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cGMP is involved in Zn tolerance through the modulation of auxin redistribution in root tips

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ABSTRACT

Excess zinc (Zn) inhibits primary root (PR) growth but induces lateral root (LR) formation. Both auxin and cGMP play a role in controlling root growth in plants. However, whether and how their interaction is involved in Zn-regulated root development remain unclear. Here, we reported that excess Zn leads to auxin accumulation in root tips, as indicated by DR5:GUS expression. Further study showed that excess Zn represses PIN4:GFP abundance in root tips and that PR elongation and LR formation in the *pin4* mutant is insensitive to excess Zn. Excess Zn also elevates cyclic guanosine monophosphate (cGMP) production in seedlings. Supplementation with the exogenous cGMP donor 8-bromoguanosine 3′,5′-cyclic guanosine monophosphate (8-Br-cGMP) increased PR elongation and LR formation in Zn-treated seedlings, whereas the guanylate cyclase (GC) inhibitor LY83583 decreased these processes. Additional physiological and genetic analyses indicated that PIN4 is involved in cGMP-modulated root development in Zn-treated seedlings. Taken together, these results indicate that Zn-regulated cGMP production plays an important role in modulating root development by maintaining PIN4 abundance in excess Zn-treated roots and subsequent adaptation to Zn toxicity.

1. Introduction

Zinc (Zn) is an essential micronutrient for all organisms and serves as a cofactor for more than 300 enzymes (González-Guerrero et al., 2005; Li et al., 2013). In plants, Zn is involved in modulating a wide range of physiological processes, including antioxidative defenses, respiration, auxin biosynthesis, and cell proliferation; thus, Zn plays an important role in regulating plant growth and development. However, excess Zn is toxic to plants. High Zn concentration in soil (150–300 μg g^{-1}) is strongly toxic and its phytotoxicity depends on plant type and plant development stage (Yadav, 2010; Baran, 2013). Increased Zn contents in seedlings are correlated with decreased fresh weight as well as inhibition of net photosynthetic rate, the rate of apparent photosynthetic electron transport, transpiration and stomatal conductance in bean seedlings (Vassilev et al., 2011). Decreased soil pH caused by acid rain and the use of soil-acidifying ammonia fertilizers aggravates Zn toxicity in plants due to the increase in the solubility of Zn^{2+} in the soil solution (Kim et al., 2009). The symptoms of Zn toxicity in plants are visible at Zn concentration of ≥300 μg g^{-1} DW in the leaves, although some crops are more sensitive to Zn toxicity at over 100 μg g^{-1} DW in the leaves (Shanmugam et al., 2011). Treatment with 50 μM $ZnSO_4$ for 12 d decreased the shoot mass to 75% in 1/2 MS medium-grown *Arabidopsis* seedlings and the Zn concentration reaches about 240 μg g^{-1} DW in its leaves (Shanmugam et al., 2011). Zn toxicity inhibits plant growth and development by disequilibrating the uptake and redistribution of nutrients and by disturbing the antioxidant defense system and metabolic processes such as photosynthesis, transpiration, and protein biosynthesis (Xu et al., 2010).

Excess Zn leads to the displacement of Fe^{2+} from its protein-binding sites and disrupts protein functions; therefore, plants exposed to excess Zn become Fe deficient (Wintz et al., 2003). Tennstedt et al. (2009) reported that Zn toxicity induced phytochelatin (PC) formation and provided the driving force for the accumulation of Zn. In our previous study, we found that Zn toxicity inhibits primary root (PR) growth and induces lateral root (LR) formation by an NO-induced ROS signaling pathway in the Zn hyperaccumulator *Solanum nigrum* (Xu et al., 2010).

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However, the underlying physiological and molecular mechanisms of Zn-modulated root system development remain unclear.

Auxin plays a central role in modulating root system architecture (RSA) in plants (Tian and Reed, 1999; Gray et al., 2001; Rogg et al., 2001; Yang et al., 2004; Tatematsu et al., 2004; Uehara et al., 2008; Nan et al., 2014). The maintenance of a steep auxin gradient in root tips is essential for auxin to regulate PR elongation and LR initiation (Laskowski et al., 2008; Shi et al., 2015). Auxin gradients are established and maintained by plasma membrane-localized carriers. The auxin influx carrier AUX1 modulates PR growth and LR initiation (De Smet et al., 2007). The rice loss-of-function mutant *osaux1* shows longer PR and shorter root hairs and exhibits increased sensitivity to cadmium (Cd) toxicity, suggesting that AUX1 plays a role in the plant response to Cd stress (Yu et al., 2015). The auxin efflux carrier mutants *pin2* and *pin3* show reduced LR initiation (Dubrovsky et al., 2009). Giehl et al. (2012) reported that local Fe supply in LR improved AUX1 abundance and thereby sustained auxin gradients in LR apices and subsequent LR elongation. Li et al. (2015) further demonstrated that AUX1 and PIN2 protect LR formation in *Arabidopsis* during the early stages of iron (Fe) stress. Arsenite (As^{3+}) toxicity inhibits auxin transport. The auxin carrier mutants *aux1*, *pin1*, and *pin2* are more sensitive to As^{3+} than *col-0* control (Krishnamurthy and Rathinasabapathi, 2013). Aluminum (Al) toxicity induces local auxin accumulation in the transition zone of root tips by specifically inducing expression of the auxin biosynthesis genes *TAA1* (Yang et al., 2004) and *YUCCA* (*YUC*) (Liu et al., 2016a, 2016b) in the zone, thereby inhibiting PR growth. Al toxicity also inhibits root elongation by altering auxin distribution via disruption of PIN2 and AUX1-mediated auxin transport (Shen et al., 2008; Sun et al., 2009). Wu et al. (2014) further demonstrated that overexpression of OsPIN2 alleviated Al-induced inhibition in rice roots. Al toxicity-induced root elongation inhibition is associated with the alteration of auxin metabolism and transport in alfalfa (*Medicago sativa*) roots (Zhou et al., 2014; Wang et al., 2016a, 2016b). Ruíz-Herrera and López-Bucio (2013) found that LR formation in response to Al toxicity and P deficiency may involve common signaling mechanisms and both *ARF7* and *ARF19* are important for Al-mediated PR growth inhibition. Wang et al. (2016a, 2016b) found that exogenous IAA increased Al-induced citrate exudation by upregulating the gene expression of *GmMATE* and increasing the phosphorylation of the plasma membrane H^+-ATPase in soybean roots. These studies indicate that modulation of auxin redistribution by auxin carriers in roots is necessary for root growth in response to heavy metal stresses.

Cyclic guanosine 3′,5′-monophosphate (cGMP) is an important secondary messenger in animals and plants and has the multiple functions in regulating growth and development. cGMP is synthesized *in vivo* from GTP by catalysis through guanylate cyclase (GC). There are six proteins that show the GC activity, including *AtGC1*, *AtWAKL10*, *AtBRI1*, *AtPepR1*, *AtPSKR*, and *AtNOGC1*, in *Arabidopsis* (Wong and Gehring, 2013). cGMP is involved in modulating root development, seed germination, stomatal movement, and stress responses in plants (Teng et al., 2010; Joudoi et al., 2013; Nan et al., 2014). Several studies indicated that cGMP is also involved in auxin-mediated PR growth, LR and adventitious root formation, and the gravitropic response (Hu et al., 2005; Bai et al., 2012). cGMP modulates auxin-dependent gene expression and Aux/IAA protein degradation through the stimulation of cGMP-dependent protein kinases (PKGs) activity (Nan et al., 2014).

