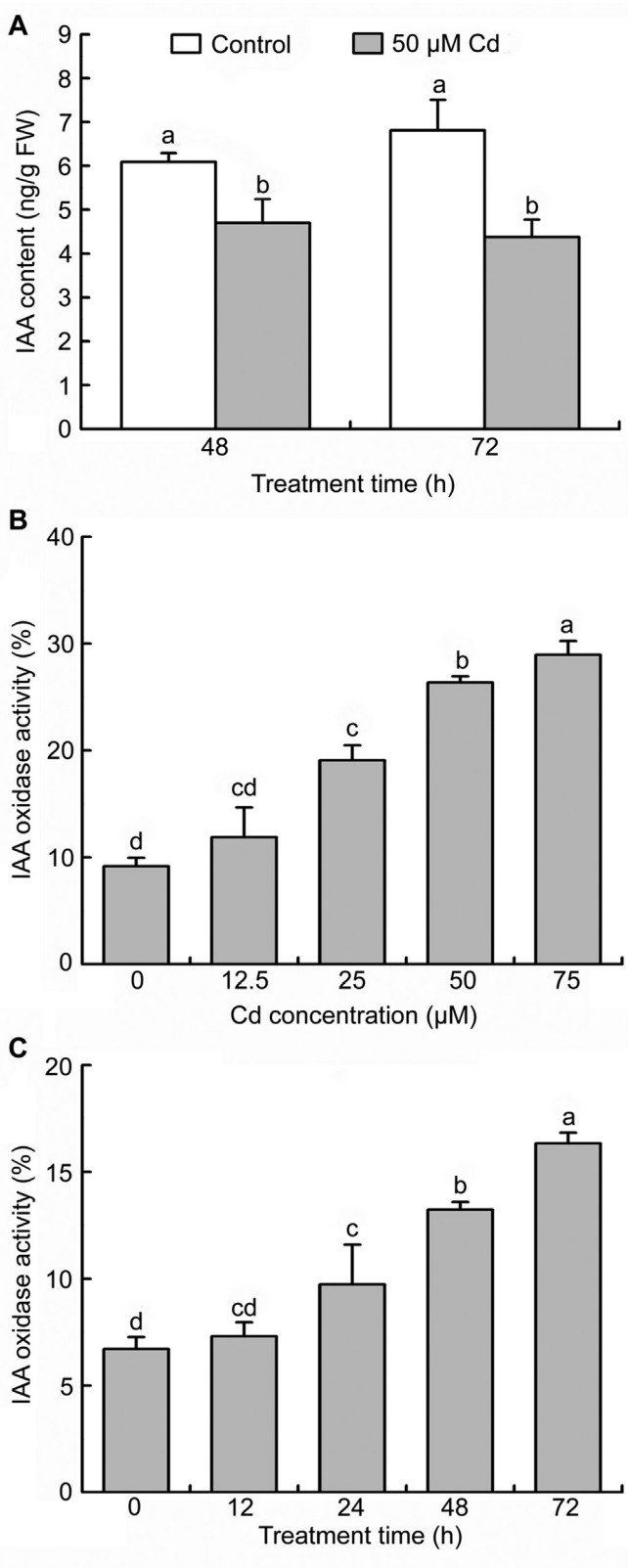

Fig. 3. Effects of Cd stress on the auxin content and activity of IAA oxidase in *Arabidopsis* seedlings (Col-0). (A) 5-day-old *Arabidopsis* (Col-0) seedlings were treated with 50 μM Cd for 48 h and 72 h and then used for IAA content determination. (B) 5-day-old *Arabidopsis* (Col-0) seedlings were treated with various concentrations of Cd for 72 h and used for IAA oxidase activity determination. (C) 5-day-old *Arabidopsis* (Col-0) seedlings were treated with 50 μM Cd for 0, 12, 24, 48 and 72 h, then used for IAA oxidase activity determination. Data points represent averages ± SE ($n = 6$). Different letters represent significant differences according to Duncan's test ($p < 0.05$).

