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Manganese Treatment Alleviates Zinc Deficiency Symptoms in Arabidopsis Seedlings

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Plant phenotypes caused by mineral deficiencies differ depending on growth conditions. We recently reported that the growth of *Arabidopsis thaliana* was severely inhibited on MGRL-based zinc (Zn)-deficient medium but not on Murashige–Skoog-based Zn-deficient medium. Here, we explored the underlying reason for the phenotypic differences in Arabidopsis grown on the different media. The root growth and chlorophyll contents reduced by Zn deficiency were rescued by the addition of extra manganese (Mn) during short-term growth (10 or 14 d). However, this treatment did not affect the growth recovery after long-term growth (38 d). To investigate the reason for plant recovery from Zn deficiency, we performed the RNA-seq analysis of the roots grown on the Zn-basal medium and the Zn-depleted medium with/without additional Mn. Principal component analysis of the RNA-seq data showed that the gene expression patterns of plants on the Zn-basal medium were similar to those on the Zn-depleted medium with Mn, whereas those on the Zn-depleted medium without Mn were different from the others. The expression of several transcription factors and reactive oxygen species (ROS)-related genes was upregulated in only plants on the Zn-depleted medium without Mn. Consistent with the gene expression data, ROS accumulation in the roots grown on this medium was higher than those grown in other conditions. These results suggest that plants accumulate ROS and reduce their biomass under undesirable growth conditions, such as Zn depletion. Taken together, this study shows that the addition of extra Mn to the Zn-depleted medium induces transcriptional changes in ROS-related genes, thereby alleviating short-term growth inhibition due to Zn deficiency.

Keywords: Arabidopsis • Manganese • ROS • Zinc.

Introduction

Zinc (Zn) is an essential micronutrient for all living organisms. Zn is a cofactor or part of the catalytic site of many enzymes and proteins. Most metabolic pathways include Zn-dependent steps. In plants, Zn is required for the activities of >80 enzymes, such as alcohol dehydrogenase and glutamate dehydrogenase, and hydrolases, such as alkaline phosphatase, carbonic anhydrase, Cu/Zn superoxide dismutase (SOD) and RNA polymerase (Marschner 1995). Plants obtain all their Zn from the soil. Zn is taken up into the roots and then transported throughout the plant. However, under Zn deficiency, plant physiological activity cannot be maintained and growth is inhibited. Zn-deficient plants exhibit symptoms, such as suppressed leaf blade and internode elongation, brown spots on petioles and veins and marked chlorosis between veins (Marschner 1995). Zn deficiency is caused by insufficient Zn absorption, which can be due to inadequate absolute amounts of Zn in the soil or the poor availability of Zn, caused by high soil pH or excessive application of phosphoric acid (Alloway 2009).

Intracellular Zn concentrations are maintained by Zn transporters that mediate Zn influx and efflux in plant cells. Among the many Zn transporters identified in plants, only members of the Zn-regulated transporter/iron-regulated transporter (IRT)-related protein (ZIP) family transport Zn into cells (Sinclair and Krämer 2012). Fifteen ZIP genes have been identified in *Arabidopsis thaliana* (Mäser et al. 2001). The expression of most ZIP genes is upregulated under Zn-deficient conditions, which promotes Zn uptake into cells (Grotz et al. 1998, Krämer et al. 2007). Some of these proteins also transport divalent cations, such as iron (Fe) and manganese (Mn).

Plants maintain Zn homeostasis under Zn-depleted conditions by regulating the expression of ZIP family genes via transcription factors. The expression of some of the ZIP transporter genes is regulated by basic leucine zipper (bZIP) transcription factors (Assunção et al. 2010). bZIP19 and bZIP23 bind to a *cis*-element called the Zn-deficiency response element, which is present in the promoter regions of some ZIP family genes (Assunção et al. 2010).

We previously reported that *bzip19-1*, a loss-of-function T-DNA insertion mutant, showed severe growth inhibition on the

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MGRL-based Zn-depleted medium (hereafter, Zn0) but grew normally on the Murashige–Skoog (MS)-based Zn-deficient medium (hereafter, MS-Zn0) (Inaba et al. 2015). Furthermore, Colombia (Col-0) plants grew well on the MS-Zn0 medium but were sensitive to the Zn0 medium (Inaba et al. 2015). The MGRL medium was designed for use in hydroponics and has a relatively low mineral content (Fujiwara et al. 1992). In contrast, the MS medium was designed for plant cell culture and its mineral content is higher than that of MGRL (Murashige and Skoog 1962). These results suggest that the relatively high concentrations of some elements in the MS-Zn0 medium could alleviate the effects of Zn deficiency in Col-0 and *bzip19-1* plants.

In this study, we found that the addition of Mn to the Zn0 medium reversed the Zn-deficiency growth defect in Col-0 seedlings. RNA-seq analysis revealed that the gene expression patterns of roots grown on the Zn0 medium differed from those grown on the MGRL basal medium, and the addition of Mn to the Zn0 medium prevented these changes in gene expression. Among them, several peroxidase and respiratory oxidase homolog (RBOH) genes were upregulated in plants grown on the Zn0 medium but were expressed at basal levels on the Zn0 medium with extra Mn. In addition, reactive oxygen species (ROS) accumulation was prevented when Mn was added to the MGRL-Zn0 medium. Our findings indicate that additional Mn supplementation to the Zn-deficient medium can alleviate the Zn-deficiency symptoms and restore normal gene expression patterns during seedling development.