Several studies on cGMP activity have suggested that the nitric oxide (NO)/cGMP-mediated signaling pathway broadly exists in animals and plants (Wendehenne et al., 2001; Lamattina et al., 2003; Pagnussat et al., 2003; Prado et al., 2004; Jacobi et al., 2007). Prado et al. (2004) demonstrated that NO and cGMP are the two important signaling molecules that are involved in Ca^{2+}-dependent cellular development within pollen. The interaction between NO and cGMP modulates root elongation and the gravitropic response in *Arabidopsis* (Jacobi et al., 2007). The cell-permeable cGMP derivative 3′,5′-cyclic guanosine monophosphate (8-Br-cGMP) further promotes auxin- and NO-induced root development, whereas the GC inhibitor LY83583 reversed the effects, which suggests that cGMP operates downstream of auxin and NO to modulate root development in plants (Pagnussat et al., 2003). Arazi et al. (2000) found that the interaction between cyclic nucleotide monophosphate (cNMP) and calmodulin in modulating heavy metal tolerance in tobacco. Romero-Puertas et al. (2007) has also found that the involvement of cGMP in Cd tolerance in pea plants. Nonetheless, the physiological and molecular mechanisms underlying cGMP-mediated root system development in response to heavy metal stresses remain poorly understood.

In this study, we investigated the roles of auxin and cGMP in excess Zn-regulated root system development. Our results indicate cGMP plays an important role in modulating PR growth and LR formation by maintaining PIN4 abundance and subsequent auxin redistribution in excess Zn-treated roots. Potential mechanisms involved in this process are discussed.

2. Materials and methods

2.1. Plant materials and growth conditions

In this study, we used transgenic and mutant *Arabidopsis* lines *DR5:GUS* (Ulmasov et al., 1997), *pAUX1:AUX1-YFP* (Swarup et al., 2004), *pPIN1:PIN1-GFP* (Benkova et al., 2003), *pPIN2:PIN2-GFP* (Blilou et al., 2005), *pPIN4:PIN4-GFP* (Blilou et al., 2005), *pCYCB1;1: CYCB1;1-GUS* (Colon-Carmona et al., 1999), *QC25:GUS* (Sabatini et al., 1999), *J2341* (Sabatini et al., 1999), *pPLT2:PLT2-GFP* (Matsuzaki et al., 2010), *pSHR:SHR-GFP* (Sabatini et al., 2003), and *pin4-3* (CS9368). Seeds were sterilized with 50% bleach for 5 min and then washed three times with sterile water. Sterilized seeds were germinated on 1/2 MS medium (Murashige and Skoog, 1962) containing 1% agar and 1.5% sucrose (pH 5.7) and incubated vertically using a 16 h light/8 h dark cycle. Five-day-old seedlings were transferred to fresh 1/2 MS medium supplemented with various components and subsequently grown for 2-4 days. The chemicals LY83583 and 8-bromoguanosine 3′,5′-cyclic guanosine monophosphate (8-Br-cGMP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Images of the seedlings were digitized using a scanner (Epson Perfection 1670, Japan) after stress treatment. The growth of the seedlings was measured using ImageJ software, version 1.38 (http://rsbweb.nih.gov/ij/download.htmlhttp://rsbweb.nih.gov/ij/download.html). At least 30 seedlings were used in each experiment. For the statistical analysis, we used Duncan's test ($P < 0.05$).

2.2. GUS staining and measurement of fluorescence microscopy

β-Galactosidase Reporter Gene Staining (GUS staining) was performed using GUS buffer (containing 1 mg/mL X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid cyclohexyl-ammonium), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, and 50 mM sodium phosphate buffer at pH 7.0) in the substrate at 37 °C for 1–5 h in the dark. Seedlings were washed and then examined under the microscope (Axioskop, Zeiss, Jena, Germany).

Endogenous NO production in the roots was detected using the specific NO fluorescence probe DAF-2 DA (Beyotime, China) according to the manufacturer's instructions. Fluorescence was then viewed using a confocal laser scanning microscope (Zeiss, excitation wavelength at 495 nm and emission wavelength at 515 nm). GFP fluorescence was detected using a confocal laser scanning microscope (Zeiss) with excitation and emission wavelengths of 488 and 520 nm, respectively.

2.3. QRT-PCR analysis

Five-day-old *Arabidopsis* seedlings were transferred to fresh media that contained or did not contain 200 μM $ZnSO_4$. After 1 d of treatment, the RNA was isolated from whole seedlings using an RNAiso Plus kit (TaKaRa) according to the manufacturer's instructions and the RNA concentration

was quantified using spectrophotometry. Reverse transcription was performed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa). The quantitative reverse transcription (qRT)-PCR was performed in a 7500 Real Time System (Applied Biosystems) using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). *ACTIN2* (AT3G18780) and *EF1α* (AT5G60390) were used as internal controls for qRT-PCR normalization using GeNorm (Czechowski et al., 2005). The specific primers are: 5′ATGTCTTCCTCTATGAACGTAGC3′ and 5′ACAGCGGTAAATTGGTATAAGG3′ for *AtASA1*, 5′ATGGTTATTGCGGTGGCGAC3′ and 5′ATCGTCGCCGACTCAATGTC3′ for *AtPAT1*, 5′CGGACTTACTCCAATGGCTCAG3′ and 5′GCTGCTGCAGGAGAACGCAACC3′ for *AtAMI1*, 5′GACCACCAAGGTGTTACAATCC3′ and 5′ATTATTGTGGCAGGGTCAGG3′ for *AtSUR1*, 5′CTCCAAGATCACAGGCCACGCTGGG3′ and 5′GACTCCTTAGACACACCAATCGAGTTC3′ for *AtTAA1*, 5′GGTGACACGGATCGGTTAGGGT3′ and 5′TGCCGAATAATGCATTACCCGT3′ for *AtYUC2*, 5′CTTGAGATTGATTCCGTTATTC3 and 5′GGAGAAGAAGTCGTTGTC3′ for *AtYUC3*, 5′TGCCTGTTCCAGCAACAATG3′ and 5′TAAGCAGAACACCGCCATTG 3′ for *AtAAO1*, 5′GGAGTCAGCGAGGTGGAAGT3′ and 5′TGCTCCTTCGGTCTGTCCTAA3′ for *AtAAO3*, 5′CACGATGATGCTCGCGAGACT3′ and 5′TCACTTCACCGTCGGGTAGAGA3′ for *AtCYP79B2*. All primer pairs were detected by only one peak in DNA melting curves, indicating a high specificity of the primers. The qRT-PCR analysis was performed on three biological replicates, and there were three technical repetitions.

2.4. Quantification of IAA content

Five-day-old *Arabidopsis* seedlings were transferred to fresh media that contained or did not contain 200 μM $ZnSO_4$ for 1 d. After treatment, the roots (0.1 g of fresh weight) were collected and immediately frozen in liquid nitrogen. IAA contents were quantified according to the methods of Gao et al. (2014). After extraction, IAA was purified, methylated, and resuspended in 100 μL of ethyl acetate. Endogenous IAA contents were analyzed using GC–MS (Liu et al., 2016a, 2016b).

2.5. Measurement of the production of cGMP

The frozen tissues (0.1 g) were immersed in 0.5 ml of 0.1 M HCl and were homogenized on ice. The supernatant was collected, and the cGMP content was analyzed using a cGMP ELISA kit (BioVision, USA) according to manufacturer's instructions.

3. Results

3.1. Effects of Zn toxicity on root growth and development in arabidopsis

To investigate the effects of excess Zn on root growth and development, five-day-old *Arabidopsis* seedlings were transferred to fresh 1/2 MS medium (containing 15 μM $ZnSO_4$) supplemented with 0, 100, 200, 400, or 600 μM $ZnSO_4$. After 3 d of treatment, primary root (PR) elongation (Fig. 1A), lateral root (LR) number (Fig. 1B), and total LR primordia number (Fig. 1C) were measured. The PR growth was inhibited by 30.1% in 200 μM $ZnSO_4$-, 53.9% in 400 μM $ZnSO_4$-, and up to 73.6% in 600 μM $ZnSO_4$-treated seedlings. Excess Zn markedly induced LR formation, and the maximum LR number was observed under 200 μM $ZnSO_4$ conditions. This LR number gradually decreased with increasing Zn concentration.