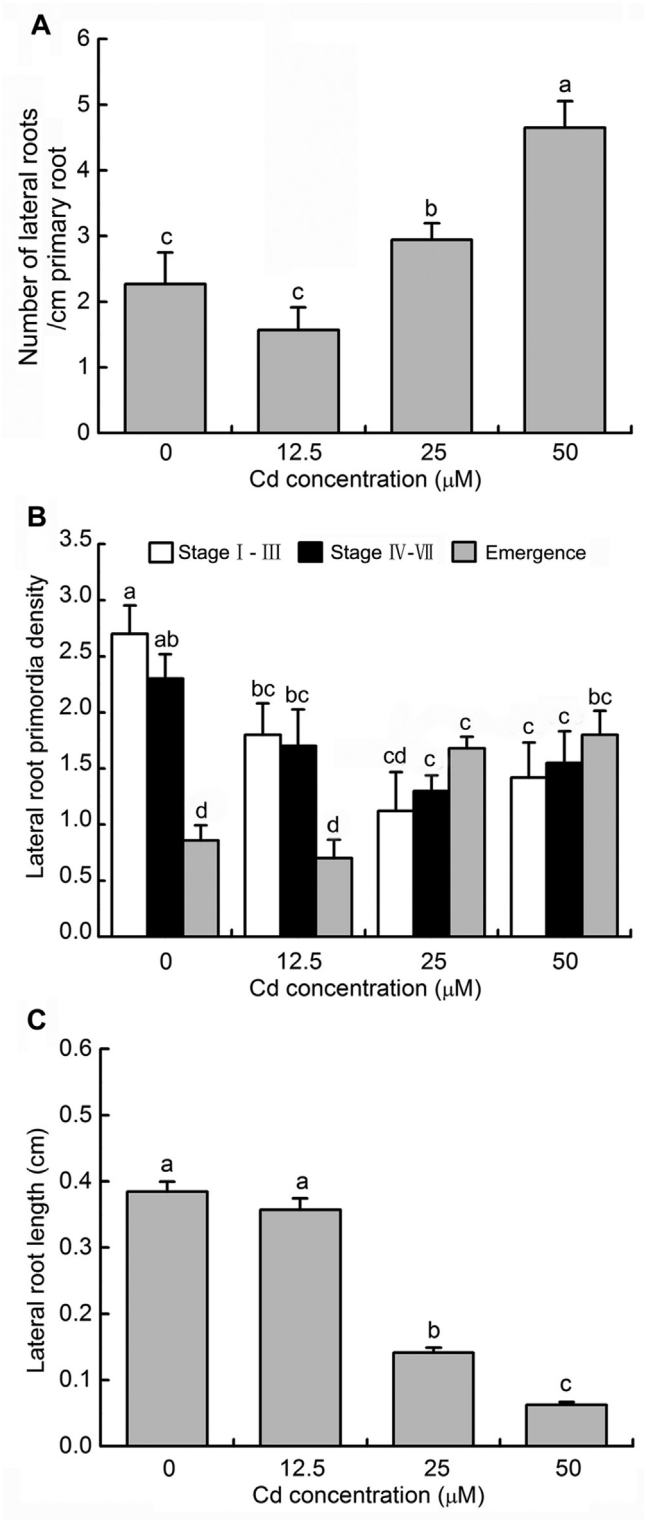

Fig. 4. Effects of Cd stress on LR development. 5-day-old *Arabidopsis* (Col-0) seedlings were treated with 0, 12.5, 25 and 50 μM Cd for another 5 days and analyzed for: (A) the density of LRs, (B) the density of LRP and (C) lateral root length. Data are mean ± SE ($n = 20$). Different letters are used to indicate significant differences at $p < 0.05$ (Duncan's multiple range tests).

