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RESEARCH PAPERS

# Combined Treatment with Cadmium and Zinc Enhances Lateral Root Development by Regulating Auxin Redistribution and Cell-Cycle Gene Expression in Rice Seedlings[1, 2]

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**Abstract**—Enhanced lateral root (LR) development is of critical importance for rice plants adapting to heavy-metal-stress conditions. LR development is affected by heavy metals, such as aluminium (Al), copper (Cu), lead (Pb), zinc (Zn), chromium (Cr) and cadmium (Cd), or metals in combination, such as Cd and As. However, it has not been reported yet whether the combination of Cd and Zn affect LR growth in rice. Here, we studied the associations between LR growth, auxin signaling, and the cell cycle in the combination of Cd and Zn-treated rice (*Oryza sativa* L. cv. Zhonghua no. 11). Combined treatment with Cd and Zn significantly enhances LR development in rice seedlings. Cd levels decreased and Zn levels increased in the lateral root development regions (LRDRs) with the treatment of (Cd + Zn) compared to the treatment of Cd alone. Zn counteracted over-accumulation of auxin caused by Cd- and (Cd + Zn)-treatment significantly promoted LR growth by maintaining appropriate auxin distribution in the roots. Experiments using TIBA (2,3,5-triiodobenzoic acid, an inhibitor of polar auxin transport), BFA (brefeldin A, a protein transport inhibitor), IBA (indole-3-butyric acid), MG132 (a protein degradation inhibitor) and *DR5-GUS* staining revealed that (Cd + Zn)-treatment influences the distribution of auxin through polar auxin transport and protein transport/degradation pathways. By evaluating expression levels of some key auxin-signaling genes and cell-cycle-related genes in roots treated with (Cd + Zn) or Cd alone, we found that (Cd + Zn)-treatment affects specific genes involved in auxin signaling and the cell cycle compared with Cd alone, and the treatment duration of 7 and 9 days showed different regulated manner. Our findings should help to elucidate how the effects of (Cd + Zn)-treatment on auxin signaling and the cell cycle influence LR growth.

*Keywords: Oryza sativa*, auxin redistribution, auxin signaling, combined cadmium and zinc, cell cycle

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## INTRODUCTION

Cadmium (Cd) is an environmental pollutant; at high concentrations it induces physiological and ultrastructural changes in plants [1]. Zinc (Zn) whereas at low concentration plays important roles in plant metabolic, physiological and transcriptional regulatory processes. However, excess Zn results in significant retardation of plant growth [2]. In plants, metal uptake and transport are regulated by several genes, such as *metal-transporting ATPases, metal-nicotianamine transporter YSL (YSL)*, *ABC transporters*, *metal-chelators, nicotianamine synthase* (*NAS*), *vacuolar iron transporters* (*VIT*), etc. [3]. Among these, $P_{1B}$-type heavy metal ATPases (HMAs) and zinc-regulated transporter and iron-regulated transporter-like proteins (ZIPs) are associated with Cd and Zn uptake and transport [4, 5].

Lateral roots (LRs) are important components of the rice (*Oryza sativa* L.) root system, and LR growth plasticity is critical to allow rice plants to adapt to stress conditions [6]. An increasing body of evidence indicates that LR development is affected by heavy metals, plant hormones, and the cell cycle. Exposure to lower or medium concentrations of heavy metals such as aluminium (Al), copper (Cu), lead (Pb), zinc (Zn), chromium (Cr) and cadmium (Cd) stimulates LR development [7–10]. For example, Al inhibited embryonic root (ER, also called primary root) growth but led to increased numbers of LRs in *Zea mays* [10]. In *Arabidopsis*, Cu, Zn, and Cd separately or in combination suppressed root elongation but increased the number of LRs [9, 11].

[1] The article is published in the original.
*Abbreviations:* AR—adventitious root; BFA—brefeldin A; ER—embryonic root (primary root); GUS—ß-glucuronidase; IAA—indole-3-acetic acid; IBA—indole-3-butyric acid; LR—lateral root; LRDR—lateral root development region; TIBA—2,3,5-triiodobenzoic acid.

[2] Supplementary materials are available for this article at https://doi.org/10.1134/S1021443719040162 and are accessible for authorized user.

Auxin is one of the key phytohormones involved in the regulation of LR development, and its role has been extensively studied [6, 12, 13]. LR formation is stimulated by exogenous auxin application, but is suppressed by inhibition of polar auxin transport [6]. *YUCCA*s, *PIN*s, *ARF* and *IAA* are key auxin-signaling gene families involved in LR growth. For instance, LR numbers decreased in some auxin-related *Arabidopsis* mutants such as *iaa28*, *aux1*, and *pin1/3/4/7*, while LR numbers increased in mutants such as *sur1*, and *arf8* [12]. The involvement of auxin biosynthesis-related genes *YUCs* and transport-related genes *PIN*s were reported to be involved in LR development in *Arabidopsis* in response to heterogeneous phosphorus availability [13]. Changes in auxin distribution patterns during LR development were also observed in rice [14], and LR developmental changes were associated with auxin redistribution in Al-, Cd-, or Cu-stressed plants [8, 11, 15]. Taken together, these data strongly suggest that auxin signaling plays an important role in controlling LR growth.

Cell-cycle regulation plays a crucial role in LR growth [10, 11], and several core cell-cycle genes may act as regulators of LR initiation. For example, a D-type-cyclin gene (*CYCD4*;1) is expressed during LR initiation [16], and over-expression of KRP2, a CDK inhibitor, reduced the number of LRs produced [17]. Auxin-regulated LR initiation is closely related to the cell cycle: in *Zea mays*, Al enhanced LR initiation by stimulating cell division [10], and the increased number of LRs formed in Cu-stressed *Arabidopsis* was associated with mitotic activity [11].

Metals can be present simultaneously in the environment and plant hormones are involved in the plant response to many metals [18]. Metals' interplay affects cadmium accumulation, further affecting root development in plants. Arsenic (As) and Cd, alone or combined, influence quiescent center formation in *Arabidopsis* through disruption of auxin homeostasis [19]. Combination of Cd and As also influence root development in rice by interrupting auxin biosynthesis and transport [1]. Zn plays important roles in Cd accumulation in plants. Supplementation of Zn in tomato plants reduced Cd accumulation and increased Zn concentration simultaneously [20]. In wheat, transcriptome profiles of (Cd + Zn) revealed that many transporters, such as cadmium-transporting ATPase, metal-nicotianamine transporter YSL (YSL), ABC transporters, are all involved in mutual inhibition of the Cd/Zn uptake in the roots [3]. In our previous studies, the treatment of Cd on rice caused a decrease in Zn content, and compared to Cd alone, the treatment of (Cd + Zn) on rice caused a decrease in Cd accumulation [21, 22].

The study of plants under one or more heavy metals stress will help to learn plant molecular and physiological responses and may improve its productivity. LR growth plasticity is critical to allow rice plants to adapt to stress conditions; however, the links between LR development, auxin signaling, and the cell cycle in (Cd + Zn)-treated rice remain unclear, and the aim of this study was therefore to investigate these relationships.

## MATERIALS AND METHODS

**Plant materials and treatments**. The relationship between LR growth, auxin signaling, and the cell cycle under (Cd + Zn)-treatment conditions was investigated using 0.2 mM $Cd(NO_3)_2$ and 0.3 mM $Zn(SO_4)_2$.