Excess Zn inhibited PR growth. Therefore, we next analyzed the effects of excess Zn on the meristematic cell division potential using the transgenic line *pCYCB1;1:CYCB1;1-GUS*, a marker used to monitor the cell cycle in roots. Excess Zn repressed the GUS activity in pCYCB1;1:CYCB1;1-GUS (Fig. 2A) seedlings, indicating that excess Zn inhibited PR growth by reducing the meristematic cell division potential. We then tested whether the excess Zn-inhibited PR elongation was caused by reducing stem cell niche activity using the quiescent center (QC)-specifically expressed *QC25:GUS*, columella stem cell (CSC)-specific marker *J2341*, *pPLETHORA2 (PLT2):PLT2-GFP* reporter, and *pSHORT ROOT (SHR): SHR-GFP* reporter lines (Fig. 2B–E). All these marker lines and reporter lines were normally expressed in roots, suggesting that excess Zn did not affect the stem cell niche activity.

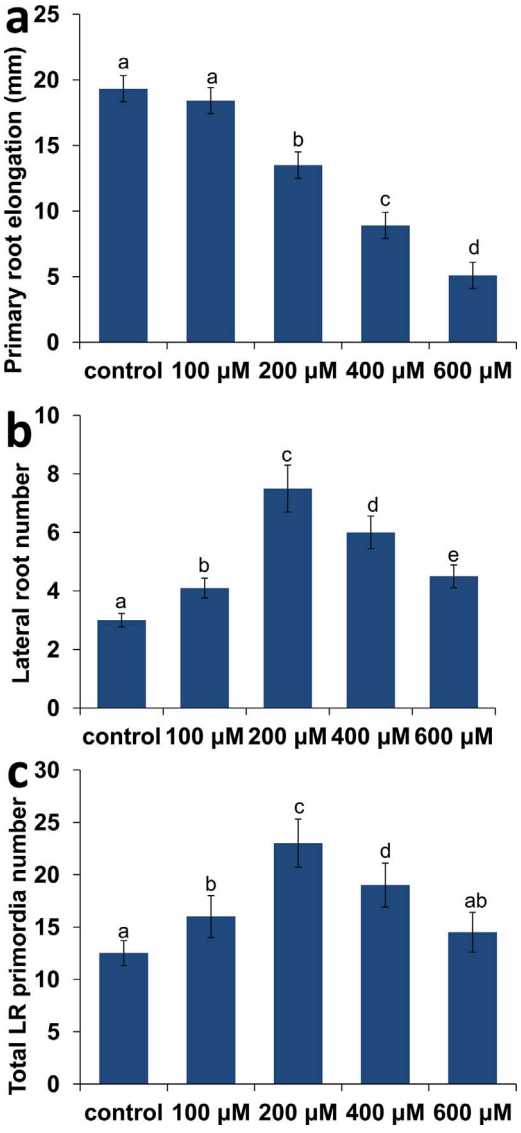

Fig. 1. Excess Zn inhibited PR growth and induced LR formation. Five-day-old seedlings were transferred to 1/2 MS medium supplemented with 0–600 μM $ZnSO_4$ for 2 d. The PR elongation (a), LR number (b) and total LR primordia number (c) were determined. Error bars represent the SD. Different letters indicate that they were significantly different at $P < 0.01$ according to Tukey's test.

3.2. PIN4 is involved in excess Zn-disrupted auxin transport in root tips

Auxin is a key regulator of root system development (Li and Jia, 2013). Therefore, we examined auxin distribution in root tips exposed to excess Zn using the auxin-responsive *DR5:GUS* transgenic marker line. Excess Zn increased DR5:GUS expression in the meristem and elongation zones of the root tips, and the maximum DR5:GUS expression was observed in roots treated with 200 μM $ZnSO_4$ for 6 h compared with that in control seedlings (Fig. 3A and B). We then measured IAA contents in Zn-treated roots using gas chromatography-mass spectrometry (GC–MS). As shown in Fig. 3C, excess Zn elevated IAA levels in *Arabidopsis* roots. These data indicate that excess Zn induced auxin accumulation in the roots.

To elucidate how Zn increased auxin accumulation in the roots, we

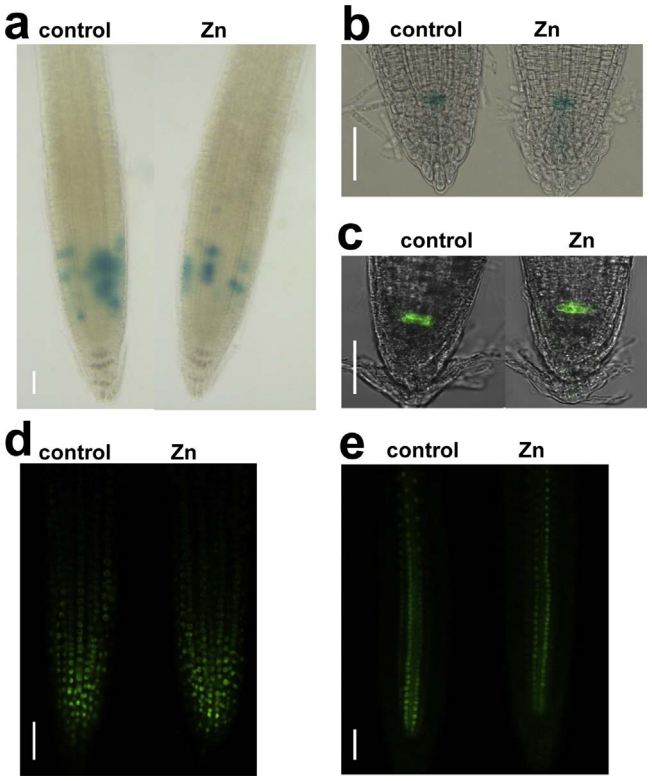

Fig. 2. Excess Zn inhibited PR growth through the repression of meristematic cell division potential but not stem cell niche activity. Five-day-old seedlings were transferred to 1/2 MS medium supplemented with 200 μM $ZnSO_4$ for 1 d. Images of GUS staining of *pCYCB1;1:CYCB1;1-GUS* (a) and *QC25:GUS* (b) root tips and GFP fluorescence in J2341 (c), *pPLT2:PLT2-GFP* (d) and *pSHR: SHR-GFP* (e) root tips are shown. Bars = 50 μm.

analyzed the transcript levels of auxin biosynthesis-related genes using quantitative reverse transcription (qRT)-PCR analysis. Consistent with the observation of increased DR5:GUS expression and IAA contents in the roots, the qRT-PCR results indicated that excess Zn significantly induced the expression of many auxin biosynthesis-related genes, including *ATP SULFURYLASE ARABIDOPSIS1* (*ASA1*) (Logan et al., 1996), *SUPERROOT 1* (*SUR1*) (Boerjan et al., 1995), *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* (*TAA1*) (Stepanova et al., 2008), *YUCCA2* (*YUC2*), *YUC3* (Cheng et al., 2006), *CYTOCHROME P450* (*CYP79B2*) (Mikkelsen et al., 2000), and *ABSCISIC ALDEHYDE OXIDASE3* (*AAO3*) (Seo et al., 1998); however, the expression of *PHOSPHORIBOSYLANTHRANILATE TRANSFERASE 1* (*PAT1*) (Rose and Last, 1997) was repressed and *AMIDASE-LIKE PROTEIN 1* (*AMI1*) (Pollmann et al., 2003) and *AAO1* (Seo et al., 1998)were unaffected (Fig. 3D). Auxin carriers play a role in modulating auxin distribution in root tips and affecting root system architecture (RSA). We thus examined the effect of excess Zn on the expression of auxin carriers using transgenic marker lines that express *pAUX1:AUX1-YFP* and *pPIN1/2/4: PIN1/2/4-GFP*. Excess Zn significantly reduced PIN4 abundance in the root tips, as indicated by PIN4:GFP fluorescence (Fig. 4A and B), whereas the expression patterns of AUX1, PIN1, and PIN2 were almost unaffected (Fig. 5). To further investigate the role of PIN4 in excess Zn-regulated root system development, we examined the PR elongation in the *pin4-3* mutant under excess Zn. The PR elongation was less inhibited in the *pin4-3* mutant compared to that of the wild-type *col-0* seedlings (Fig. 4C and D). These data indicate that PIN4 is involved in Zn-mediated root system development in *Arabidopsis*.