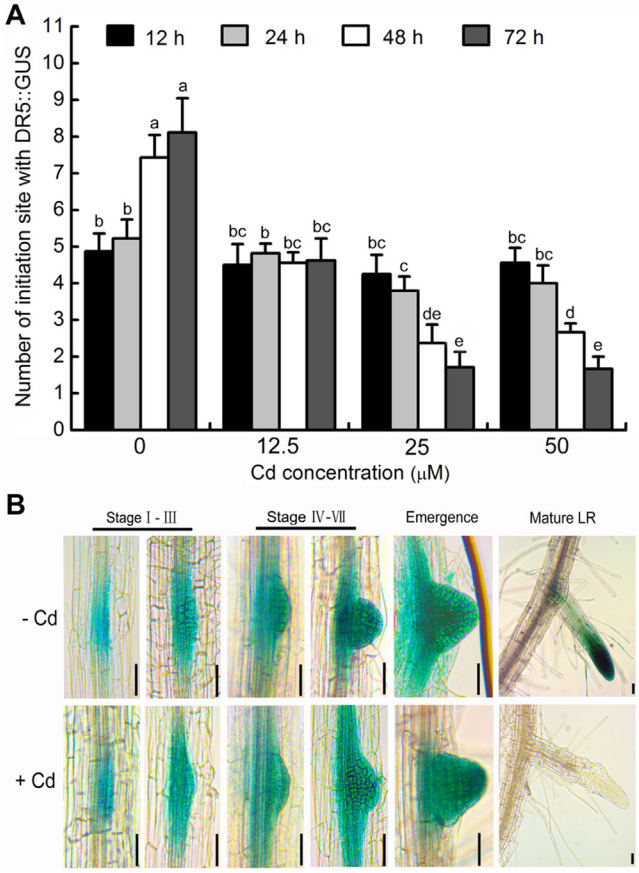

Fig. 5. (A) Number of LR initiation sites after 12, 24, 48 and 72 h of Cd treatment. (B) Expression patterns of *DR5::GUS* in LRP in 5-day-old *Arabidopsis DR5::GUS* seedlings treated with 50 μM Cd for 0, 12, 24 and 48 h. Images shown are representative of each treatment ($n = 20$). Scale bar = 50 μm. Data in A show the mean ± SE ($n = 20$). The pictures in (B) are representatives of similar results in three independent experiments. Different letters represent significant differences according to Duncan's multiple range tests ($p < 0.05$).

growth (Fig. 6A) and LR formation of WT plants (Fig. 6B). However, induction of LR formation by 50 μM Cd was also markedly suppressed by the addition of NPA (Fig. 6B). As shown in Fig. 6C, root growth on MS medium containing 10 μM 1-NOA was not significantly inhibited (Fig. 6C). Whereas simultaneous application of 50 μM Cd and 10 μM 1-NOA resulted in significant reduction of root growth, and primary root length decreased by 58.7%, compared with untreated plants (Fig. 6C). Moreover, the density of LR was markedly decreased in the presence of Cd and NPA, compared with the Cd treatment alone (Fig. 6D).

We further explored the effects of Cd stress on root development of *Arabidopsis* mutants defective in auxin influx (*aux1-7*) or efflux (*pin2*). After various concentrations of Cd treatment for 5 days, *aux1-7* and *pin2* showed a WT level of sensitivity to Cd in primary root growth inhibition (Fig. 7A). However, the LR density in WT and mutants exhibited different sensitivity to Cd treatment (Fig. 7B). When exposed to 25 and 50 μM Cd treatments, the density of LR was increased significantly in WT plants, but *aux1-7* and *pin2* mutants remained unchanged in LR density compared to untreated mutant seedlings (Fig. 7B).

Cd stress affects the expression of auxin-related genes involved in auxin homeostasis

Since endogenous IAA levels were altered in Cd-treated *Arabidopsis* seedlings, we investigated its effects on the expression levels of genes coding for enzymes involved in IAA biosynthesis