Rice (*Oryza sativa* L. cv. Zhonghua No. 11) seeds were germinated for 2 days and transferred onto agar-solidified MS medium with or without 0.2 mM $Cd(NO_3)_2$ (Cd) or 0.3 mM $Zn(SO_4)_2$ (Zn) only, or in combination (Cd + Zn), in a growth chamber under 200 μmol/($m^2$ s) illumination, 14-h photoperiod, with day/night temperatures of 26/20°C, and relative humidity levels of 60/80% for 7–11 days. The MS medium used contains 0.03 mM $Zn(SO_4)_2$, so minor Zn exists in all treatments. At the end of the treatments, the roots of the seedlings were used for further analyses. Each treatment was performed at least in triplicate using at least four 100-mL containers with 50 seedlings per container.

For the analysis of the effect of (Cd + Zn)-treatment on LR growth and auxin transport, rice seeds were germinated for 2 days and transferred onto agar-solidified MS medium with or without (Cd + Zn) plus 1 μM TIBA (2,3,5-triiodobenzoic acid, an inhibitor of polar auxin transport) or 10 nM IBA (indole-3-butyric acid), and incubated for 11 days under the same conditions as described above. Each treatment was performed at least in triplicate.

**Characterization of LR growth**. The length and number of LRs that developed on the ERs and adventitious roots (ARs) were measured and scored under a microscope with a digital camera. In each replicate of every treatment, the roots of 20 plants were analyzed, and the results were expressed per plant.

**Determination of Cd and Zn concentration**. For determination of Cd and Zn concentrations, roots were used following various treatments for 9 days. The harvested roots (LRDRs from the ERs and ARs) were washed first in distilled water and then in 0.01 mM EDTA solution, and dried at 80°C until the materials reached constant weights. The Cd and Zn contents in the tissue extracts were measured using inductively coupled plasma mass spectroscopy (ICPS). The results were based on the average of three replicate determinations [22].

**Indole-3-acetic acid (IAA) analyses.** To analyze IAA content in ERs (basal, middle, and tip regions) treated with various compounds for 9 days, aliquots (250 mg) of ERs (1 cm of each region was excised from at least 100 seedlings for each treatment) were immediately powdered in liquid nitrogen and homogenized

in 2.5 mL mixture of cold 2-propanol : $H_2O$ : 37% HCl (2 : 1 : 0.002, v/v/v). The content of IAA was determined by high-performance liquid chromatography coupled with mass spectrometry (HP1100 HPLC, Agilent Inc.; esquire 2000 MS Bruker Daltonics Inc., United States), according to Sofo et al. [23]. Internal labeled standards of IAA (Sigma, United States) dissolved in methanol were used.

**Histochemical and quantitative analysis GUS (ß-glucuronidase) activity**. The *DR5-GUS* transgenic rice seeds (in an *Oryza sativa* L. cv. Zhonghua No. 11 background) were germinated for 2 days and transferred onto agar-solidified MS medium with or without Cd, Zn and (Cd + Zn), and incubated for 7–9 days under the same conditions as above. Roots were submerged in a GUS staining buffer [22]. The roots for all treatments were incubated at 35°C for 10 h and cleared with 70% (v/v) ethanol and 3% (v/v) sodium hypochlorite. The seedlings of 20 plants were used for each treatment. Images were taken using a dissecting microscope (Nikon SMZ1500, Japan) with a digital camera (Nikon D5000).

For further quantitative analysis of GUS activity in ERs (basal, middle, and tip regions) treated with various compounds for 9 days, approximately 50 mg of ERs (1 cm of each region was excised from at least 100 seedlings for each treatment) were powdered in liquid nitrogen and homogenized in GUS extraction buffer [24]. Fluorescence was determined with a spectrofluorometer (RF-5301PC, Shimadzu, Japan) calibrated with a standard curve of known concentrations of 4-methylumbelliferone (4-MU; Sigma). Protein concentrations of root extracts were determined by the method of Bradford [25]. The GUS activities were expressed as fluorescence 4-MU/(μg protein h). Each treatment was performed at least in triplicate.

For the analysis of auxin transport, the *DR5-GUS* transgenic rice seeds were germinated for 2 days and transferred onto agar-solidified MS medium with or without (Cd + Zn) plus 1 μM TIBA or 10 nM IBA, and incubated for 9 d under the same conditions as above. Each treatment was performed at least in triplicate. The seedlings of 20 plants were used for each treatment.

To further test the effects of BFA (brefeldin A, a protein transport inhibitor) and MG132 (a protein degradation inhibitor) on GUS activity, the *DR5-GUS* transgenic rice seeds were germinated for 2 days and transferred onto agar-solidified MS medium and incubated for 7 days under the same conditions as above. Seedlings were transferred to Hoagland's nutrient solution and pretreated with or without 50 μM BFA or MG132 for 3 h, and then transferred to new Hoagland's nutrient solution with or without (Cd + Zn), and incubated for 12 h under the same conditions as above. GUS activity was further quantitative analyzed in ER tip regions (1 cm). Each treatment was performed at least in triplicate. The seedlings of 20 plants were used for each treatment.

**Analysis using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)**. Total RNA was extracted from the LRDRs of ER and AR after the 7- or 9-day treatment (roots of 20 plants in each replicate), and was immediately powdered in liquid nitrogen using the Trizol reagent (Invitrogen, Germany) according to the manufacturer's instructions. The cDNA was synthesized using 1 μg of total RNA with an RNA PCR Kit (AMV) V3.0 (Takara Biotechnology, Japan) according to the manufacturer's instructions. Equal amounts of cDNA were used in each reaction. Each PCR pattern was verified using independent triplicate experiments under identical conditions. The rice actin gene was used as an internal control. Gene primers are listed in the Supplementary Table S1. A semi-quantitative RT-PCR analysis of specific gene expression was performed using the Gel-Pro Analyzer software (Media Cybernetics, United States). The relative transcription activity of each gene was calculated in reference to the control, which was set at 1.0. Transcription alterations between different treatments ≥0.3 were considered significant. Expression values of the tested genes are listed in the Supplementary Table S2. In this study, we mainly focused on the differential expression of tested genes following Cd- and (Cd + Zn)-treatments but not after Zn-treatment alone (because no significant effects of Zn alone on LR growth were observed under this experimental condition).

**Statistical analysis of data**. The three independent experiments for each treatment were compared using one-way ANOVA, followed by multiple comparisons test (SPSS, 16.0, SPSS Inc., United States). Differences were considered significant at $P \leq 0.05$. Results are presented as the mean ± SE.

## RESULTS

### *Effect of Combined Treatment with Cd and Zn on Lateral Root Development in Rice Seedlings*

To test the effects of Cd and Zn on LR growth in rice, seedlings were treated with Cd or Zn only, or in combination, for 7–11 days. After this period, the number (Fig. 1a) and length (Fig. 1b) of LRs on the ERs in the combination treatment group significantly exceeded those in the control and Cd- or Zn-only groups ($P < 0.05$). Similarly, the numbers and length of LRs on the ARs (Figs. 1c–1d), in the combination treatment group were significantly higher than those in the control and Cd- or Zn-alone groups when treated for 9–11 days ($P > 0.05$). These results indicate that under the current experimental conditions, (Cd + Zn)-treatment enhanced LR development in rice seedlings.