Results

Seedling growth was rescued by supplementation of additional Mn to the Zn-depleted medium

We previously reported that Col-0 seedlings grew better on the MS-Zn0 medium than on the Zn0 medium (Inaba et al. 2015). Furthermore, *bzip19-1* had no distinct phenotype on the MS-Zn0 medium, even though the mutant showed a severe Zn-deficiency phenotype on the Zn0 medium (Inaba et al. 2015). In this study, we examined the cause of the difference in phenotypes on the two Zn-deficient media by focusing on the differences in the elemental composition of the MGRL and MS media. MS-Zn0 has more than five times the Fe, Mn, potassium (K) and molybdenum (Mo) concentrations in the Zn0 medium (Inaba et al. 2015). Therefore, we examined the growth recovery of Col-0 seedlings grown on the Zn0 medium supplemented with these elements. The MS-Zn0 medium contains approximately 100, 100, 14,000 and 0.7 μM more Fe, Mn, K, and Mo, respectively, than the Zn0 medium. Thus, we hypothesized that one or more of these elements were responsible for the normal growth of seedlings on the MS-Zn0 medium.

To determine which elements caused growth recovery, Col-0 was grown on the Zn0 medium supplemented with 100 μM Fe, 100 μM Mn, 14,000 μM K or 0.7 μM Mo (hereafter Zn0 + Fe100, Zn0 + Mn100, Zn0 + K14000 and Zn0 + Mo0.7, respectively). In the case of K and Mo, we used two different salts because of the possibility that nitrate or ammonium present in the usual K and Mo sources might be a source of nitrogen (N), which would affect growth recovery. The growth phenotypes of Col-0 on the Zn0 + K14000 (KNO_3), Zn0 + K14000 (K_2SO_4), Zn0 + Mo0.7 [$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$] and Zn0 + Mo0.7 ($MoCl_5$) media were similar to those on the Zn0 medium (**Fig. 1**). Compared with that on the Zn0 medium, the shoot and root growth of Col-0 was inhibited on the Zn0 + Fe100 medium. In contrast, the root growth inhibition of Col-0 on the Zn0 medium was reversed on the Zn0 + Mn100 medium (**Fig. 1**). Thus, the addition of supplementary Mn had a positive effect on plant growth under the Zn-depleted condition.

As an excess amount of Mn can inhibit plant growth, we next determined the optimal concentration of Mn to prevent Zn-deficiency symptoms. Col-0 seedlings were grown on the Zn0 medium supplemented with an additional 50–150 μM Mn (**Fig. 2**). The roots of Col-0 grown on the Zn0 + Mn75 medium were almost as long as those grown on the basal medium, and the addition of more Mn did not further rescue the growth inhibition phenotype (**Fig. 2A, B**). In addition, the basal

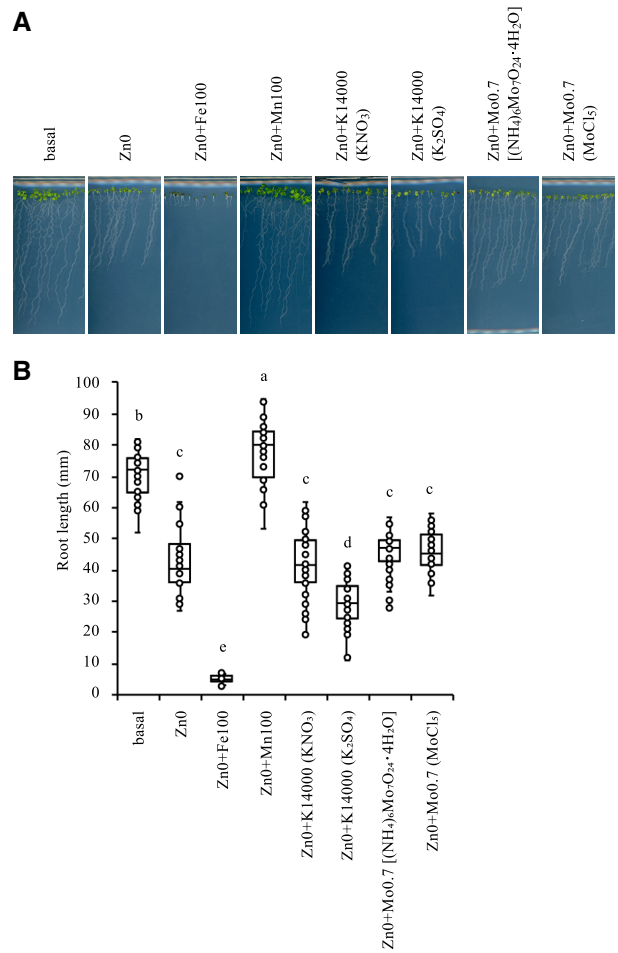

Fig. 1 Phenotypic recovery from Zn-deficient symptom by the addition of minerals. (A) Phenotypes of Col-0 grown on basal, Zn0, Zn0 + Fe100, Zn0 + Mn100, Zn0 + K14000 (KNO_3), Zn0 + K14000 (K_2SO_4), Zn0 + Mo0.7 [$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$] and Zn0 + Mo0.7 ($MoCl_5$) media grown for 10 d. (B) The box plot shows the root lengths of Col-0 shown in (A). Error bar shows standard error ($n = 30$). Significant differences indicated by the alphabet were determined by Tukey's HSD test ($P < 0.05$).

medium supplemented with 75 μM Mn did not affect Col-0 growth (Fig. 2A, B). Our results indicate that 75 μM is the optimal concentration of extra Mn to be added to prevent Zn-deficiency symptoms on the Zn0 medium. Therefore, we further examined the growth differences of Col-0 grown on the basal, Zn0 and Zn0 + Mn75 media.