3.3. Excess Zn induced cGMP production in roots

In our previous study, we found that excess Zn induced NO over-accumulation in *Solanum nigrum* roots (Xu et al., 2010). NO could activate guanylate cyclase (GC), an enzyme that catalyzes cGMP biosynthesis (Pagnussat et al., 2003). cGMP is an important signal molecule that modulates root system development (Nan et al., 2014). We thus investigated the production of NO and cGMP in *Arabidopsis* roots. Similar to *Solanum nigrum* seedlings, excess Zn significantly induced NO accumulation in *Arabidopsis* roots. The NO production markedly increased at 6 h and peaked at 12 h after treatment with 200 μM $ZnSO_4$ (Fig. 6). Excess Zn also significantly induced cGMP production in roots (Fig. 7A). The levels of cGMP began to increase at 12 h and peaked at 1 d after Zn treatment, implying that Zn-induced cGMP production is a late stage response to Zn stress.

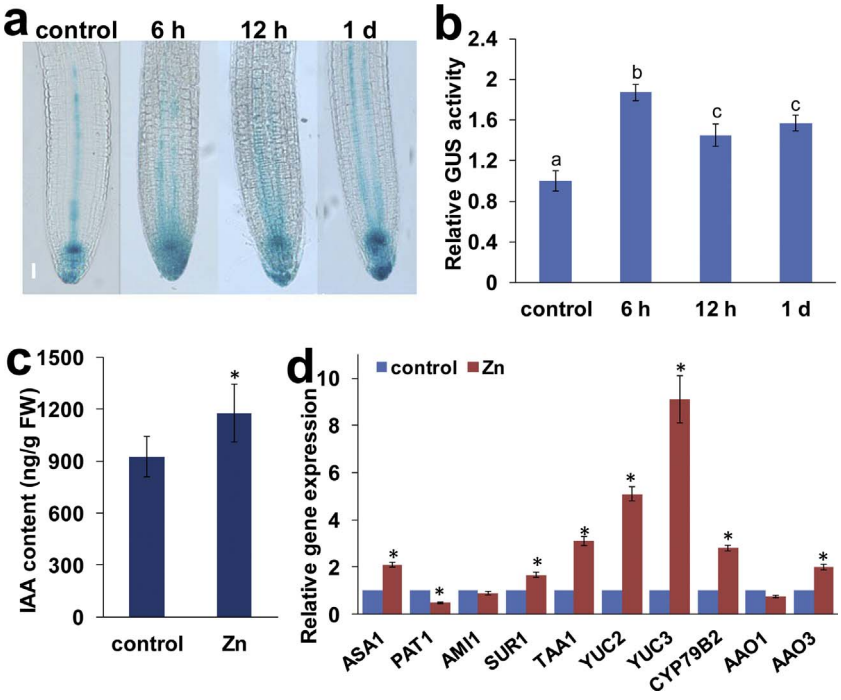

Fig. 3. Excess Zn induced auxin accumulation in root tips. (a, b) Images of GUS staining of *DR5:GUS* (a) and the relative GUS activity (b) of five-day-old seedlings that were transferred to 1/2 MS medium supplemented with 200 μM $ZnSO_4$ for 6 h–1 d. Bars = 50 μm. (c) IAA contents were determined in the roots treated with or without 200 μM ZnSO4 for 1 d. (d) qRT-PCR analysis of the expression of auxin biosynthesis-related genes in wild-type seedlings treated with or without 200 μM $ZnSO_4$ for 1 d. Error bars represent the SD. Different letters indicate that they were significantly different at $P < 0.01$ according to Tukey's test. *$P < 0.01$ according to Tukey's test.

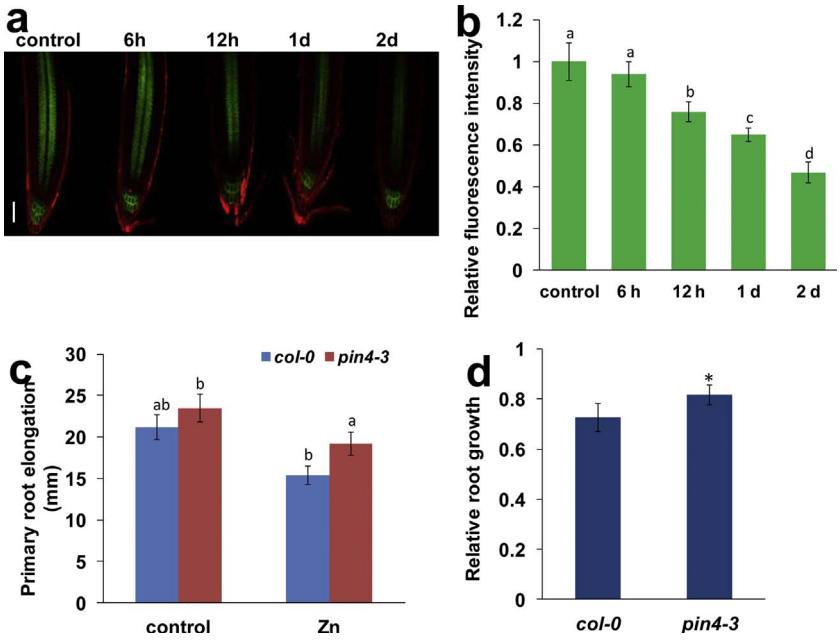

Fig. 4. PIN4 is involved in excess Zn-induced PR growth inhibition. (a, b) GFP fluorescence (a) and relative fluorescence intensity (b) in 5-d-old *PIN4:GFP* roots treated with 200 μM $ZnSO_4$ for 6 h-2 d. Bars = 50 μm. (c, d) PR growth (c) and relative root growth (d) in 5-d-old *col-0* and *pin4-3* seedlings treated with or without 200 μM $ZnSO_4$ for 2 d. Error bars represent the SD. Different letters indicate that they were significantly different at $P < 0.01$ according to Tukey's test. *$P < 0.01$ according to Tukey's test.