### *Effect of (Cd + Zn) Stress on Cd and Zn Accumulation in Rice Lateral Root Development Regions*

To examine whether treatment with (Cd + Zn)-induced changes in Cd and Zn absorbability in the lat-

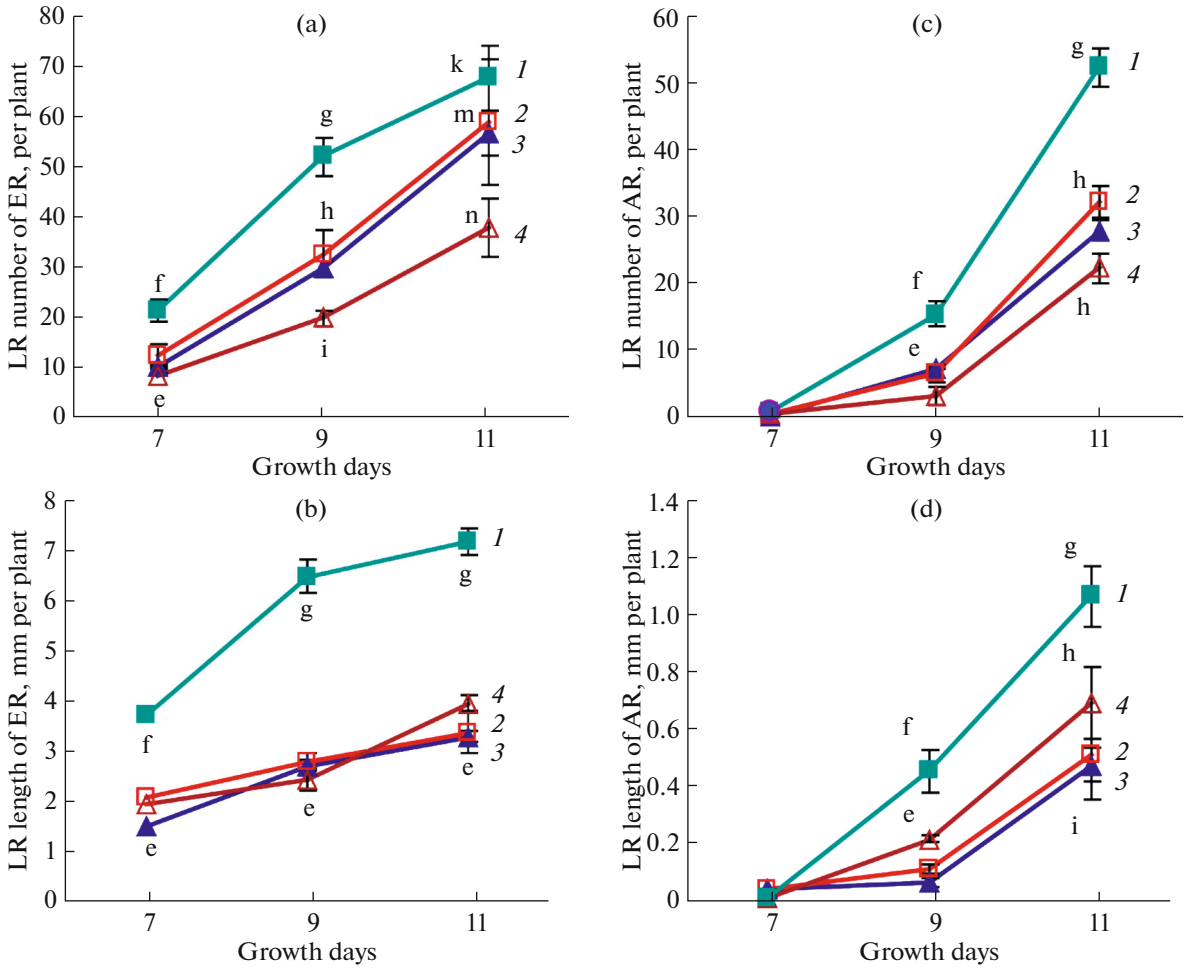


**Fig. 1.** Variations in the LR growth during 7–11 days of exposure to different treatments. (a) LR number on the ER; (b) LR length on the ER; (c) LR number on the AR; (d) LR length on the ARs. (*1*) (Cd + Zn)-treatment; (*2*) Zn-treatment; (*3*) control; (*4*) Cd-treatment. Values represent means ± SE of three independent experiments. Different letters indicate that one treatment or treatment time was significantly different from the other at $P < 0.05$.

eral root development regions (LRDRs) of rice roots, metal contents were assessed in treated 9-day-old seedlings. As shown in Fig. 2a, the Cd content in the root treated with (Cd + Zn) was significantly lower than that treated with Cd alone. Zn content was higher in both Zn- and (Cd + Zn)-treated roots than in control roots, but was lower ($P < 0.01$) in Cd-treated roots than in control roots. In (Cd + Zn)-treated roots, the Zn content was 5.7-fold ($P < 0.01$) higher than in Cd-treated roots. This suggests that an antagonistic effect of Zn on Cd occurred in the rice roots under the (Cd + Zn) stress conditions applied in this study.

### *Influence of OsHMA and OsZIP Expression Changes in the LRDRs of Rice Roots on Cd and Zn Uptake under (Cd + Zn)-Treatment Conditions*

It is reported that Zn could reduce Cd toxicity in plants [20]. In our study, we found an antagonistic effect of Zn on Cd toxicity in rice roots both in LR development and Cd content under the (Cd + Zn) conditions. Transporters of Cd and Zn may be involved in this process. HMAs and ZIPs are reported to be involved in metal uptake and transport in plants [4, 5]. To further examine the relationship between Cd and Zn absorbability and the expression of *OsHMA* and *OsZIP* family genes, the transcription levels of *OsHMAs* (nine genes) and *OsZIPs* (nine genes) were examined in the LRDRs of treated roots. The genes that were differentially expressed in response to Cd-and (Cd + Zn)-treatment are shown in Fig. 2b. Seven and nine days after initiation of treatment, the transcript levels of *OsHMA9*, *OsZIP3*, and *OsZIP6* were higher in the roots of (Cd + Zn)-treated plants than in those of Cd-treated plants, but *OsHMA4* expression was reduced. *OsHMA9* and *OsHAM4* belong to $P_{1B}$-type heavy metal ATPases involved in Cd and Zn efflux from root cells and in xylem loading [4]; *OsZIP3* and *OsZIP6* belong to zinc-regulated transporter and iron-regulated transporter-like proteins involved in Zn uptake [5]. These results may indicate

that Cd and Zn uptake and transport in (Cd + Zn)-treated rice roots is related to the altered expression of a subset of *OsHMA* and *OsZIP* genes.

### *Effect of Combined Treatment with Cd and Zn on Auxin Transport in Rice Lateral Root Development*

Auxin plays an important role in regulating LR growth. To determine whether LR growth stimulated by (Cd + Zn)-treatment is associated with auxin levels and transport, seedlings were treated with (Cd + Zn) plus TIBA or IBA for 11 days. As shown in Table 1, growth and formation of LRs on the ERs and ARs was markedly inhibited by treatment with TIBA only and with (Cd + Zn + TIBA) when compared with roots treated only with (Cd + Zn) ($P < 0.001$). However, LR development was not significantly different between plants treated with (Cd + Zn) alone and plants treated with (Cd + Zn + IBA) or IBA only ($P > 0.05$). These results indicate that the enhancement of LR development under (Cd + Zn)-treatment conditions is linked to auxin transport in rice plants.