The purity of Mn did not affect Zn-deficiency symptoms in plants

As we used 99% purified Mn reagent in the Zn0 + Mn medium, it was possible that the reagent contained a small amount of Zn as an impurity. Indeed, inductively coupled plasma-mass spectrometry analysis revealed that 10 ppm of the 99% $MnSO_4$ solution contained <3.0 ppb Zn, although Zn was not detected in 10 ppm of the 99.9% $MnSO_4$ solution. To test whether the very small amount of Zn had an effect on plant growth, Col-0 was grown on the Zn0 + Mn75 medium that was prepared using 99% or 99.9% purified Mn reagent. Both purified Mn reagents prevented the growth inhibition due to Zn deficiency, and the root lengths of Col-0 were almost the same between the Zn0 + Mn75 (99%) and Zn0 + Mn75 (99.9%) treatments, suggesting that the small amount of Zn in the 99% $MnSO_4$ reagent had no effect on the growth recovery (Supplementary Fig. S1). These results indicate that the supplementation of additional Mn recovered the plant growth under the Zn-depleted condition, because the plant growth recovered even when the 99.9% $MnSO_4$ reagent was used, which has a Zn concentration below the detectable limit. Therefore, we used 99% purified Mn reagent for the subsequent experiments.

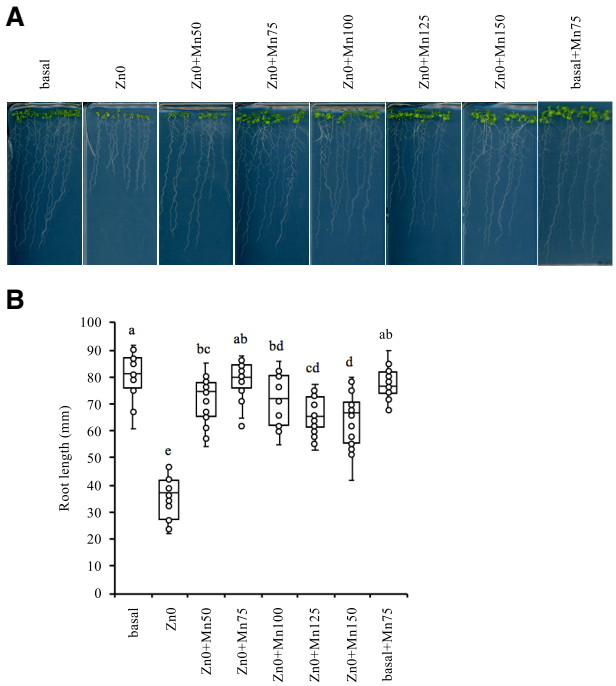

Fig. 2 Determination of optimal Mn concentration for phenotypic recovery from Zn-deficient symptom. (A) Phenotypes of Col-0 grown on basal, Zn0, Zn0 + Mn50, Zn0 + Mn75, Zn0 + Mn100, Zn0 + Mn125, Zn0 + Mn150 and basal + Mn75 media for 10 d. (B) The box plot shows the root lengths of Col-0 shown in (A). Error bar shows standard error (n = 20). Significant differences indicated by the alphabet were determined by Tukey's HSD test ($P < 0.05$).

Supplementation of the Zn0 medium with additional Mn had no effect on intracellular Zn concentrations in Col-0

Supplementation of the Zn0 medium with 75 μM Mn might have caused changes in the concentrations of other elements in the plants, which could have led to the recovery of plant growth. To determine if Mn supplementation affected the elemental concentration in the plants, we grew Col-0 on basal, Zn0 and Zn0 + Mn75 media for 10 d and measured the elemental concentrations in the shoots and roots. The average Zn concentrations were significantly lower in both the shoots and roots of seedlings grown on the Zn0 and Zn0 + Mn75 media than those of seedlings grown on the basal medium (Fig. 3A, B, Supplementary Table S1). These results indicate that additional Mn supplementation had little effect on Zn concentrations in plants grown on the Zn-depleted media. The average Mn concentration in the shoots and roots grown on the basal and Zn0 media were similar but were significantly higher in those grown on the Zn0 + Mn75 medium (Fig. 3C, D, Supplementary Table S1). The concentrations of some other minerals were also affected by Mn supplementation, but these changes were small (Supplementary Table S1). Therefore, we concluded that supplying additional Mn to plants grown on the Zn-depleted medium did not significantly affect the intracellular concentrations of minerals other than Mn.

Additional Mn supplementation had a small effect on Zn-deficiency symptoms during long-term growth

Next, we tested whether supplementation of the Zn0 medium with additional Mn had a long-term effect on plant growth. Col-0 plants were grown on the basal, Zn0, and Zn0 + Mn75 solid media for 10 d and then transplanted to liquid media of the same composition with rock wool and grown for 4 more weeks. The main axes of plants grown in the Zn0 and Zn0 + Mn75 media were significantly shorter than those of plants grown in the basal media (Fig. 4). Furthermore, the leaves of plants grown in Zn0 + Mn75 were as yellowed as those of plants grown in the Zn0 medium (Fig. 4). In contrast, bolting rates of the plants grown in the Zn0 + Mn75 liquid medium were almost the same as those of plants grown in the basal liquid medium, but the rate of those in Zn0 was substantially lower than those of plants grown in the basal liquid medium (Supplementary Table S2). These results suggest that additional Mn supplementation under Zn-depleted conditions can alleviate deficiency symptoms during vegetative growth state and the transition to reproductive growth, but not during reproductive growth state.