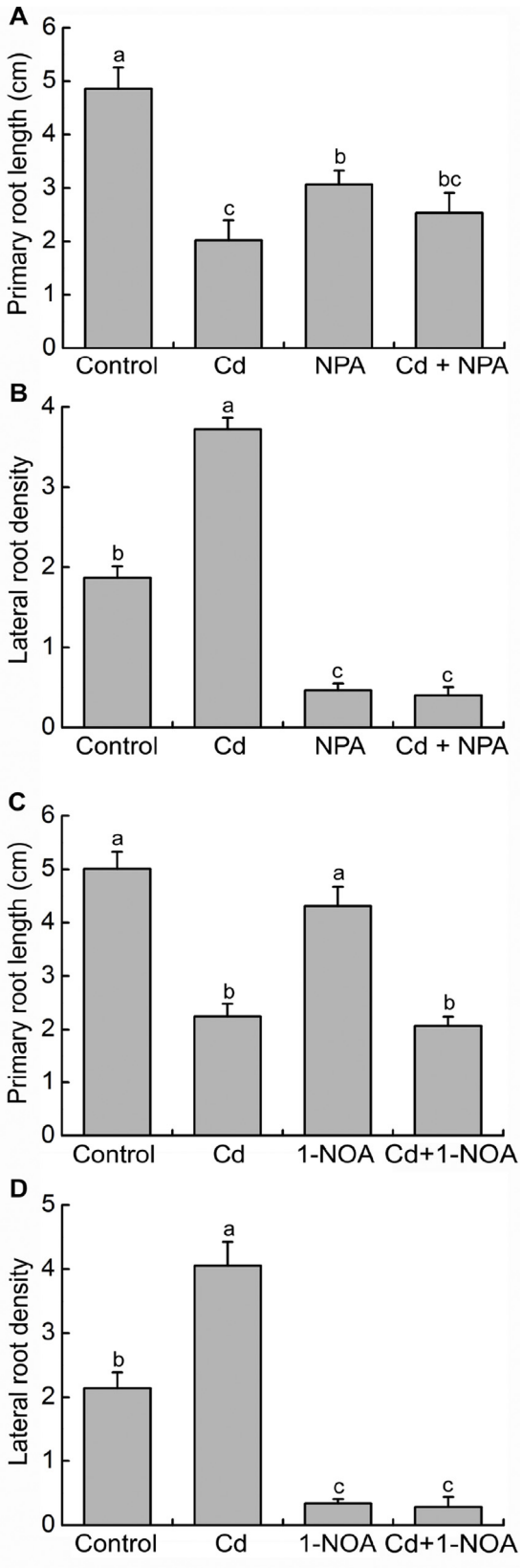

Fig. 6. Effect of auxin transport inhibitors and Cd stress on root architecture in *Arabidopsis* seedlings (Col-0). 5-day-old *Arabidopsis* seedlings were treated with 50 μM Cd in the presence or absence of 10 μM NPA or 10 μM 1-NOA for another 5 days. The effects of 10 μM NPA and 50 μM Cd on primary root length (A) and lateral root density (B) were examined. The effects of 10 μM 1-NOA and 50 μM Cd on primary root length (C) and lateral root density (D) were checked. Data points represent averages ± SE (n = 20). Different letters are used to indicate significant differences at $p < 0.05$ (Duncan's multiple range tests).

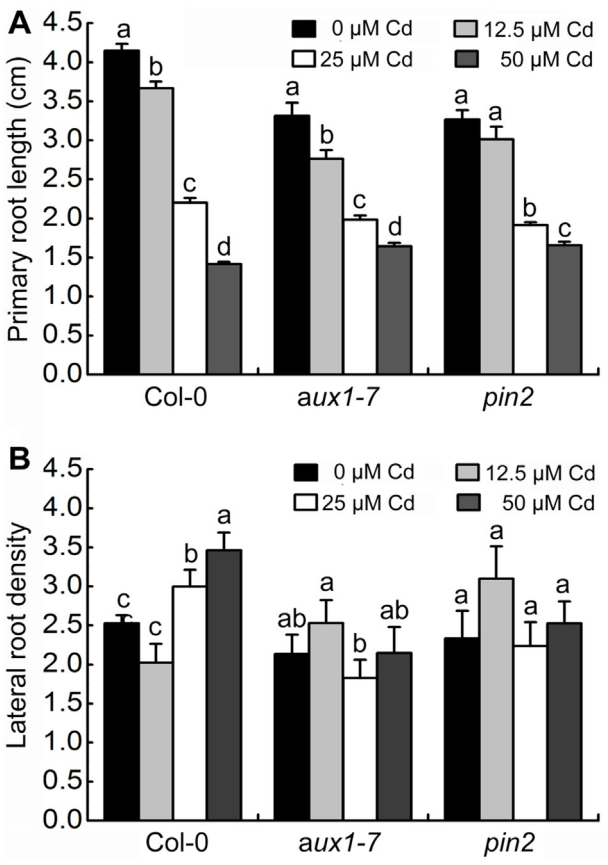

Fig. 7. Effects of Cd stress on root architecture in *Arabidopsis* seedlings (Col-0) and auxin transport mutant *aux1-7* and *pin2*. 5-day-old *Arabidopsis* seedlings were treated with different concentrations of Cd for another 5 days to examine: (A) primary root length, (B) lateral root density. Values shown are the mean ± SE (n = 20). Different letters indicate significant differences between treatments within a given genotype (Duncan's multiple range tests, $p < 0.05$).

and catabolism. The results showed that the expression of *PAT1*, a gene of Trp biosynthesis, increased significantly in roots after 6 h of Cd treatment; unexpectedly, it decreased significantly in shoots after 3–48 h of Cd treatment compared to the control. The transcript levels for genes that code for enzymes involved in IAA biosynthesis through the Trp-dependent pathway were also affected in the roots or grounded parts of Cd-treated seedlings at specific phases (Fig. 8). For example, *CYP79B2* and *CYP79B3* genes expression both in roots and shoots were down-regulated by Cd treatment. The expression of *NIT1* and *NIT2* was significantly up-regulated after 24 h and 48 h of Cd treatment in roots, whereas in shoots they were markedly up-regulated after 12 h and 24 h of Cd treatment, respectively (Fig. 8B). In Cd-treated roots, *YUCCA1* showed significant decrease in transcript levels at 24 h and 48 h of treatment, but its expression in shoots was inhibited at 6, 12 and 48 h. In contrast, Cd treatment markedly increased the expression of *YUCCA5* in roots at 6 h, and then inhibited its expression at 24 h and 48 h. However, unlike the pattern of expression in roots, the expression of *YUCCA5* in shoots was significantly up-regulated at 48 h (Fig. 8). For *TAA1*, a very similar pattern of gene expression as *YUCCA5* in roots was observed after Cd treatment, but a marked decrease in *TAA1* expression was also found in shoots after Cd treatment (Fig. 8).