### *Effect of Combined Treatment with Cd and Zn on Auxin Distribution in Rice Lateral Root Development*

Auxin distribution is one of the key processes regulating LR development. To determine whether the LR growth mediated by (Cd + Zn)-treatment involves the redistribution of auxin, transgenic rice containing *DR5-GUS*, which is widely used as a marker for monitoring the endogenous distribution of auxin [22], was used. Transgenic seedlings were treated with Cd, Zn or (Cd + Zn) for 7–9 days and then examined for GUS expression (representative ERs are shown in Figs. 3a, 3b). Lower levels of GUS activity were observed in seedlings treated with (Cd + Zn), particularly in the ER and AR tips, than in seedlings treated with Cd only. However, in plants treated with Zn alone, GUS staining was similar to that in control plants (Figs. 3a, 3b). GUS activity was quantitatively analyzed in 9-day samples from basal, middle and tip regions of ERs. As shown in Table 2, GUS activity in these regions, particularly in the tip, was lower in (Cd + Zn)-treated plants than in plants treated with Cd alone ($P < 0.05$), while similar levels of GUS activity were observed in the control and Zn-treated plants. Moreover, in experiments with non-transgenic rice, indole-3-acetic acid (IAA) contents in ERs were also markedly lower in (Cd + Zn)-treated plants than in Cd-alone-treated plants ($P < 0.05$) (Table 2). IAA content in ERs exposed to Zn was no different from that in control plants. The changes in IAA content brought about by the various treatments were similar to the changes in GUS activity. These results confirm that the LR growth affected by (Cd + Zn)-treatment is closely associated with a redistribution of auxin. The data also suggest that Cd stimulates the accumulation of auxin in roots, whereas Zn counteracts it. The (Cd + Zn) interaction is important for appropriate auxin homeostasis, the distribution of auxin, and LR development, at least under the experimental conditions used in this study.

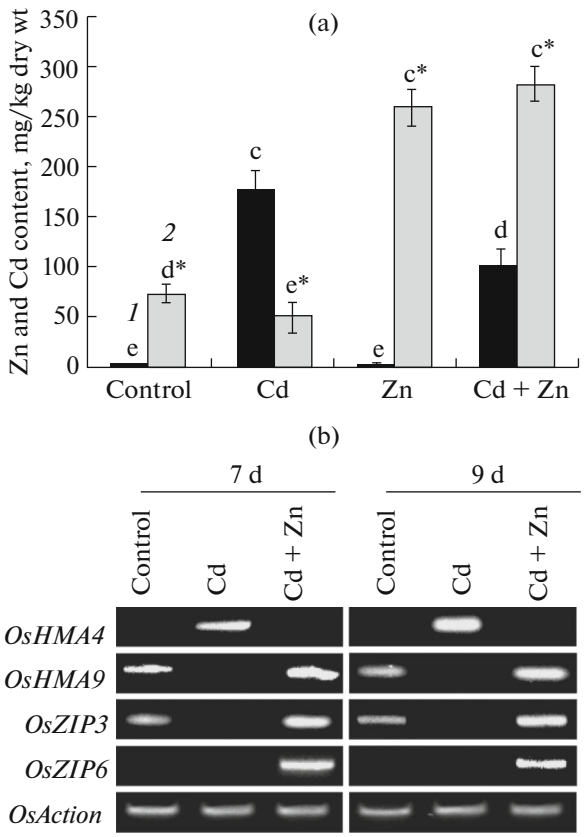


**Fig. 2.** Accumulation of Cd and Zn (a) and the expression of *OsHMA* and *OsZIP* genes (b) in the LRDRs exposed to different treatments. (a) Accumulation of Cd and Zn in the LRDRs exposed to different treatments for 9 days. (*1*) Cd content; (*2*) Zn content. Values represent means ± SE of three independent experiments. Different letters indicate that one treatment was significantly different from the other at $P < 0.05$; Asterisk (*) means that Zn was significantly different from Cd at $P < 0.01$; (b) expression profiles of *OsHMA* and *OsZIP* genes in the LRDRs exposed to (Cd + Zn)-treatments for 7 or 9 days. The *OsActin* gene was used as an internal control. The relative transcript levels of *OsHMA* and *OsZIP* genes were listed in Supplementary Table S2.

### *Mediation of Auxin Redistribution in Combined Treatment with Cd and Zn through Polar Auxin Transport and Protein Transport/Degradation Pathways*

Auxin distribution involves auxin signaling and polar auxin transport. Polar auxin transport is mediated by OsPIN auxin transport proteins. The exocytic trafficking of OsPIN proteins from endosomes to the plasma membrane could be inhibited by BFA, therefore affecting auxin transport. OsPIN, auxin transport protein, BFA inhibits the exocytic trafficking of OsPIN proteins from endosomes to the plasma membrane and therefore affects auxin transport. Auxin sig-

**Table 1.** Influences of TIBA and IBA on the growth of lateral roots exposure to (Cd + Zn)-treatment for 11 days

| Treatments | LR number of the ER, per plant | LR length of the ER, mm per plant | LR number of the ARs, per plant | LR length of the ARs, mm per plant |
|---|---|---|---|---|
| Cd + Zn | $67.89 \pm 8.5^{a}$ | $7.0 \pm 0.4^{a}$ | $52.46 \pm 2.7^{a}$ | $1.06 \pm 0.2^{a}$ |
| TIBA | $10.7 \pm 0.5^{e}$ | $1.3 \pm 0.1^{c}$ | $3.0 \pm 0.4^{e}$ | $0.08 \pm 0.01^{c}$ |
| Cd + Zn + TIBA | $19.95 \pm 1.1^{d}$ | $1.74 \pm 0.4^{c}$ | $9.05 \pm 1.2^{d}$ | $0.12 \pm 0.03^{c}$ |
| IBA | $65.2 \pm 9.7^{a}$ | $4.5 \pm 0.2^{b}$ | $45.6 \pm 4.1^{a}$ | $0.83 \pm 0.18^{a}$ |
| Cd + Zn + IBA | $71.15 \pm 3.2^{a}$ | $7.67 \pm 0.7^{a}$ | $50.1 \pm 3.2^{a}$ | $0.97 \pm 0.11^{a}$ |

LR—lateral root; ER—embryonic root; AR—adventitious root. In each repeat of every treatment, the roots of 20 plants were used, and the results are reported per plant. Control—MS medium; Cd—0.2 mM $Cd(NO_3)_2$; Zn—0.3 mM $Zn(SO_4)_2$; TIBA—1 μM; IBA—10 nM. Values represent means ± SE of three independent experiments. Different letters indicate that one treatment was significantly different from the other at $P < 0.05$.