To quantify the yellowing of leaves, the total short- and long-term chlorophyll content in Col-0 grown on the basal, Zn0 and Zn0 + Mn75 media was measured. The total chlorophyll content in Col-0 seedlings grown on the basal, Zn0 and Zn0 +

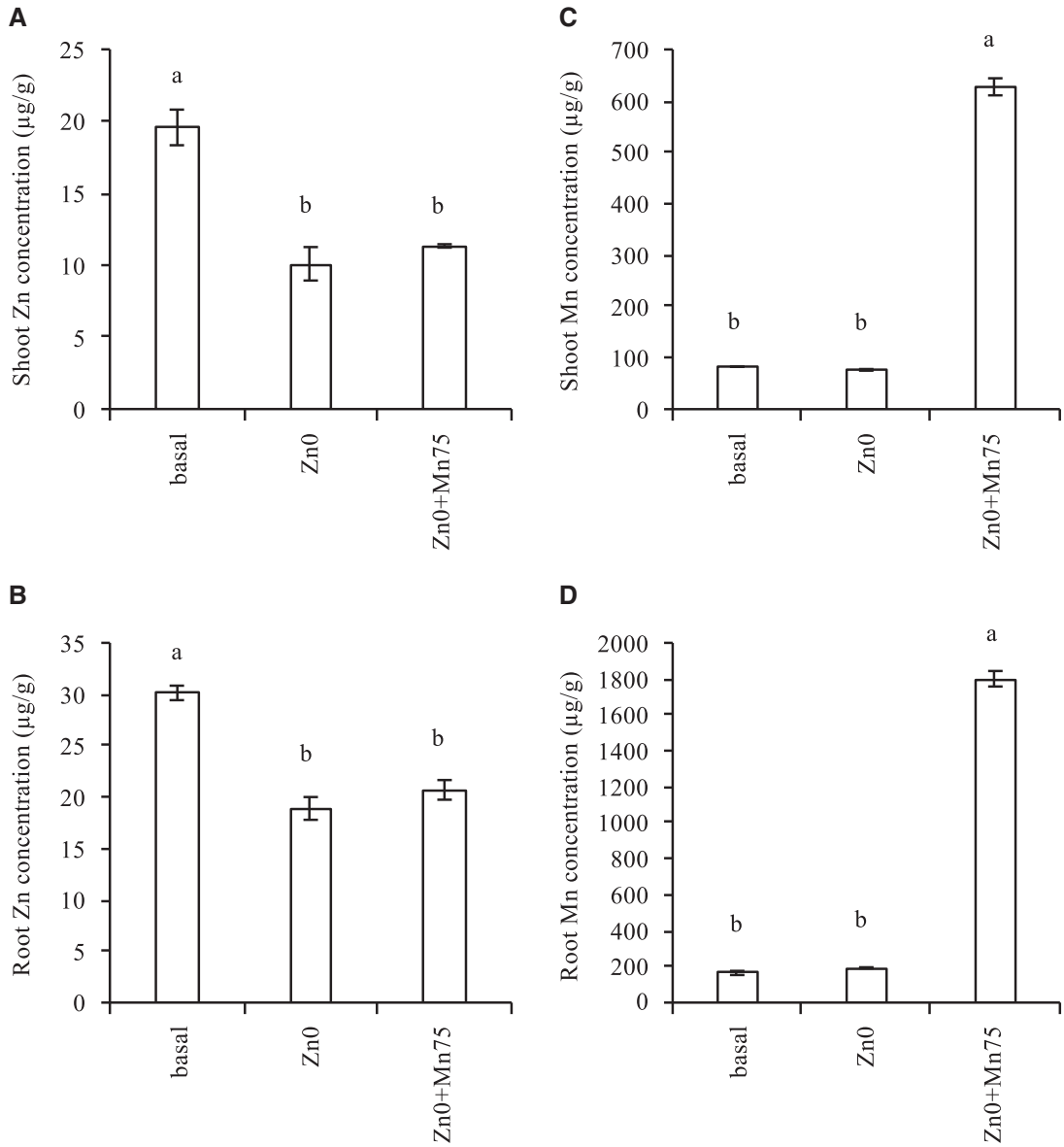

Fig. 3 Concentrations of Zn and Mn in the shoot and root of Col-0. Zn concentrations in the shoot (A) and root (B) of Col-0 grown for 10 d on basal, Zn0 and Zn0 + Mn75 media were determined. Mn concentrations in the shoot (C) and root (D) of Col-0 grown for 10 d on basal, Zn0 and Zn0 + Mn75 media were determined. Error bar shows standard error ($n = 3$). Significant differences indicated by the alphabet were determined by Tukey's HSD test ($P < 0.05$).

Mn75 media was 494.5, 324.9 and 531.9 μg Chl/g fresh weight (FW), respectively (**Fig. 5A**). These results suggest that additional Mn supplementation relieved the loss of chlorophyll caused by Zn deficiency. In the long-term growth experiments, however, the average total chlorophyll content of Col-0 grown in the basal, Zn0 and Zn0 + Mn75 media was 1,034.6, 889.4 and 621.8 μg Chl/g FW, respectively (**Fig. 5B**). Zn deficiency slightly decreased the chlorophyll content compared with basal media, and the addition of extra Mn to the Zn0 medium did not prevent the loss of chlorophyll during long-term growth. These results are consistent with the phenotypes observed in the short- and long-term growth experiments.