In addition, it was also found that the expressions of *GH3* genes coding for enzymes involved in catalyzing the synthesis of IAA amide conjugates were affected by Cd treatment. As shown in Fig. 8, the expression of *GH3.17* decreased significantly in roots at 24 h and 48 h of Cd treatment, whereas *GH3.9* gene expression

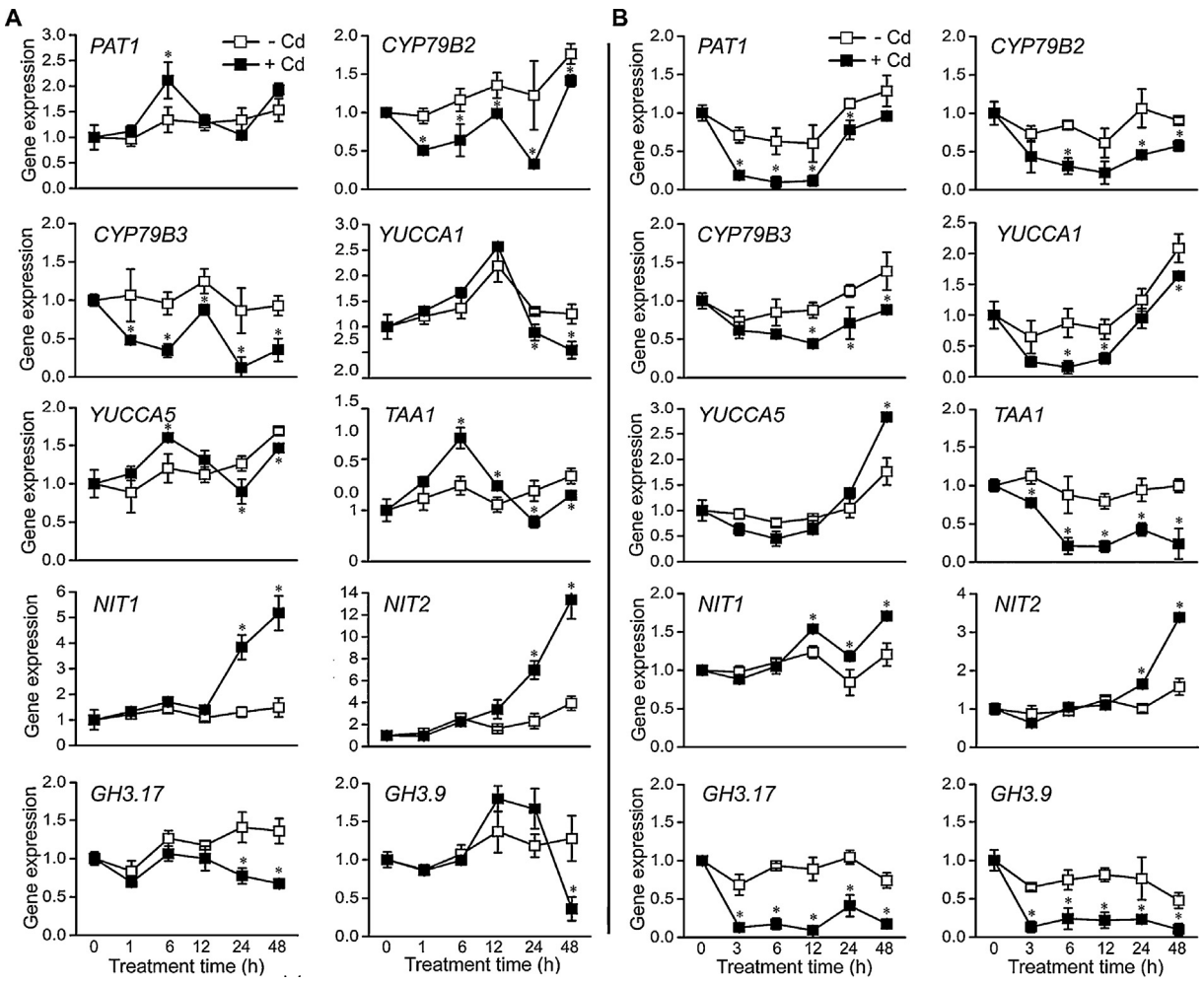

Fig. 8. qRT–PCR analysis of auxin biosynthesis or metabolism genes in *Arabidopsis* (Col-0) seedlings. (A) Relative gene expression in roots. (B) Relative gene expression in shoots. Relative expression levels are normalized to *ACTIN2*. Bars represent SE ($n = 4$). The asterisk denotes means that significantly differ between control and Cd treatment conditions (Student's *t*-test, $p < 0.05$).