**Table 2.** Effects of (Cd + Zn) on GUS activity and IAA content in different regions of ER after treatment for 9 days

| Treatments | GUS activity, fluorescence 4-MU/(μg protein h) | | | IAA content, nmol/g fr wt | | |
|---|---|---|---|---|---|---|
| | tip region | middle region | basal region | tip region | middle region | basal region |
| Control | $7.67 \pm 1.2^{b}$ | $4.54 \pm 0.4^{c}$ | $9.95 \pm 0.3^{b}$ | $22.02 \pm 2.2^{b}$ | $15.87 \pm 2.7^{b}$ | $28.67 \pm 1.3^{c}$ |
| Cd | $31.25 \pm 1.7^{a}$ | $18.92 \pm 1.4^{a}$ | $25.13 \pm 2.2^{a}$ | $60.74 \pm 7.3^{a}$ | $44.28 \pm 3.4^{a}$ | $51.87 \pm 4.3^{a}$ |
| Zn | $7.87 \pm 1.4^{b}$ | $6.02 \pm 1.1^{c}$ | $10.15 \pm 1.2^{b}$ | $23.78 \pm 1.3^{b}$ | $17.32 \pm 1.8^{b}$ | $30.02 \pm 2.4^{bc}$ |
| Cd + Zn | $8.52 \pm 1.3^{b}$ | $14.97 \pm 1.8^{b}$ | $13.16 \pm 1.6^{b}$ | $25.06 \pm 1.5^{b}$ | $18.46 \pm 3.9^{b}$ | $37.35 \pm 4.1^{b}$ |

For quantitative analysis of GUS activity (*DR5-GUS* transgenic rice) and IAA content (non-transgenic rice) in basal, middle and tip regions of ERs, 1 cm of each region were excised at least from 100 seedlings for each treatment. Control—MS medium; Cd—0.2 mM $Cd(NO_3)_2$; Zn— 0.3 mM $Zn(SO_4)_2$. Values represent means ± SE of three independent experiments. Different letters indicate that one treatment was significantly different from the other at $P < 0.05$.

naling is associated with auxin biosynthesis, transport and response, which are linked to protein metabolism. MG132 inhibits protein degradation and thereby mediates auxin distribution. To determine whether the redistribution of auxin observed after (Cd + Zn)-treatment is associated with polar auxin transport, transgenic *DR5-GUS* seedlings were treated with (Cd + Zn) plus TIBA or IBA for 9 days and were analyzed for GUS expression. As shown in Fig. 3c, similar patterns of GUS staining were observed in roots treated with (Cd + Zn + TIBA) and with TIBA only, and the GUS activity levels were higher than in (Cd + Zn)-treated roots, particularly in the root tips. However, the pattern of GUS staining was similar between plants treated with (Cd + Zn), IBA alone, and (Cd + Zn + IBA). These results confirm that the regulation of the redistribution of auxin by (Cd + Zn)-treatment is closely associated with polar auxin transport.

The link between the effects of (Cd + Zn)-treatment on auxin redistribution, and on protein transport and metabolism was further investigated by treating seedlings with BFA or MG132. Seven-day-old *DR5-GUS* rice plants were pretreated with BFA or MG132 for 3 h, after which the seedlings were additionally treated with (Cd + Zn) for 12 h. GUS activity was lower in both ERs and ARs in plants treated with (Cd + Zn + BFA) than in those treated with (Cd + Zn) only. Representative roots and GUS activity in ER tip regions are shown in Figs. 3d–3f. Similarly, GUS expression in ERs and ARs of plants treated with (Cd + Zn + MG132) for 12 h was significantly lower than in those of plants treated with (Cd + Zn) alone (Figs. 3d–3f). Together, these results suggest that (Cd + Zn)-treatment influences auxin redistribution through polar auxin transport, and protein transport and degradation pathways.

### *Effect of Combined Treatment with Cd and Zn on the Specific Genes Expression in the Auxin-Signaling Pathway in the LRDRs of Rice Roots*

To further investigate the relationship between the changes in LR development induced by (Cd + Zn)-treatment and auxin signaling in rice roots, a comprehensive expression analysis of 67 key genes in the auxin-signaling pathway was performed using semi-quantitative RT-PCR. The genes under investigation comprised 7 *OsYUCCA* family genes (involved in auxin biosynthesis), 9 *OsPIN* genes (pin-formed, auxin efflux carriers), 25 *OsARF* genes (auxin-response factors), and 26 *OsIAA* genes (auxin-response regulators). Expression data are provided in Supplementary Table S2. First, the expression of plants treated for 7 days with (Cd + Zn) or with Cd alone was compared. In (Cd + Zn)-treated roots, the expression of ten genes (*OsYUCCA6*, *OsYUCCA7*, *OsPIN5a*, *OsARF10*, *OsARF15*, *OsARF16*, *OsIAA7*, *OsIAA18*, *OsIAA21* and *OsIAA24*) was higher and the expression