To examine the effects of Zn deficiency on seed quality in the next generation, we harvested seeds from Col-0 plants grown in the basal, Zn0, or Zn0 + Mn liquid media, sowed them on solid media of the same type used to grow their parents and measured the germination rate and root length. Seeds harvested from Col-0 grown in a mineral-rich medium were used as a control. Compared with control seeds, the seeds of plants grown on the basal medium were slightly smaller and lighter in color but did not show a significant difference in shape (**Fig. 6A**). In contrast, the seeds of plants grown in the Zn0 and Zn0 + Mn75 media were mostly distorted, as well as browner and smaller than the control seeds (**Fig. 6A**). Abnormal seed formation was observed under Zn-depleted conditions even after the addition of extra Mn to the medium. In addition, the seeds showed poor germination and growth under all growth conditions. The germination

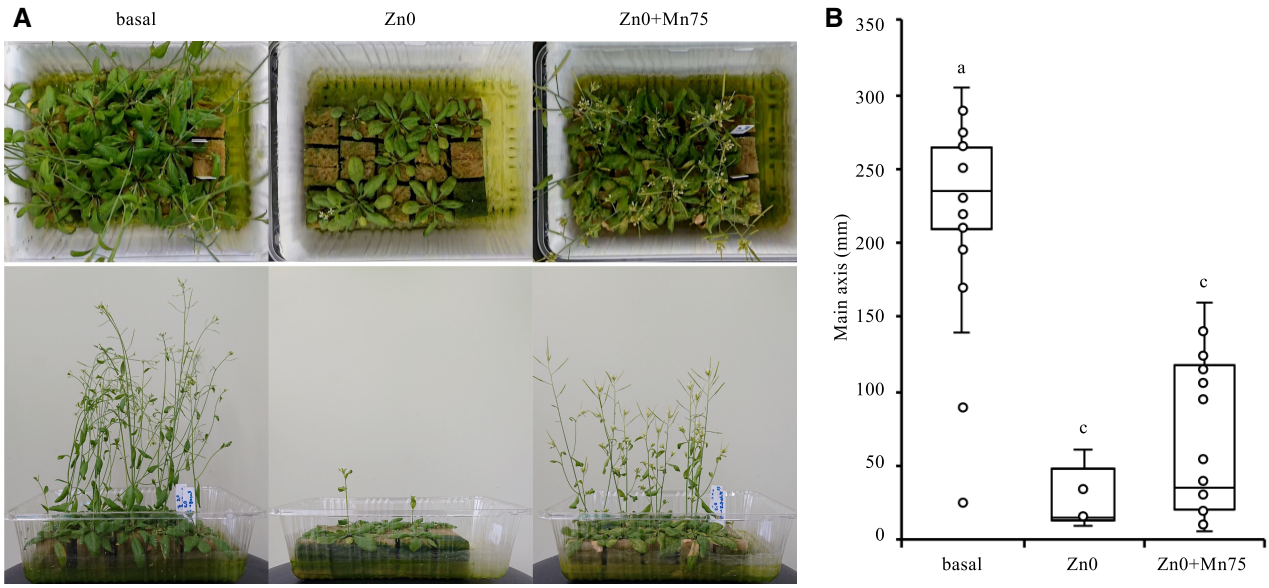

Fig. 4 Phenotypes of Col-0 in hydroponic culture for long term. (A) Col-0 grown on basal, Zn0 and Zn0 + Mn75 media for 10 d and then transplanted to hydroponic media of the same composition and grown for 4 more weeks. The pictures were taken from above and side. (B) Box plot shows the main axis lengths of Col-0 shown in (A) (basal: $n = 23$, Zn0: $n = 5$ and Zn0 + Mn75: $n = 26$). Significant differences indicated by the alphabet were determined by Tukey's HSD test ($P < 0.05$).

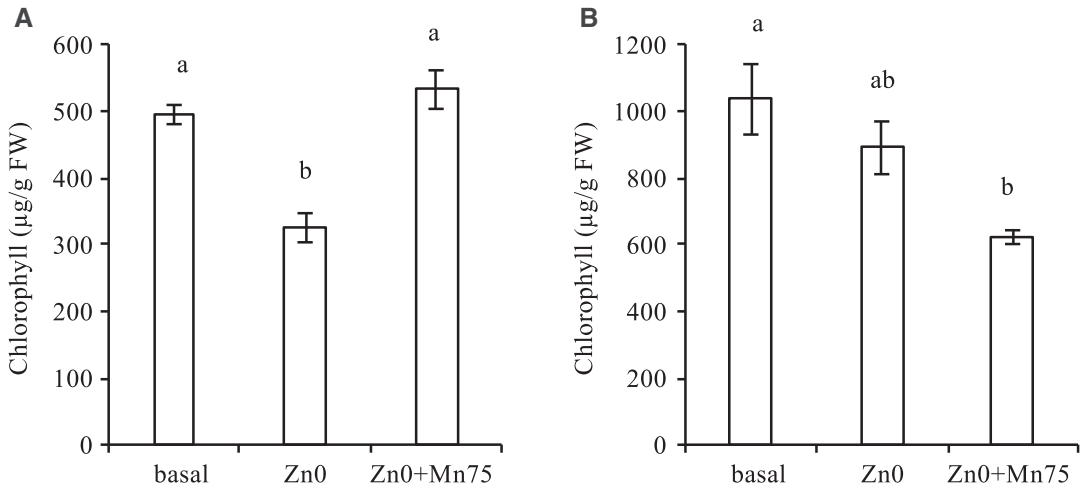

Fig. 5 Total chlorophyll content in Col-0 shoots. (A) Chlorophyll content in Col-0 shoots grown for 14 d on basal, Zn0 or Zn0 + Mn75 media. (B) Chlorophyll content in Col-0 leaves grown on basal, Zn0 or Zn0 + Mn75 liquid media for 10 d and then grown in basal, Zn0 or Zn0 + Mn75 hydroponic culture for 24 d, respectively. Error bar shows standard error ($n = 3$). Significant differences indicated by the alphabet were determined by Tukey's HSD test ($P < 0.05$).