was significantly suppressed at 48 h (Fig. 8A). Moreover, Cd treatment also resulted in the significant decrease in the transcript level of *GH3.17* and *GH3.9* in shoots (Fig. 8B), suggesting that Cd stress affects auxin catabolism. We also examined the effect of Cd stress on the expression of auxin transport genes in *Arabidopsis* roots. Data revealed that Cd treatment stimulated *PIN1* expression at 6 h and the expression of *PIN2* from 1 h to 12 h, but *PIN3* expression level was markedly inhibited after 48 h of treatment (Fig. 9). The expression of *PIN4* was significantly up-regulated by Cd treatment at 12 h and 24 h, however, down-regulation of *PIN7* was observed after 12 h, 24 h and 48 h of treatment (Fig. 9). The auxin influx carrier gene *AUX1* was significantly up-regulated by Cd treatment at 1 h and 6 h (Fig. 9).

Discussion

Plants possess a wide variety of hormones that, in addition to regulating growth and development, are involved in biotic and abiotic stress responses. Over-production of ethylene and methyl jasmonate was proposed to regulate the induction of pathogenesis-related protein in plants in order to protect proteins from Cd toxicity-related damages (Rodriguez-Serrano et al., 2009). It has also been observed that excess Cu causes the changes in the distribution of auxin and cytokinin in *Arabidopsis* roots (Lequeux et al., 2010). Recently, it was found that Cd stress triggered increases in GH3 activities which, in turn, decreased auxin concentrations in wood and thereby shunted the metabolism to enhanced formation of lignin in poplar (Elobeid et al., 2012). Villiers et al. (2012) reported that a modulation of the BR content in *Arabidopsis* seedlings affects their response to Cd. These findings provided evidence that, except for some biological processes, such as changes in photosynthesis, nutrient uptake, ROS levels, cell elongation and cell division, alterations in hormonal homeostasis may be involved in the response to metal stresses. The purpose of this work is to determine whether auxin homeostasis is involved in the response of *Arabidopsis* seedlings to Cd stress.

DR5-GUS has been used in many studies as an indirect indicator of the levels and distribution of auxin (Benková et al., 2003; Sun et al., 2010; Xu et al., 2010b). In this work, we found that *DR5* gene expression was markedly decreased in *Arabidopsis* root tips and cotyledons after Cd treatment (Fig. 2). These results are in agreement with previous reports (Xu et al., 2010b) that Cd stress reduces auxin levels in *Arabidopsis* seedlings. Interestingly, the observation that excess Cu increases the *DR5* expression in the root apical meristem and cotyledons of *Arabidopsis* seedlings (Lequeux et al., 2010; Peto et al., 2011), together with the findings that treatment with Al^{3+} enhances the level of *DR5::GUS* in the *Arabidopsis* root apices (Sun et al., 2010), suggests that the discrepancies may be attributed to differences in metal species and concentrations used and in time points in these studies; on the other hand, it also implies that the modulation of auxin level is required for plant development under metal stresses. ELISA kits have previously been validated by HPLC

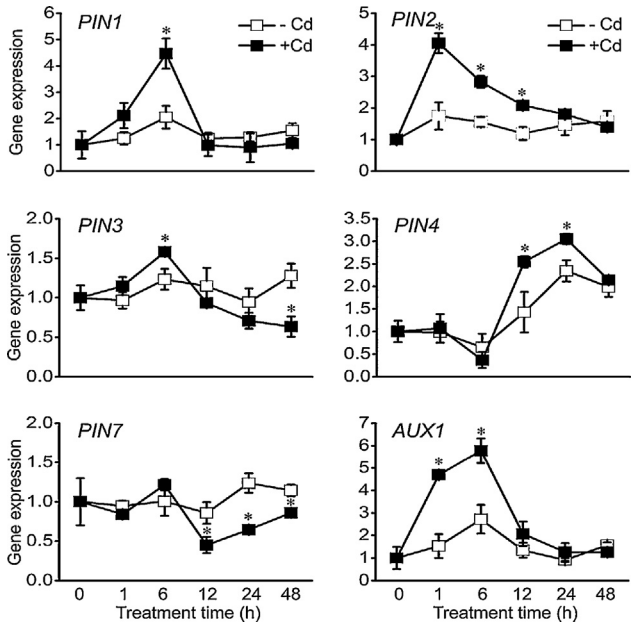

Fig. 9. qRT–PCR analysis of auxin transport genes in *Arabidopsis* (Col-0) roots. Relative expression levels are normalized to *ACTIN2*. Data shown are means ±SE ($n = 4$). The asterisk represents means that significantly differ between control and Cd treatment conditions (Student's *t*-test, $p < 0.05$).