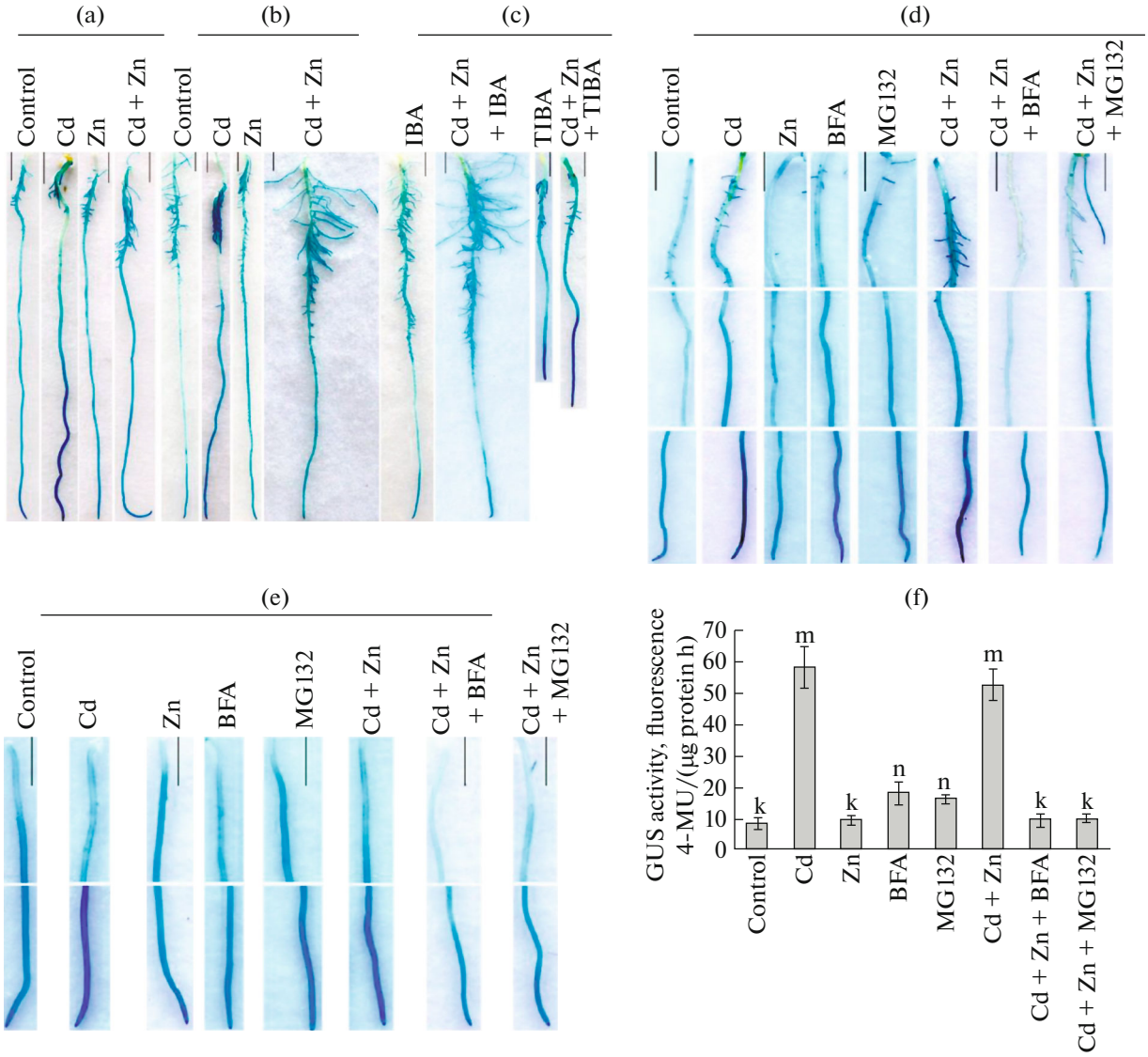


**Fig. 3.** GUS activity in DR5-GUS rice seedlings exposed to treatment with various compounds. (a, b) GUS activity in DR5-GUS rice seedlings during 7–9 days of development in response to treatment with (Cd + Zn). (a) ERs on the seventh day; (b) ERs on the ninth day; (c) ERs after treatment with (Cd + Zn + TIBA) or IBA for 9 days; (d) ERs (basal, middle and apex regions); (e) ARs (basal and apex regions) and (f) fluorometric assays of GUS activity of ER tips of 7-day-old seedlings after treatment with (Cd + Zn + BFA) or MG132 for 12 h. Scale bars for (a–e) are 5 mm. Values represent means ± SE of three independent experiments. Different letters indicate that one treatment was significantly different from the other at $P < 0.05$ (f).

of 18 genes (*OsYUCCA1*, *OsPIN1a*, *OsPIN1c*, *OsPIN5b*, *OsPIN10b*, *OsARF1*, *OsARF7*, *OsARF9*, *OsIAA8*, *OsIAA13*, *OsIAA14*, *OsIAA15*, *OsIAA16*, *OsIAA19*, *OsIAA20*, *OsIAA23*, *OsIAA30*, and *OsIAA31*) was lower than in roots treated with Cd alone (Fig. 4). Expression levels were also compared after treatment for 9 days. Compared to the Cd alone treatment, the expression levels of four genes (*OsYUCCA6*, *OsYUCCA7*, *OsIAA14*, and *OsIAA23*) were lower in the (Cd + Zn)-treated group and the expression levels of seven genes (*OsYUCCA5*, *OsPIN1b*, *OsPIN1c*, *OsPIN5b*, *OsPIN10b*, *OsARF15*, and *OsIAA16*) were higher (Fig. 4).

These results indicate that the transcription of key components in the auxin-signaling pathway is different depending on whether the roots are subjected to Cd-treatment or (Cd + Zn)-treatment. Furthermore, many of the auxin genes showed different levels of expression at 7 and 9 days, suggesting that the effect of (Cd + Zn)-treatment on auxin gene expression is closely related to the LR developmental stage.

### *Influence of Combined Treatment with Cd and Zn on the Specific Cell-Cycle Genes Expression in the LRDRs of Rice Roots*

LR formation and growth is associated with cell division. To study the link between LR developmental changes induced by (Cd + Zn)-treatment and cell-

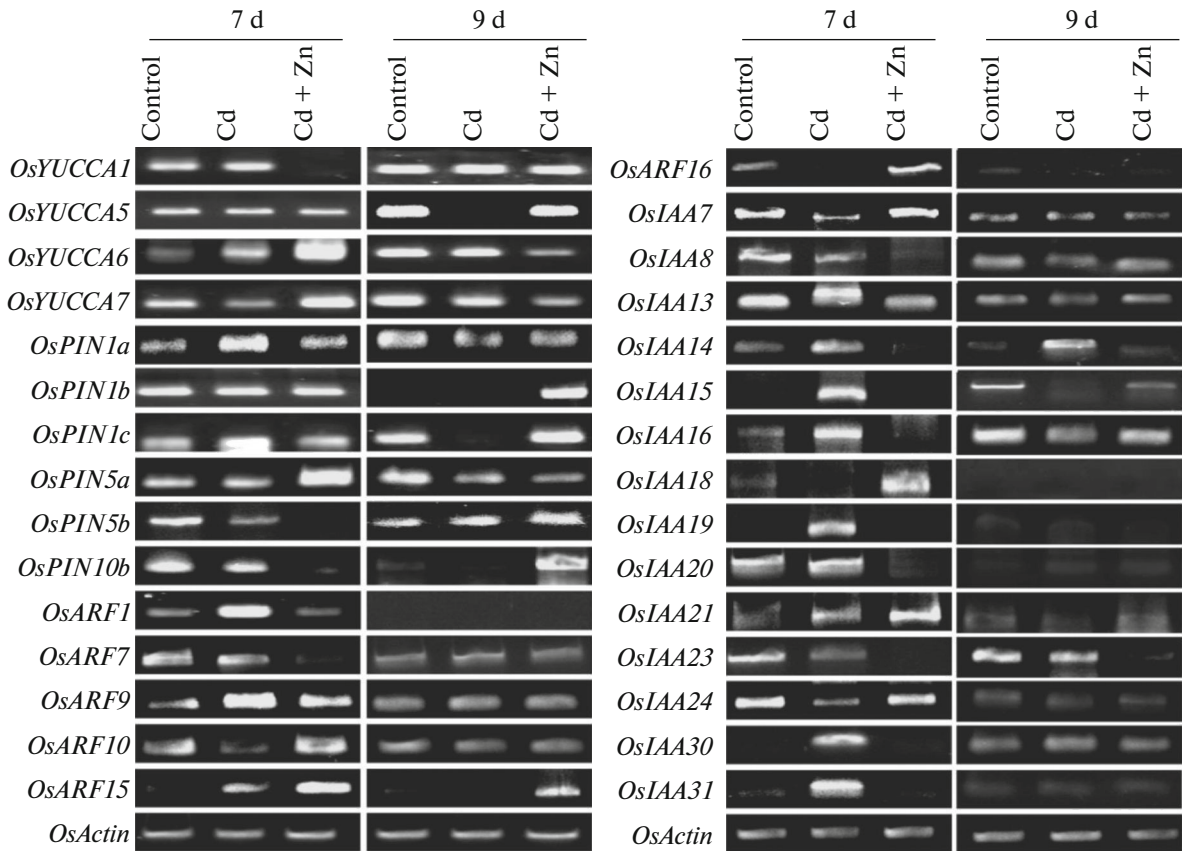


**Fig. 4.** Expression profiles of *OsYUCCA*, *OsPIN*, *OsARF*, and *OsIAA* genes in the LRDRs exposed to (Cd + Zn)-treatment for 7 or 9 days. Expression profiles were analyzed using RT-PCR. The *OsActin* gene was used as an internal control. The relative transcript levels of these genes were listed in Supplementary Table S2.