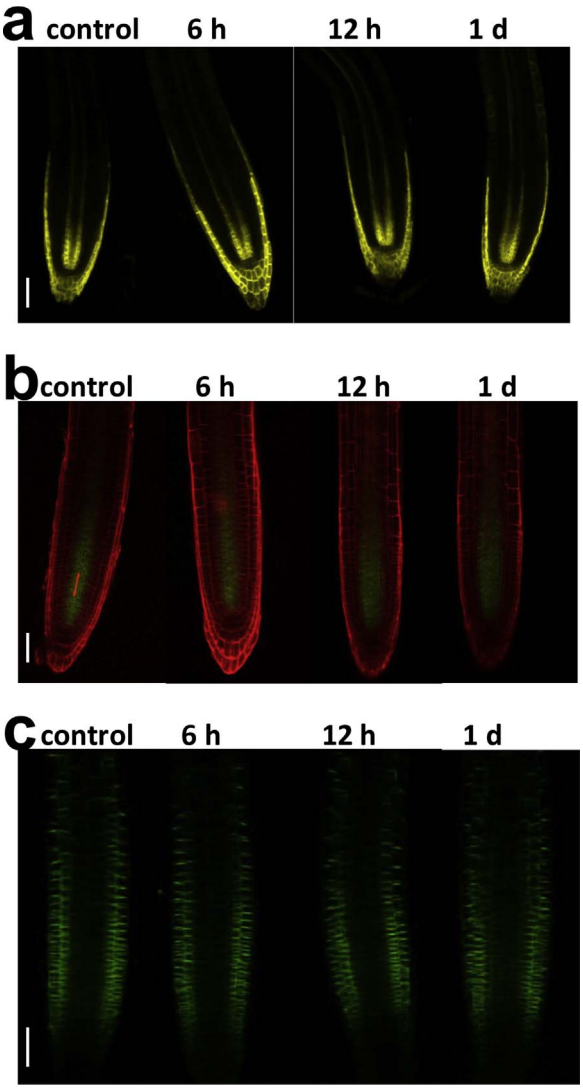

Fig. 5. YFP/GFP images of 5-d-old (a) *AUX1:YFP*, (b) *PIN1:GFP*, and (c) *PIN2:GFP* seedlings treated with or without 200 μM $ZnSO_4$ for 6 h-1 d. Bars = 50 μm.

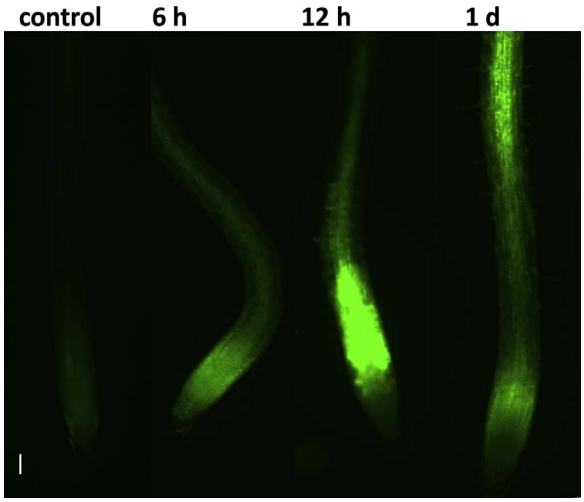

Fig. 6. Excess Zn induced NO accumulation in root tips. Detection of NO production in the roots of 5 day-old wild-type seedlings treated with or without 200 μM $ZnSO_4$ for 6 h-1 d using the NO-specific fluorescent probe DAF-2 DA. Bars = 50 μm.

3.4. Involvement of cGMP in Zn-regulated root system development through auxin pathway

The above results indicate that excess Zn improves cGMP levels in roots. Therefore, we next investigated the role of Zn-induced cGMP production in root growth and development. As shown in Fig. 7B and C, supplementation with the exogenous cGMP donor 8-Br-cGMP alleviated Zn-induced PR growth inhibition and further increased the LR number in excess Zn-treated seedlings, whereas supplementation with the GC inhibitor LY83583 further repressed PR growth and reduced the LR number. These data indicate that cGMP is involved in excess Zn-regulated root system development.

It has been previously demonstrated that cGMP is involved in regulating the expression of auxin carriers and that exogenous auxin increases cGMP levels in roots (Li and Jia, 2013; Nan et al., 2014). We then tested the hypothesis that cGMP regulates root development in excess Zn-treated roots by modulating auxin transport in root tips. For this purpose, we investigated the expression pattern of PIN4:GFP and found that supplementation with 8-Br-cGMP significantly increased the

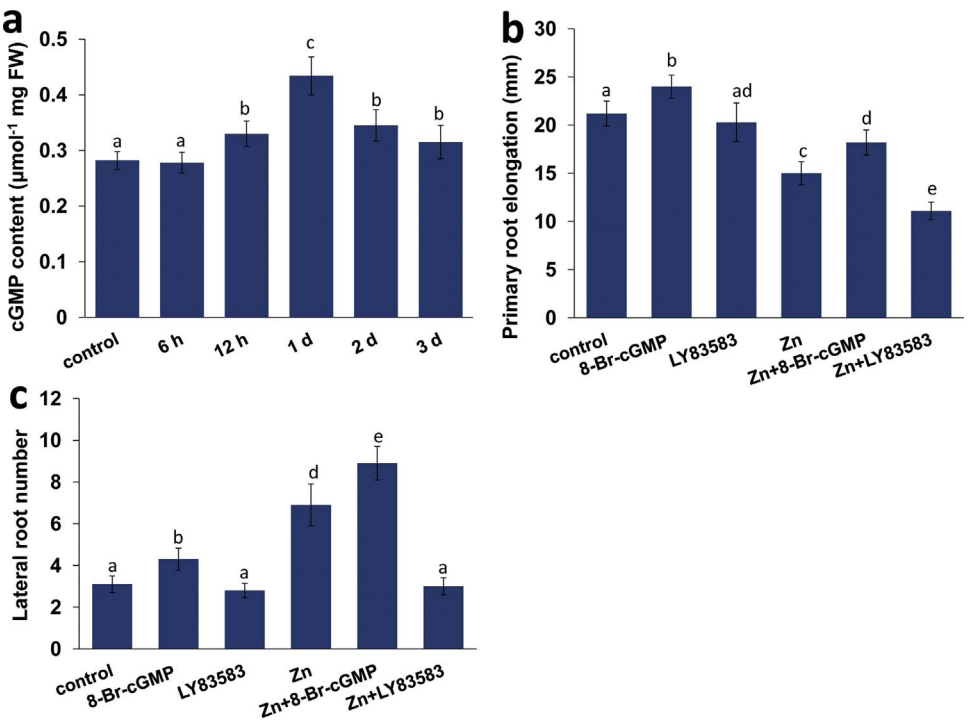

Fig. 7. cGMP is involved in excess Zn-mediated RSA remodeling. (a) cGMP content in 5-d-old wild-type seedlings treated with 200 μM ZnSO4 for 6 h-3 d. (b) PR elongation and (b) LR number in wild-type seedlings treated with 200 μM $ZnSO_4$ supplemented with 40 μM 8-Br-cGMP or 0.5 μM LY83583 for 2 d. Error bars represent the SD. Different letters indicate that they were significantly different at $P < 0.01$ according to Tukey's test.

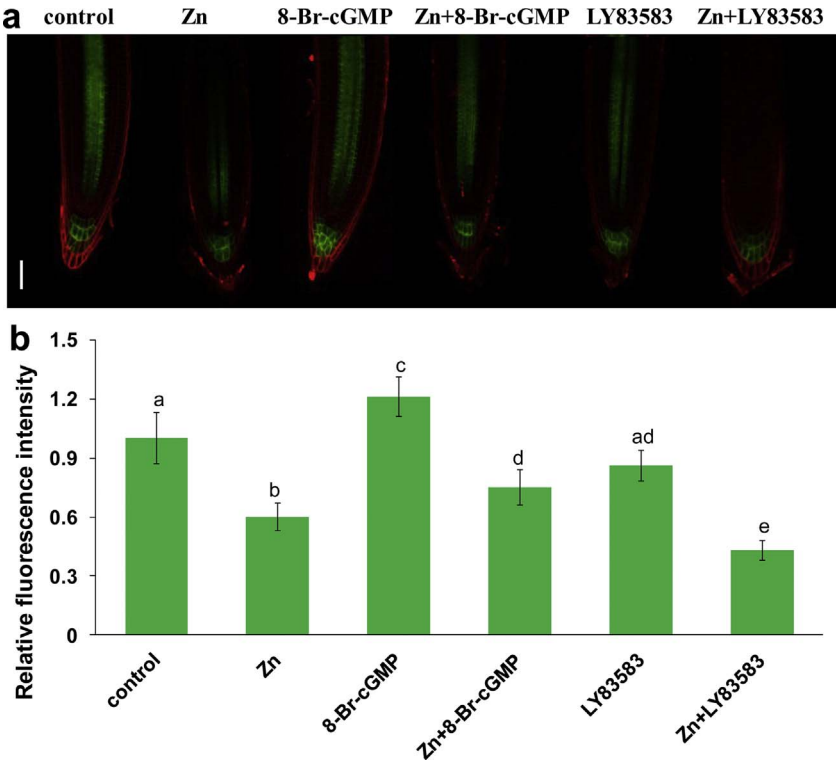

Fig. 8. (a) GFP fluorescence and (b) relative fluorescence intensity in 5-d-old *PIN4:GFP* roots treated with 200 μM $ZnSO_4$ supplemented with 40 μM 8-Br-cGMP or 0.5 μM LY83583 for 1 d. Bars = 50 μm.

expression, but supplementation with LY83583 further repressed PIN4 abundance in the roots of Zn-treated seedlings, as indicated by PIN4:GFP fluorescence (Fig. 8A and B). These data indicate that cGMP sustains PIN4 abundance and thus promotes root growth and development in Zn-treated seedlings.