numbers of control seeds and seeds harvested from plants grown in basal liquid media were 19–20/20 seeds (**Supplementary Table S3**). In contrast, the germination numbers of seeds from plants grown in Zn0 and Zn0 + Mn75 were 1–5/20 seeds. Furthermore, the root lengths of Zn0 and Zn0 + Mn75 seedlings were markedly shorter than those of the control and basal seedlings (**Fig. 6B, C**). We reasoned that the growth conditions might have affected seed development, thereby influencing the germination rate and seed phenotype. Altogether, these results indicate that additional Mn supplementation to the Zn-deficient medium is effective for improving short-term growth but not long-term growth.

Gene expression patterns in roots after short-term growth were similar on the basal and Zn0 + Mn75 media but not on Zn0

To explore why Zn-deficiency symptoms were alleviated by the addition of extra Mn, we performed the RNA-seq analysis of the roots of Col-0 grown on the basal, Zn0 and Zn0 + Mn75 media for 10 d. Based on the principal component analysis (PCA) of gene expression patterns, genes expressed in the roots of plants grown on basal vs. Zn0 medium belonged to different clusters, whereas genes expressed in the roots of plants grown on the Zn0 + Mn75 medium belonged to the same cluster as those for

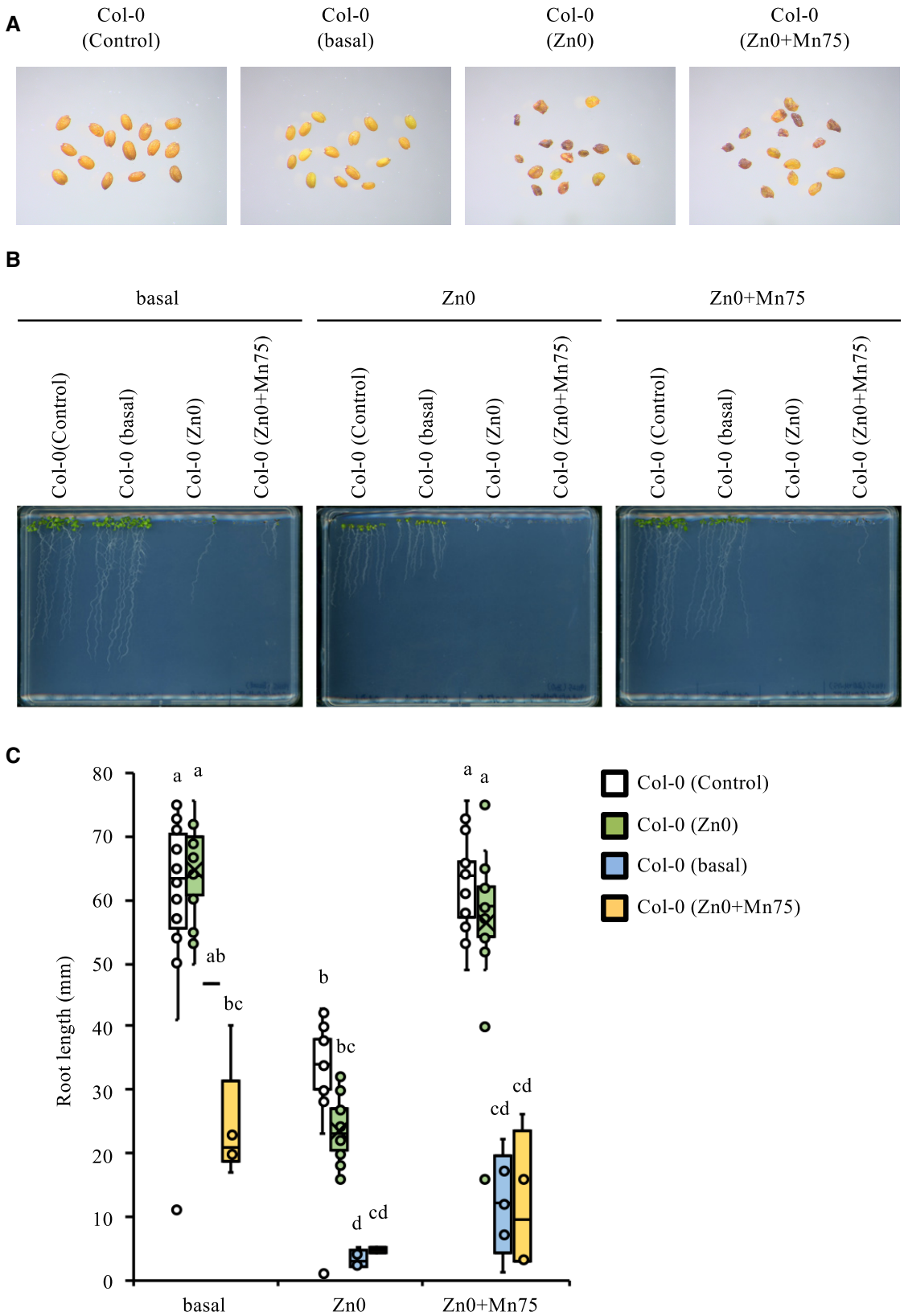

Fig. 6 Phenotypic observations of Col-0 seeds grown on basal, Zn0 or Zn0 + Mn75 liquid media. (A) Stereomicroscopic images of 15 Col-0 seed harvested in each growth condition were obtained. (B) The harvested Col-0 seeds from the plants grown on basal, Zn0 or Zn0 + Mn75 liquid media, or soil cultivation with the mineral-rich condition (control) grown on basal, Zn0 or Zn0 + Mn75 media. (C) The box plot shows the root lengths shown in (B) ($20 \geqq n \geqq 1$). Significant differences indicated by the alphabet were determined by Tukey's HSD test ($P < 0.05$).

the plants grown on the basal medium (Fig. 7). Therefore, the expression levels of many genes were comparable in plants grown on the basal medium vs. the Zn-depleted medium supplemented with extra Mn, which likely led to healthy phenotypes during short-term growth.