cycle gene expression, a comprehensive expression analysis of core cell-cycle genes (60 genes) [26] was performed using semi-quantitative RT-PCR. Expression data are provided in Supplementary Table S2. The cell-cycle genes affected by treatment with Cd or (Cd + Zn) at 7 or 9 days are presented in Fig. 5 and summarized below. For the 7-day treatment, ten of the sixty cell-cycle genes had higher expression levels in (Cd + Zn)-treated roots than in Cd-treated roots (*Oryza;CycF1;4*, *Oryza;CycF2;3*, *Oryza;CycT1;1*, *Oryza;CycU2;1*, *Oryza;CycU4;3*, *Oryza;CDKG;1*, *Oryza;CKL2*, *Oryza;CKL7*, *Oryza;DEL1*, and *Oryza;KRP4*) whereas six genes had lower expression (*Oryza;CycA1;1*, *Oryza;CycB1;1*, *Oryza;CycD4;2*, *Oryza;CKL1*, *Oryza;CKL10*, and *Oryza;RB2*). For the 9-day treatment, 11 of the 60 cell-cycle-related genes had lower expression levels in the (Cd + Zn)-treated roots than in the Cd-treated plants (*Oryza;CycA1;1*, *Oryza;CycD4;2*, *Oryza;CycU2;1*, *Oryza;CDKD;1*, *Oryza;CDKF;3*, *Oryza;CKL8*, *Oryza;CKS1*, *Oryza;DEL1*, *Oryza;DP1*, *Oryza;KRP1*, and *Oryza;KRP4*), and nine genes showed higher expression (*Oryza;CycB1;1*, *Oryza;CycB2;2*, *Oryza;CycD2;2*, *Oryza;CycT1;1*, *Oryza;CDKC;1*, *Oryza;CKL1*, *Oryza;CKL2*, *Oryza;CKL6*, and *Oryza;RB1*) (Fig. 5).

These data show that key cell-cycle genes are differentially affected by Cd- and (Cd + Zn)-treatment in rice roots. Moreover, many of the cell-cycle genes showed different levels of expression at 7 and 9 days, suggesting that the regulatory effect of (Cd + Zn)-treatment on cell-cycle gene expression is associated with the LR developmental stage.

## DISCUSSION

Cd levels are lower, and Zn levels are higher, in roots treated with (Cd + Zn) than in roots treated with Cd alone [20]. Similar results were obtained in the current study (Fig. 2a). *HMA* and *ZIP* gene families are involved in metal efflux, uptake and homeostasis in plants [4, 5]. In this study, expression levels of *OsHMAs* and *OsZIPs* such as *OsHMA4* and *OsZIP6* differed between (Cd + Zn)-treated and Cd-treated roots (Fig. 2b). The high accumulation of Zn and Cd in (Cd + Zn)-treated roots may be a consequence of the increased expression of *OsZIP*s, which participate in the absorption of Zn and Cd [26], or a consequence of the decreased expression of *OsHMA9,* which take in Zn and Cd efflux from root cells [4]. Our data indicate that rice plants may use varied molecular mechanisms for the

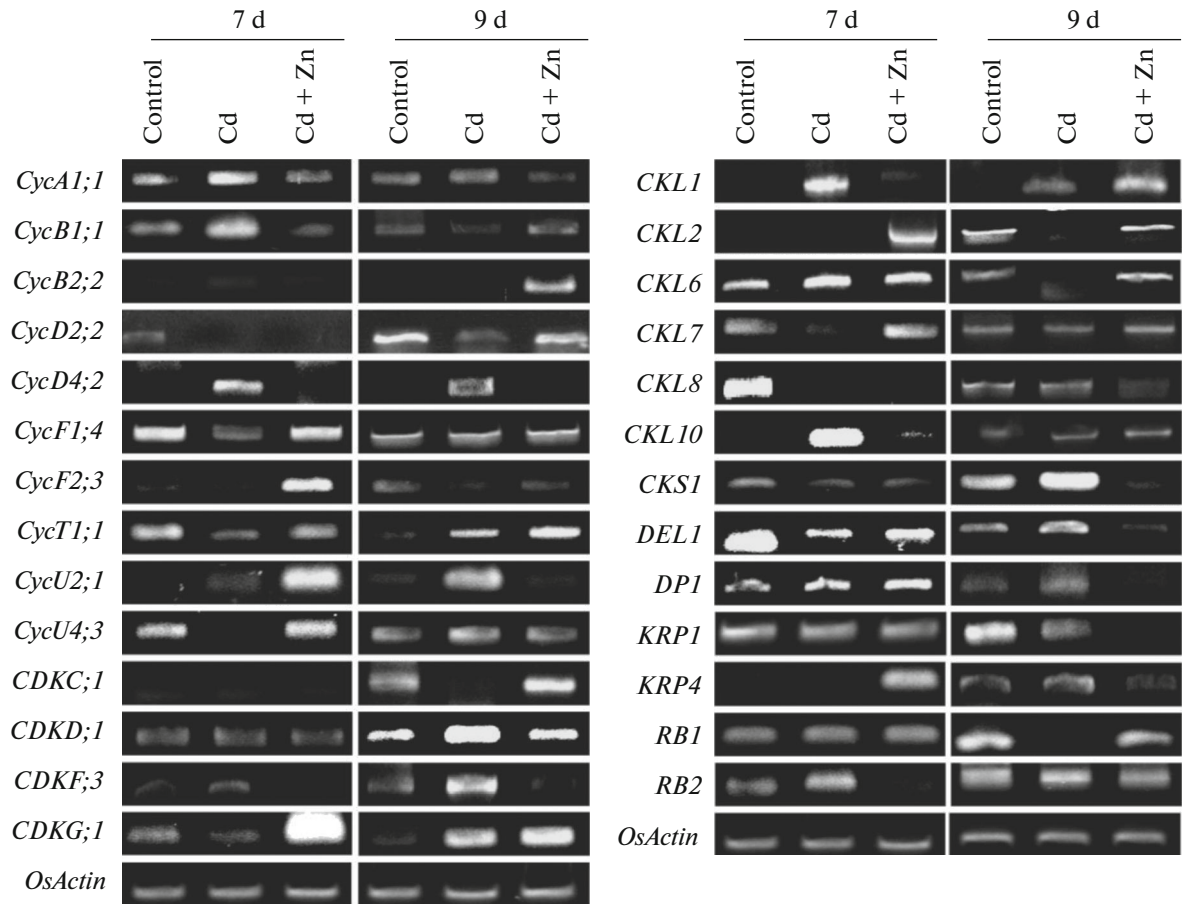


**Fig. 5.** Expression profiles of core cell-cycle genes in the LRDRs exposed to (Cd + Zn)-treatment for 7 or 9 days. Expression profiles were analyzed using RT-PCR. The *OsActin* gene was used as an internal control. The relative transcript levels of these genes were listed in Supplementary Table S2.

regulation of Cd and Zn uptake in response to Cd and (Cd + Zn) stress.

Data shown in Figs. 1 and 2a indicate that (Cd + Zn)-treatment and Zn-treatment increased accumulation of Zn; however, (Cd + Zn)-treatment significantly enhanced LR growth, but Zn alone did not. Therefore, the promotion of LR growth by (Cd + Zn)-treatment cannot be attributed to an increase in the Zn level. We therefore propose three pathways for the mediation of (Cd + Zn)-stimulated LR development.