and GC-MS analysis and have been widely used to determinate the contents of endogenous phytohormone in plants (Yang et al., 2001; Xu et al., 2010a; Janicka-Russak and Kabała, 2012). ELISA assays further indicated that free IAA levels were significantly lower in Cd-treated plants than in untreated control seedlings (Fig. 3A), similar to that found by Xu et al. (2010a), demonstrating that the alteration in auxin levels in *Arabidopsis* seedlings occurs under Cd stress. One possibility could be that changes in auxin homeostasis induced by Cd stress affect auxin levels.

The alteration of IAA levels was found to be related to the activity of IAA oxidase under Cd stress (Chaoui and Ferjani, 2005; Xu et al., 2010a). Moreover, optimal salt supplementation has been proven to improve the accumulation of IAA in Cd-stressed plants by decreasing the IAA oxidase activity (Xu et al., 2010b). In the present study, we hypothesized that there appears to be a relationship between the reduction of IAA levels and the activity of IAA oxidase in Cd-treated plants. Consistent with previous reports (Xu et al., 2010a; b) that Cd treatment induces significant elevation of the IAA oxidase activity in *Arabidopsis* seedlings (Fig. 3B and C), our results suggest that the increase of the IAA oxidase activity may contribute to the reduction of auxin levels in *Arabidopsis* seedlings under Cd stress. Transcriptomic analysis of Cu^{2+}-regulated genes demonstrated that the expression of auxin biosynthetic genes (e.g. IAA amide synthase, tryptophan synthase) is induced in response to Cu^{2+} treatment (Zhao et al., 2009), which might be responsible for an increase in auxin levels under excess Cu^{2+} (Peto et al., 2011). Another hypothesis would be that the reduction of IAA levels is due to the altered expression of auxin biosynthetic genes caused by Cd stress. It has been proven that Trp is converted into indole-3-acetaldoxime (IAOx) by two cytochrome P450 proteins (*CYP79B2/CYP79B3*), IAOx is then converted into indole-3-acetonitrile (IAN) which is finally converted into IAA by nitrilases (*NIT*) expressed in *Arabidopsis* roots (Normanly, 2010). We found that Cd treatment down-regulated the expression of *CYP79B2* and *CYP79B3* genes and up-regulated the expression of *NIT1* and *NIT2* genes both in roots and shoots (Fig. 8). The *YUCCA* gene family of flavin monooxygenases is reported to be a rate-limiting step in one of the Trp-dependent auxin biosynthetic pathways (Zhao et al., 2001). Our results showed that the expression of *YUCCA1* and *YUCCA5* genes decrease in roots after 24 h and 48 h of treatment, with the exception of *YUCCA5* at 6 h (Fig. 8A). In shoots, *YUCCA1* is down-regulated at 6, 12, and 48 h, while *YUCCA5* is up-regulated at 48 h (Fig. 8B), suggesting that the effects of Cd stress on the expression of these genes are tissue-specific. All together, these results indicated that Cd could affect auxin levels in *Arabidopsis* seedlings through modulating the expressions of some key auxin biosynthetic genes. In *Arabidopsis*, overproduction of a GH3 class gene *WES1*, encoding an amide-type auxin-conjugating enzyme, increased biotic and abiotic stress tolerance in *Arabidopsis* (Park et al., 2007). Similarly, enhanced resistance to biotic stress was observed in transgenic rice over expressing *OsGH3.1* (Domingo et al., 2009). It was also discovered that levels of IAA-Glc and IBA-Glc increase in *Arabidopsis* seedlings under osmotic stress (Tognetti et al., 2010). In addition, *GH3* genes are up-regulated in *Brassica juncea* L (Minglin et al., 2005) and poplar (Elobeid et al., 2012) under Cd stress. These studies illustrate that stress-induced changes in auxin conjugation can adjust the internal auxin balance, and the GH3 auxin conjugate synthases play an important role in plant stress adaptation. However, our observations showed that Cd treatment down-regulated the expression of *GH3.9* and *GH3.17* in *Arabidopsis* seedlings, which are contrary to the reports by Minglin et al. (2005) and Elobeid et al. (2012). The discrepancies might be due to differences in plant species in these studies. Additionally, it is also possible that *Arabidopsis* uses the auxin conjugate pool by decreasing GH3 auxin conjugate synthases to balance out the effects of Cd on auxin metabolism.