Based on the RNA-seq results, we summarized the differentially expressed genes (DEGs, *q*-values of <0.05, Supplementary Tables S4 and S5). The comparison between the Zn0 and basal media revealed 1,278 DEGs; 860 and 418 genes were upregulated and downregulated by Zn deficiency, respectively. On the other hand, a comparison between the Zn0 + Mn75 and basal media revealed 45 DEGs; 24 and 21 genes were upregulated and downregulated by the addition of extra Mn to the Zn-deficient condition, respectively.

Among these, we found that the expression of five transcription factors, *WRKY38* (AT5G22570), *WRKY51* (AT5G64810), *BHLH19* (AT2G22760), *WRKY62* (AT5G01900) and *BHLH51* (AT2G40200), was markedly upregulated by more than 5-fold in response to Zn deficiency (Supplementary Table S4). However, the expression levels of these genes in plants grown on the Zn0 + Mn75 medium were comparable to the levels observed when grown on the basal medium. These results suggest that the altered expression patterns of some transcription factor genes might contribute to the upregulated expression of target genes in plants grown on the Zn0 medium and that their expression levels were restored in response to the addition of extra Mn, thereby leading to phenotypic recovery.

Conversely, the expression levels of the low-affinity nitrate transporter *NPF7.3/NRT1.5* (AT1G32450) and the high-affinity nitrate transporters *NRT2.6* (AT3G45060) and *NRT2.4* (AT5G60770) were markedly downregulated by Zn deficiency but were restored by the addition of extra Mn (Supplementary Table S5). However, they were still downregulated by <0.5-fold on Zn0 + Mn75. *NPF7.3/NRT1.5* and *NRT2.4* uptake N from roots in response to N starvation (Tsay et al. 1993, Lin et al. 2008, Kiba et al. 2012). In contrast, *NRT2.6* cannot import N into roots (Dechorgnat et al. 2012), although it has a role in plant growth promotion by the rhizospheric bacterium STM196 (Kechid et al. 2013). These results raise the possibility that Zn deficiency alters N concentration in seedlings. To investigate whether Zn deficiency affects N content, total N concentrations were measured in both the shoots and roots grown for 10 d on the basal, Zn0 and Zn0 + Mn75 media. Total N concentrations did not change in the roots under any growth conditions, although it was decreased by Zn deficiency in the shoots (Supplementary Fig. S2).

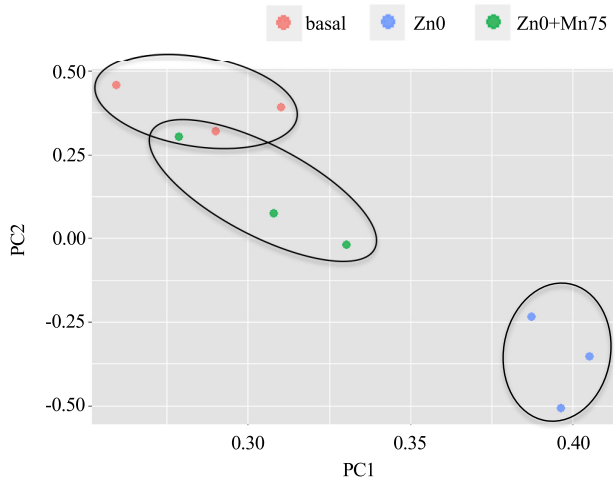

Fig. 7 PCA of RNA-seq analysis. RNA-seq analysis was performed using RNA extracted from Col-0 roots grown on basal (red circles), Zn0 (blue circles) or Zn0 + Mn75 media (green circles). Each three independent RNA-seq data was analyzed by PCA.

Zn and Mn transporter genes in the RNA-seq data

Twelve ZIP, five heavy metal ATPases (HMA), five cation exchanger (CAX), five natural resistance-associated macrophage protein (NRAMP) and three metal tolerance protein (MTP) transporters were identified in the RNA-seq analysis as Zn and/or Mn transporters (Supplementary Table S6). Some of these transporters have been shown to transport both Zn and Mn (Alejandro et al. 2020). Among them, *ZIP12* was markedly upregulated by 177- and 19-fold on the Zn0 and Zn0 + Mn75 media, respectively. The expression level was downregulated by the addition of extra Mn to the Zn0 medium. Nevertheless, *ZIP12* maintained higher expression levels due to Zn deficiency. *ZIP9*, *IRT2* and *IRT3* were also upregulated by approximately 2-fold on the Zn0 and Zn0 + Mn75 media. However, other transporters were largely not affected by Zn deficiency and supplementation of additional Mn to the Zn0 medium.