Auxin signaling is involved in regulating LR development under both normal and stress conditions [12, 15]. In *Arabidopsis,* LR numbers are lower in some auxin mutants such as *iaa28*, *aux1*, and *pin1/3/4/7*, while LR numbers are higher in mutants such as *sur1* and *arf8* [12]. The role of *YUCs* and *PIN*s in LR development in *Arabidopsis* was reported by Liu et al. [13]. In this study, we found that changes in the expression patterns of genes associated with auxin signaling and transport are important for LR growth under (Cd + Zn)-treatment conditions. The results indicate that (Cd + Zn) affects LR growth by regulating auxin signaling in two ways. First, (Cd + Zn) influences auxin distribution. Changes in the distribution of auxin correlate well with LR formation in heavy-metal-stressed plants [8, 11, 15]. Similar results were obtained in this study when plants were treated with (Cd + Zn) but not when they were treated with Cd only. Quantitative analysis of GUS activity and IAA content in different regions of ERs treated with (Cd + Zn) confirmed the notion that (Cd + Zn) influences auxin distribution in rice plants (Table 2). (Cd + Zn) stimulated LR development by maintaining appropriate auxin homeostasis and distribution, at least under the current experimental conditions (Figs. 1a–1d, 3a, 3b). Auxin distribution involves both auxin transport and metabolism. In this study, we confirmed that the redistribution of auxin observed in (Cd + Zn)-treated plants is a consequence of a change in auxin transport by using a polar auxin transport inhibitor, TIBA, and a protein transport inhibitor, BFA (Table 1, Figs. 3c–3f). Furthermore, we found that the transcription of certain *OsPIN* genes (such as *OsPIN5a* and *OsPIN10b*), which encoded auxin efflux carriers that control polar auxin transport [21, 22], was differentially influenced by (Cd + Zn)

and Cd, and differed depending on the treatment duration (7 or 9 days). Therefore, the differences in polar auxin transport observed between roots treated with (Cd + Zn) and those treated with Cd may be a consequence of changes in the expression of these *OsPIN* genes (Figs. 3 and 4). The link between the auxin redistribution in (Cd + Zn)-treated roots and auxin metabolism was confirmed by using different IAA content and the protein degradation inhibitor MG132 (Table 2, Figs. 3d–3f). Moreover, (Cd + Zn)-treated and Cd-treated roots showed differences in the expression of several *OsYUCCA* genes (such as *OsYUCCA6* and *OsYUCCA7*), and the expression of these genes varied depending on the treatment time (7 or 9 days) (Fig. 4).

Second, (Cd + Zn)-treatment affected the expression of auxin-response genes. Cd has already been shown to influence the expression of auxin-response genes belonging to *OsARF* and *OsIAA* families [21, 22]. In this study, among these genes, seven (such as *OsARF10* and *OsIAA18*) were upregulated and 13 (such as *OsARF1* and *OsIAA20*) were downregulated under (Cd + Zn)-treatment conditions when the expression levels were compared to those under Cd-treatment conditions during the 7-day treatment. During the 9-day treatment, only four genes (such as *OsARF15* and *OsIAA23*) were up- or downregulated by (Cd + Zn)-treatment compared to Cd-treatment (Fig. 4). The expression of most auxin genes changed between 7 and 9 days in roots treated with (Cd + Zn), indicating that (Cd + Zn) affects specific genes involved in auxin signaling in a developmentally regulated manner. Collectively, these results suggest that the effects of (Cd + Zn)-treatment on LR development in rice are a consequence of the redistribution of auxin brought about by changes in auxin-related gene expression.

Cell-cycle regulation plays a crucial role in LR growth [11, 17]. A number of genes control the progression of the plant cell cycle [27], and increased LR formation in heavy-metal-stressed plants is associated with mitotic activity [11]. However, the relationship between LR growth and the expression of cell-cycle genes in (Cd + Zn)-stressed rice seedlings has been unclear. In this study, 16 genes (7 days, such as *Oryza;CDKG;1* and *Oryza;CKL10*) and 20 genes (9 days, such as *Oryza;CDKC;1* and *Oryza;CKS1*) were up- or downregulated upon treatment with (Cd + Zn) when compared to treatment with Cd alone (Fig. 5). These results suggest that (Cd + Zn)-treatment, at least in part, affects cell-cycle progression via regulation of specific cell-cycle genes. Cell-cycle genes influenced by (Cd + Zn)-treatment showed changes in expression depending on the treatment time (7 or 9 days), suggesting that the effect of (Cd + Zn)-treatment on cell-cycle gene expression is closely related to LR development. Moreover, cell-cycle gene expression was different between Cd-treated and (Cd + Zn)-treated roots, suggesting that (Cd + Zn) influences specific cell-cycle proteins related to cell division. Both auxin and the cell cycle are involved in LR growth. Therefore, the LR growth induced by (Cd + Zn)-treatment observed in this study may result from the combined effects of auxin signaling and expression of specific cell-cycle genes.

Ions transport may exhibit competition between each other, since the metal transporters usually have a broad substrate range and tend to transport the same valence state ions. The absorption of divalent cations Cd and Zn can be mediated by the same transporter, such as ZIP family Zn transporter ZIP4 [27]. In *A. thaliana*, AtHMA2 and AtHMA4 function in root-to-shoot transport of Zn and Cd [27, 28]. In tobacco, $P_{1B}$-ATPase HMA4.1 and HMA4.2 are involved in root-to-shoot translocation of Zn and Cd [28]. In rice, the expression of *OsHMA9* could be induced by a high concentration of divalent cations Cu, Zn, and Cd [4]. Zn could compete with Cd by the same transporters, and further reducing Cd toxicity by its antagonistic effects on Cd uptake and transport in the roots [3, 20–22]. In this study, we found that Cd content in the root treated with (Cd + Zn) was significantly lower than in treated with Cd alone (Fig. 2a). So, there might be the same transporters that transport Cd and Zn simultaneously, thereby decreasing Cd content and mitigate Cd toxicity under the stress of (Cd + Zn) compared with Cd only; however, the specificity for the transporters in Cd and Zn uptake need further investigation.

Redox homeostasis is important for plant growth and stress responses, and heavy metals elicit oxidative stress in plants. It has been widely studied on the relationship between metal toxicity and oxidative stress by measuring the redox metabolic components in stressed plants. In rice roots, Cd induced activation of mitogen-activated protein kinase (MAPK) may confer rice plants tolerance to Cd, and the NADPH oxidase is associated with this process [29]. Besides NADPH oxidase, – other antioxidant network components, such as SOD, ascorbate peroxidase (APX) and CAT, are involved in plant tolerance to many other metals, such as Ni, Cu and Al [29]. Our previous study in rice under Cd stress showed that hydrogen peroxide could mediate root growth via the modification of auxin signaling [22]. So the decreased Cd content may lower the oxidative stress imposed by Cd toxicity and further improve the lateral root system growth via the modification of auxin signaling.

The metal cross-talk between Cd and As also revealed that auxin biosynthesis and transport operate in lateral root primordia organization and development in rice plants [1]. Besides auxin and reactive oxygen, ABA, JA and SA have been reported functioning in the process of Cd stress response [30].

Taken together, the joint treatment of plants with (Cd + Zn) as compared to Cd alone promoted LR

growth by the decrease of Cd content, influencing auxin signaling, cell-cycle gene expression, other phytohormones and reactive oxygen species participation. Our findings should help to elucidate how the combined effect of Cd and Zn on Cd content, auxin signaling and cell cycle, further influencing LR growth. Based on our results, we propose that (Cd + Zn)-treatment decreases Cd content compared with Cd alone and further influences auxin signaling, including auxin distribution and response, by regulating the expression of auxin-related genes and protein transport/degradation. (Cd + Zn)-treatment affects cell division by altering the expression of specific cell-cycle genes either directly or via auxin-signaling pathways, thereby promoting LR growth.

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## COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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