In many cases, exposure to stress conditions leads to a common remodeling of the root system architecture characterized by the inhibition of primary root growth and the simultaneous enhanced formation of LR formation (Potters et al., 2007). Similar phenotypic alterations of *Arabidopsis* seedlings in response to Cd stress were also detected in this study. Auxin mediates many aspects of the root development, including root elongation, LR formation, and root hair proliferation (Woodward and Bartel, 2005). A role for auxin in the reorganization of the root architecture has been reported for metal stresses (Lequeux et al., 2010; Sun et al., 2010). Analysis of the *DR5::GUS* activity indicates that Cd treatment reduces the auxin level in the root apex (Fig. 2A), while it increases the auxin level in the middle part of roots, suggesting that the modulated auxin redistribution by Cd may affect the root system development. The reduced level of auxin in root tips causes reduced cell division in the meristem, resulting in subsequent growth arrest of the primary root. Potters et al. (2007) reported that there is a good correlation between Cd-induced changes in auxin distribution and LR formation. However, to date there is no direct evidence of involvement of auxin in plant LR development under Cd stress. In this work, we found that the Cd stress-stimulated formation of LR may be due to the promotion of LR emergence instead of LR initiation, because the *DR5::GUS* site assay indicates that Cd stress inhibits LR initiation (Fig. 5). LR development is initiated by asymmetric divisions in pairs of founder cells within xylem pole pericycle cells. When auxin reaches a threshold which is sensed and transduced by the xylem pole pericycle cells, the cell cycle machinery becomes activated, resulting in progression to S phase (Benková et al., 2003). Previous studies have confirmed that the formation of LR in phosphorus-or sulfur-starved plants is linked to altered auxin metabolism (Potters et al., 2007). Based on our results, Cd stress decreases the auxin contents and, when auxin falls below the threshold, cells lose their competence for organ initiation, defaulting to a pericycle tissue identity.

Subsequently, another piece of evidence in favor of the involvement of auxin in the root architecture response to Cd stress can be drawn from the comparison with auxin transport inhibitor effects on LR development and the analysis of auxin transport mutants

under Cd treatment. Application of Cd led to increased LR density in wild-type *Arabidopsis* seedlings and this effect was reversed in NPA- or 1-NOA- treated plants (Fig. 6), indicating that auxin transport may play important roles in the promotion of LR formation under Cd stress. If the effect of Cd on LR formation involves the auxin transport, the density of LR in mutants defective in auxin transport should differ from WT plants under Cd treatment. Compared to the WT seedlings, the induction in LR density by Cd stress was abolished in *aux1-7* and *pin2* mutants (Fig. 7). This suggests that auxin transport is required for Cd-modulated LR induction. Indeed, many studies reported that stress can impact on auxin distribution or transport via effects on the expression of auxin carrier genes (Pasternak et al., 2005; Sun et al., 2010; Li et al., 2011). We also found that Cd stress up-regulated the expression of *PIN1*, *PIN2*, *PIN4* and *AUX1* in *Arabidopsis* roots (Fig. 9). Because PIN2 and AUX1 proteins play critical roles in mobilizing auxin between root apical cells and cells in the elongation zone (Swarup et al., 2007), enhanced expression of *PIN2* and *AUX1* may be required for the changes in *DR5::GUS* activity in both apical and middle parts of roots. Although the free IAA content decreased in Cd-treated plants, Cd treatment did not significantly affect the auxin level or patterning in several stages of LR development (Fig. 5B). The GUS activity pattern, therefore, may reflect changes in auxin distribution within each plant part without any decrease in total auxin levels in stages of LR development. However, Cd stress stimulated the expression of *PIN1* and *PIN2* genes in roots would ensure establishment of a local auxin gradient in stages of LR development, this may account for the effect of Cd on *DR5::GUS* activity in stages of LR development (Fig. 5B).

In conclusion, our results provide novel insights into how Cd stress triggers changes in auxin homeostasis in *Arabidopsis* seedlings. In the present study, we showed that Cd stress results in the reduction of endogenous auxin levels through increasing the IAA oxidase activity and modulating the expressions of auxin metabolic genes. Our study provides an explanation that Cd-induced auxin redistribution in the *Arabidopsis* roots is due to the alteration in the expressions of auxin transport genes. It was also found that Cd treatment affects the architecture of root system, and it may improve the ability of the plants to take up water and nutrients. In addition, to our knowledge, this is the first genetic evidence that the auxin transport pathway is required for the adaptive remodeling of root system architecture under Cd stress. The regulative mechanism of auxin on plant growth under abiotic stress is considerably complicated. Thus, further research is required for elucidation of the detailed molecular mechanisms involved in Cd-induced auxin homeostasis changes in plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2013.02.008.

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