4. Discussion

The root system is an important organ for water and nutrient uptake in plants. Root traits are closely related to the stress tolerance of plants and are also tightly modulated by environmental cues. Zn is an essential micronutrient; however, excess Zn is harmful to plant growth and development. In our previous study, we found that Zn toxicity-induced NO production promoted reactive oxygen species (ROS) accumulation and subsequent programmed cell death (PCD) in primary root tips of hyperaccumulator *Solanum nigrum*, thereby modulating root system architecture (RSA) and activity (Xu et al., 2010). In the present study, we found that excess Zn disrupted auxin transport and distribution in

root tips by reducing PIN4 abundance, thereby inhibiting PR elongation. Our results also show that cGMP production in the roots increased Zn tolerance by increasing PIN4 abundance in root tips.

4.1. Excess Zn inhibited PR growth by reducing cell division potential in root tips

Root growth and root meristem size are tightly regulated by meristematic cell division and stem cell niche activity maintenance. Excess Zn markedly inhibited the expression of the cell cycle marker pCYCB1;1:CYCB1;1-GUS reporter in root tips, whereas the expression levels of the QC-specific marker QC25:GUS and CSC-specific marker *J2341* were unaffected. The *SHR/SCR* pathway and the *PLT* pathway are the two major pathways that regulate both stem cell niche activity and QC identity in roots. *SHR* activates *SCR* together with *WOX5* to regulate QC identity, and the *PLT* pathway is involved in auxin-dependent stem cell niche maintenance in roots (Liu et al., 2016a, 2016b). Both PLT2 and SHR abundances were unaffected by excess Zn, thereby further confirming that excess Zn inhibits PR growth through the repression of meristematic cell division potential but not stem cell niche activity.

4.2. Excess Zn affected root system development by modulating auxin expression and transport

Excess Zn markedly inhibited PR growth but promoted LR formation, thereby regulating RSA remodeling. Because a change in auxin accumulation and distribution is one of the important factors that modulate root growth and development in root responses to heavy metal stresses (Shen et al., 2008; Giehl et al., 2012; Yang et al., 2004; Wu et al., 2014; Yu et al., 2015; Li et al., 2015), we therefore investigated auxin levels in roots and found that excess Zn increased auxin distribution in root tips. Excess Zn increased DR5:GUS expression in root tips by increasing IAA biosynthesis and disrupting auxin transport in root tips. GC–MS analysis indicated that excess Zn elevated IAA contents in the roots, and the expression of auxin biosynthesis-related genes was upregulated in Zn-treated seedlings. Besides auxin biosynthesis, auxin accumulation and the auxin gradient in roots also rely on proper auxin transport (Liu et al., 2016a, 2016b). We then investigated the abundance of auxin carriers using *AUX1:YFP* and *PIN1/2/4:GFP* marker lines and found that excess Zn markedly repressed PIN4 abundance in root tips. Furthermore, the *pin4-3* mutant showed reduced sensitivity to Zn-induced PR growth inhibition, suggesting that PIN4 mediated excess Zn-regulated auxin distribution to inhibit PR growth. These data indicate that excess Zn represses PIN4 abundance in root tips; this repression therefore disrupts both auxin transport and the proper auxin gradient, resulting in auxin accumulation in root tips and ultimately inhibiting PR growth.

4.3. cGMP production that alleviates zn-induced PR growth inhibition by increasing PIN4 abundance is a late-stage adaptive response to excess Zn in roots

In this study, we also obtained evidence of the involvement of cGMP in Zn-regulated RSA remodeling. The NO/cGMP signaling pathway plays a role in modulating plant growth and development (Wendehenne et al., 2004; Prado et al., 2004; Jacobi et al., 2007). Our previous study reported that excess Zn induced NO accumulation in *Solanum nigrum* roots (Xu et al., 2010). In the present study, we found that excess Zn significantly induces NO accumulation at 6 h and peaks after 12 h of Zn treatment; however, excess Zn-induced cGMP production occurred after 12 h and peaked after 1 d of Zn treatment. This temporal elevation of NO and cGMP production suggests that, in contrast to NO production, Zn-induced cGMP production is a late-stage response to Zn stress. These data further support the results of previous studies in that NO acts upstream of cGMP to regulate plant growth and development (Pagnussat et al., 2003).

Several studies have indicated that cGMP affects auxin signaling by modulating auxin-dependent gene expression and promoting Aux/IAA protein degradation (Li and Jia, 2013; Nan et al., 2014). Excess Zn repressed PIN4 abundance, thereby resulting in auxin accumulation in root tips and subsequent PR growth inhibition. However, Zn-induced cGMP production improved PIN4 abundance, thereby alleviating PR growth inhibition in Zn-treated seedlings. This result indicates that cGMP production is an adaptive response in plants response to excess Zn. However, we can still not exclude the possibility that cGMP regulates root development by an auxin-independent pathway, which needs to be further elucidated. Taken together, our results indicate that cGMP mediates the auxin pathway not only by modulating auxin signaling but also by regulating the expression of auxin carriers, such as PIN4 thereby affecting auxin transport and ultimately modulating root system development.

5. Conclusions

Our previous study indicated that excess Zn-mediated NO production and subsequent ROS accumulation induce PCD in primary root tips (Xu et al., 2010). In this study, we found that excess Zn increases the expression of auxin biosynthesis-related genes and results in auxin accumulation in root tips. Excess Zn also induced cGMP production, and elevated cGMP improved PIN4 abundance, thereby alleviating Zn-induced PR growth inhibition and further promoting LR formation. The coordination of NO/ROS-mediated PCD and cGMP-mediated auxin redistribution modulates RSA and activity, thereby increasing Zn tolerance (Fig. 9). Modifying RSA is an effective route to improve crop tolerance and quality. Our study provides insights into novel strategies for improving Zn tolerance and such an understanding is helpful for breeding and cultivation of Zn accumulators.

Author contributions

JX conceived and designed the research, JQ, PZ, LLS, JPW, and RLW performed the experiments, JQ, PZ, SL, and JX analyzed the data, JX and JQ wrote the manuscript. All authors read, commented on- and approved the manuscript.

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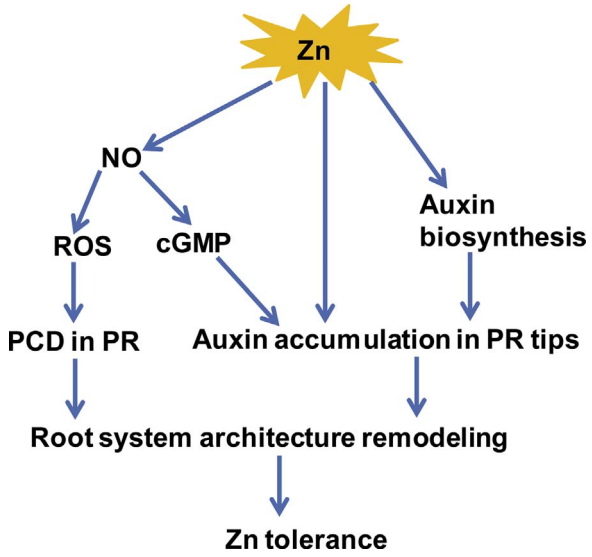

Fig. 9. A proposed model of excess Zn-mediated RSA remodeling. PR, primary roots.

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References

Arazi, T., Kaplan, B., Sunkar, R., Fromm, H., 2000. Cyclic-nucleotide-and Ca^{2+}/calmodulin-regulated channels in plants: targets for manipulating heavy-metal tolerance, and possible physiological roles. Biochem. Soc. Trans. 28, 471–475.

Bai, X., Todd, C.D., Desikan, R., Yang, Y., Hu, X., 2012. N-3-oxo-decanoyl-Lhomoserine-lactone activates auxin-induced adventitious root formation via hydrogen peroxide- and nitric oxide-dependent cyclic GMP signaling in mung bean. Plant Physiol. 158, 725–736.

Baran, A., 2013. Assessment of zea mays sensitivity to toxic content of zinc in soil. Pol. J. Environ. Stud. 22.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., Friml, J., 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115, 591–602.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., Scheres, B., 2005. The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. Nature 433, 39–44.

Boerjan, W., Cervera, M.T., Delarue, M., Beeckman, T., Dewitte, W., Bellini, C., ... Inzé, D., 1995. Superroot, a recessive mutation in *Arabidopsis*, confers auxin overproduction. Plant Cell 7, 1405–1419.

Cheng, Y., Dai, X., Zhao, Y., 2006. Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. Genes Dev. 20, 1790–1799.

Colon-Carmona, A., You, R., Haimovitch-Gal, T., Doerner, P., 1999. Technical advance: spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. Plant J. 20, 503–508.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., Scheible, W.R., 2005, 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiol. 139, 5–17.

De Smet, I., Tetsumura, T., De Rybel, B., Frey, N.F., Laplaze, L., Casimiro, I., Swarup, R., Naudts, M., Vanneste, S., Audenaert, D., Inzé, D., Bennett, M.J., Beeckman, T., 2007. Auxin-dependent regulation of lateral root positioning in the basal meristem of *Arabidopsis*. Development 134, 681–690.

Dubrovsky, J.G., Soukup, A., Napsucialy-Mendivil, S., Jeknic, Z., Ivanchenko, M.G., 2009. The lateral root initiation index: an integrative measure of primordium formation. Ann. Bot. 103, 807–817.

Gao, X., Yuan, H.M., Hu, Y.Q., Li, J., Lu, Y.T., 2014. Mutation of *Arabidopsis* CATALASE2 results in hyponastic leaves by changes of auxin levels. Plant Cell Environ. 37, 175–188.

Giehl, R.F., Lima, J.E., von Wirén, N., 2012. Localized iron supply triggers lateral root elongation in *Arabidopsis* by altering the AUX1-mediated auxin distribution. Plant Cell 24, 33–49.

González-Guerrero, M., Azcón-Aguilar, C., Mooney, M., Valderas, A., MacDiarmid, C.W., Eide, J., Ferrol, N., 2005. Characterization of a *Glomus intraradices* gene encoding a putative Zn transporter of the cation diffusion facilitator family. Fungal Genet. Biol. 42, 130–140.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O., Estelle, M., 2001. Auxin regulates SCF (TIR1)-dependent degradation of AUX/IAA proteins. Nature 414, 271–276.

Hu, X., Neill, S.J., Tang, Z., Cai, W., 2005. Nitric oxide mediates gravitropic bending in soybean roots. Plant Physiol. 137, 663–670.

Jacobi, J., Elmer, J., Russell, K., Soundur, R., Porterfield, D.M., 2007. Nitric oxide and cGMP dependent signaling in *Arabidopsis* root growth. Gravi. Space Res. 19.

Joudoi, T., Shichiri, Y., Kamizono, N., Akaike, T., Sawa, T., Yoshitake, J., Yamadac, N., Iwai, S., 2013. Nitrated cyclic GMP modulates guard cell signaling in *Arabidopsis*. Plant Cell 25, 558–571.

Kim, Y.Y., Choi, H., Segami, S., Cho, H.T., Martinoia, E., Maeshima, M., Lee, Y., 2009. AtHMA1 contributes to the detoxification of excess Zn (II) in *Arabidopsis*. Plant J. 58, 737–753.

Krishnamurthy, A., Rathinasabapathi, B., 2013. Auxin and its transport play a role in plant tolerance to arsenite-induced oxidative stress in *Arabidopsis thaliana*. Plant Cell Environ. 36, 1838–1849.

Laskowski, M., Grieneisen, V.A., Hofhuis, H., ten Hove, C.A., Hogeweg, P., Maree, A.F.M., Scheres, B., 2008. Root system architecture from coupling cell shape to auxin transport. PLoS Biol. 6, 2721–2735.

Li, J., Jia, H., 2013. cGMP modulates *Arabidopsis* lateral root formation through regulation of polar auxin transport. Plant Physiol. Biochem. 66, 105–117.

Li, Y., Zhang, Y., Shi, D., Liu, X., Qin, J., Ge, Q., Xu, L., Pan, X., Li, W., Zhu, Y., Xu, J., 2013. Spatial-temporal analysis of zinc homeostasis reveals the response mechanisms to acute zinc deficiency in *Sorghum bicolor*. New Phytol. 200, 1102–1115.

Li, G., Song, H., Li, B., Kronzucker, H.J., Shi, W., 2015. Auxin resistant1 and PIN-FORMED2 protect lateral root formation in *Arabidopsis* under iron stress. Plant Physiol. 169, 2608–2623.

Liu, G., Gao, S., Tian, H., Wu, W., Robert, H.S., Ding, Z., 2016a. Local transcriptional control of YUCCA regulates auxin promoted root-growth inhibition in response to aluminium stress in *Arabidopsis*. PLoS Genet. 12, e1006360.

Liu, Y.Y., Wang, R.L., Zhang, P., Chen, Q., Luo, Q., Zhu, Y.Y., Xu, J., 2016b. The nitrification inhibitor methyl 3-(4-hydroxyphenyl) propionate modulates root development by interfering with auxin signaling via the NO/ROS pathway. Plant Physiol. 171, 1686–1703.

Logan, H.M., Cathala, N., Grignon, C., Davidian, J.C., 1996. Cloning of a cDNA encoded by a member of the *Arabidopsis thaliana* ATP sulfurylase multigene family EXPRESSION STUDIES IN YEAST AND IN RELATION TO PLANT SULFUR NUTRITION. J. Biol. Chem. 271, 12227–12233.

Matsuzaki, Y., Ogawa-Ohnishi, M., Mori, A., Matsubayashi, Y., 2010. Secreted peptide signals required for maintenance of root stem cell niche in *Arabidopsis*. Science 329, 1065–1067.

Mikkelsen, M.D., Hansen, C.H., Wittstock, U., Halkier, B.A., 2000. Cytochrome P450 CYP79B2 from *Arabidopsis* catalyzes the conversion of tryptophan to indole-3-acetaldoxime, a precursor of indole glucosinolates and indole-3-acetic acid. J. Biol. Chem. 275, 33712–33717.

Nan, W., Wang, X., Yang, L., Hu, Y., Wei, Y., Liang, X., ... Bi, Y., 2014. Cyclic GMP is involved in auxin signalling during *Arabidopsis* root growth and development. J. Exp. Bot. 65, 1571–1583.

Pagnussat, G.C., Lanteri, M.L., Lamattina, L., 2003. Nitric oxide and cyclic GMP are messengers in the indole acetic acid-induced adventitious rooting process. Plant Physiol. 132, 1241–1248.

Pollmann, S., Neu, D., Weiler, E.W., 2003. Molecular cloning and characterization of an amidase from *Arabidopsis thaliana* capable of converting indole-3-acetamide into the plant growth hormone indole-3-acetic acid. Phytochemistry 62, 293–300.

Prado, A.M., Porterfield, D.M., Feijó, J.A., 2004. Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. Development 131, 2707–2714.

Rogg, L.E., Lasswell, J., Bartel, B., 2001. A gain-of-function mutation in IAA28 suppresses lateral root development. Plant Cell 13, 465–480.

Romero-Puertas, M.C., Corpas, F.J., Rodríguez-Serrano, M., Gómez, M., Luis, A., Sandalio, L.M., 2007. Differential expression and regulation of antioxidative enzymes by cadmium in pea plants. J. Plant Physiol. 164, 1346–1357.

Rose, A.B., Last, R.L., 1997. Introns act post-transcriptionally to increase expression of the *Arabidopsis thaliana* tryptophan pathway gene PAT1. Plant J. 11, 455–464.

Ruíz-Herrera, L.F., López-Bucio, J., 2013. Aluminum induces low phosphate adaptive responses and modulates primary and lateral root growth by differentially affecting auxin signaling in *Arabidopsis* seedlings. Plant Soil 371, 593–609.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P., 1999. An auxin-Dependent distal organizer of pattern and polarity in the *Arabidopsis* root. Cell 99, 463–472.

Sabatini, S., Heidstra, R., Wildwater, M., Scheres, B., 2003. SCARECROW is involved in positioning the stem cell niche in the *Arabidopsis* root meristem. Genes Dev. 17, 354–358.

Seo, M., Akaba, S., Oritani, T., Delarue, M., Bellini, C., Caboche, M., Koshiba, T., 1998. Higher activity of an aldehyde oxidase in the auxin-Overproducing superroot1 mutant *of arabidopsis thaliana*. Plant Physiol. 116, 687–693.

Shanmugam, V., Lo, J.C., Wu, C.L., Wang, S.L., Lai, C.C., Connolly, E.L., ... Yeh, K.C., 2011. Differential expression and regulation of iron-regulated metal transporters in Arabidopsis halleri and *Arabidopsis thaliana*-the role in zinc tolerance. New Phytol. 190, 125–137.

Shen, H., Hou, N., Schlicht, M., Wan, Y., Mancuso, S., Baluska, F., 2008. Aluminium toxicity targets PIN2 in *Arabidopsis* root apices: effects on PIN2 endocytosis, vesicular recycling, and polar auxin transport. Chinese Sci. Bull. 53, 2480–2487.

Shi, Y.F., Wang, D.L., Wang, C., Culler, A.H., Kreiser, M.A., Suresh, J., Cohen, J.D., Pan, J.W., Baker, B., Liu, J.Z., 2015. Loss of GSNOR1 function leads to compromised auxin signaling and polar auxin transport. Mol. Plant 8, 1350–1365.

Stepanova, A.N., Robertson-Hoyt, J., Yun, J., Benavente, L.M., Xie, D.Y., Dole_zal, K., Schlereth, A., Jürgens, G., Alonso, J.M., 2008. TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell 133, 177–191.

Sun, P., Tian, Q.Y., Chen, J., Zhang, W.H., 2009. Aluminium-induced inhibition of root elongation in *Arabidopsis* is mediated by ethylene and auxin. J. Exp. Bot. 61, 347–356.

Swarup, R., Kargul, J., Marchant, A., Zadik, D., Rahman, A., Mills, R., ... Tsurumi, S., 2004. Structure-function analysis of the presumptive *Arabidopsis* auxin permease AUX1. Plant Cell 16, 3069–3083.

Tatematsu, K., Kumagai, S., Muto, H., Sato, A., Watahiki, M.K., Harper, R.M., ... Yamamoto, K.T., 2004. MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in *Arabidopsis thaliana*. Plant Cell 16, 379–393.

Teng, Y., Xu, W., Ma, M., 2010. cGMP is required for seed germination in Arabidopsis thaliana. J. Plant Physiol. 167, 885–889.

Tennstedt, P., Peisker, D., Böttcher, C., Trampczynska, A., Clemens, S., 2009. Phytochelatin synthesis is essential for the detoxification of excess zinc and contributes significantly to the accumulation of zinc. Plant Physiol. 149, 938–948.

Tian, Q., Reed, J.W., 1999. Control of auxin-regulated root development by the *Arabidopsis thaliana* SHY2/IAA3 gene. Development 126, 711–721.

Uehara, T., Okushima, Y., Mimura, T., Tasaka, M., Fukaki, H., 2008. Domain II mutations in CRANE/IAA18 suppress lateral root formation and affect shoot development in *Arabidopsis thaliana*. Plant Cell Physiol. 49, 1025–1038.

Ulmasov, T., Murfett, J., Hagen, G., Guilfoyle, T.J., 1997. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9, 1963–1971.

Vassilev, A., Nikolova, A., Koleva, L., Lidon, F., 2011. Effects of excess Zn on growth and photosynthetic performance of young bean plants. J. Phytol. 3.

Wang, P., Yu, W., Zhang, J., Rengel, Z., Xu, J., Han, Q., ... Chen, Q., 2016a. Auxin enhances aluminium-induced citrate exudation through upregulation of GmMATE and activation of the plasma membrane H+-ATPase in soybean roots. Ann. Bot. 118,

933–940.
Wang, S., Ren, X., Huang, B., Wang, G., Zhou, P., An, Y., 2016b. Aluminium-induced reduction of plant growth in alfalfa (*Medicago sativa*) is mediated by interrupting auxin transport and accumulation in roots. Sci. Rep. 6.
Wendehenne, D., Durner, J., Klessig, D.F., 2004. Nitric oxide: a new player in plant signaling and defense responses. Curr. Opin. Plant Biol. 7, 449–455.
Wendehenne, D., Pugin, A., Klessig, D.F., Durner, J., 2001. Nitric oxide: comparative synthesis and signaling in animal and plant cells. Trends Plant Sci. 6, 177–183.
Wintz, H., Fox, T., Wu, Y.Y., Feng, V., Chen, W., Chang, H.S., Zhu, T., Vulpe, C., 2003. Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. J. Biol. Chem. 278, 47644–47653.
Wong, A., Gehring, C., 2013. The *Arabidopsis thaliana* proteome harbors undiscovered multi-domain molecules with functional guanylyl cyclase catalytic centers. Cell Commun. Signal 11, 48.
Wu, D., Shen, H., Yokawa, K., Baluška, F., 2014. Alleviation of aluminium-induced cell rigidity by overexpression of OsPIN2 in rice roots. J. Exp. Bot. 65, 5305–5315.
Xu, J., Yin, H., Li, Y., Liu, X., 2010. Nitric oxide is associated with long-term zinc tolerance in *Solanum nigrum*. Plant Physiol. 154, 1319–1334.
Yadav, S.K., 2010. Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. S. Afr. J. Bot. 76, 167–179.
Yang, X., Lee, S., So, J.H., Dharmasiri, S., Dharmasiri, N., Ge, L., ... Estelle, M., 2004. The IAA1 protein is encoded by AXR5 and is a substrate of SCFTIR1. Plant J. 40, 772–782.
Yu, C., Sun, C., Shen, C., Wang, S., Liu, F., Liu, Y., ... Geisler, M., 2015. The auxin transporter, OsAUX1, is involved in primary root and root hair elongation and in Cd stress responses in rice (*Oryza sativa* L.). Plant J. 83, 818–830.
Zhou, P., Yang, F., Ren, X., Huang, B., An, Y., 2014. Phytotoxicity of aluminum on root growth and indole-3-acetic acid accumulation and transport in alfalfa roots. Environ. Exp. Bot. 104, 1–8.