Genes responsive to Zn depletion in both the Zn0 and Zn0 + Mn75 media

The expression of some upregulated and downregulated genes due to Zn deficiency was not affected by the addition of extra Mn, although most changes in gene expression due to Zn deficiency were restored by additional Mn supplementation to the Zn-depleted condition. The RNA-seq analysis showed that eight and five genes were upregulated and downregulated more or less than 4-fold on not only Zn0 but also Zn0 + Mn75 media, respectively (Supplementary Table S7). We considered these genes to be significant Zn depletion-responsive genes. *ZIP12* was highly upregulated on both the Zn0 and Zn0 + Mn75 media, as shown above. This result is consistent with the lower levels of Zn in plants grown on the Zn0 and Zn0 + Mn75 media than in those grown on the basal medium (Fig. 3). These results imply that the expression of some genes was regulated by a Zn depletion signal, even though additional Mn was present in the growth condition.

Zn deficiency-mediated ROS accumulation in roots was reduced by the addition of extra Mn

The RNA-seq analysis shed light on the involvement of ROS in Zn-deficiency symptoms. The expression levels of class III peroxidase (PER) and RBOH genes in the roots were upregulated under the Zn-depleted condition but were comparable to those on the basal medium after the addition of extra Mn (Table 1). As the class III peroxidase family and RBOHs are involved in ROS homeostasis in plants, we analyzed the accumulation of ROS, such as hydrogen peroxide (H_2O_2), in the

roots grown on the basal, Zn0 and Zn0 + Mn75 media by DAB (3,3′-diaminobenzidine) staining. The roots of Col-0 grown on the Zn0 medium accumulated higher ROS than those grown on the basal medium (Fig. 8). On the other hand, ROS levels in the roots of plants grown on the Zn0 + Mn75 medium were almost the same as those of plants grown on the basal medium. These results suggest that the addition of extra Mn tends to reduce the accumulation of ROS induced by Zn deficiency.

Discussion

Addition of extra Mn to the Zn-depleted medium relieved Zn-deficiency symptoms

Essential trace elements, such as Zn and Mn, in plants cannot be replaced by other elements. These elements directly affect plant growth, and their roles are not limited to specific plants (Arnon and Stout 1939). In the current study, we demonstrated that the addition of extra Mn to a Zn-depleted medium alleviated

Table 1 Summary of peroxidase and RBOH genes with differential expression levels in RNA-seq analysis

Arabidopsis Genome Initiative (AGI) code	Fold change (Zn0/basal)	*q*-Value	fold change (Zn0 + Mn75/basal)	*q*-Value	Symbol	Gene name
AT4G33720.1	19.409	0.157	1.139	0.994		AT4g33720/T16L1_210
AT1G49570.1	**7.000**	**0.000**	2.081	0.917	PER10	Peroxidase
AT5G19890.1	**6.182**	**0.000**	3.695	0.045	PER59	Peroxidase 59
AT5G39580.1	2.822	0.066	0.648	0.927	PER62	Peroxidase 62
AT5G06720.1	**2.582**	**0.003**	0.720	0.927	PER53	Peroxidase 53
AT3G49120.1	2.330	0.159	1.058	0.991	PER34	Peroxidase 34
AT1G34510.1	2.278	0.141	1.381	0.934	PER8	Peroxidase
AT5G06730.1	**2.164**	**0.000**	1.020	0.991	PER54	Peroxidase 54
AT5G64120.1	2.095	0.222	1.061	0.990	PER71	Peroxidase
AT4G11290.1	**1.993**	**0.000**	1.030	0.977	PER39	Peroxidase 39
AT4G25980.1	1.889	0.565	1.203	0.968	PER43	Peroxidase 43
AT2G38380.1	**1.856**	**0.000**	1.139	0.927	PER22	Peroxidase 22
AT5G51890.1	**1.670**	**0.000**	1.024	0.987	PER66	Peroxidase 66
AT3G21770.1	**1.662**	**0.000**	1.010	0.992	PER30	Peroxidase 30
AT2G38390.1	**1.633**	**0.000**	0.967	0.973	PER23	Peroxidase 23
AT1G14550.1	1.616	0.784	1.218	0.973	PER5	Peroxidase 5
AT4G08770.1	1.592	0.161	1.323	0.927	PER37	Peroxidase 37
AT5G22410.1	1.590	0.091	1.455	0.828	PER60	Peroxidase 60
AT1G71695.1	1.407	0.372	1.204	0.933	PER12	Peroxidase
AT2G18150.1	1.405	0.603	0.408	0.114	PER15	Peroxidase 15
AT4G25100.1	1.363	0.130	**0.496**	**0.000**	FSD1	Superoxide dismutase [Fe] 1, chloroplastic
AT5G17820.2	1.317	0.072	1.295	0.565		Peroxidase superfamily protein
AT5G64110.1	1.292	0.826	0.963	0.993	PER70	Peroxidase
AT1G30870.1	1.288	0.509	1.115	0.949	PER7	Peroxidase 7
AT2G34060.1	1.284	0.191	0.994	0.995	PER19	Peroxidase 19
AT4G36430.1	1.267	0.609	0.666	0.764	PER49	Peroxidase 49
AT1G68850.1	1.264	0.052	1.101	0.927	PER11	Peroxidase 11
AT5G64100.1	1.229	0.087	1.156	0.909	PER69	Peroxidase
AT4G08390.1	**1.225**	**0.045**	1.049	0.947	APXS	L-Ascorbate peroxidase S, chloroplastic/mitochondrial
AT3G32980.1	1.223	0.237	1.067	0.947	PER32	Peroxidase 32
AT4G09010.3	0.916	0.947	1.354	0.927	APX4	Ascorbate peroxidase 4
AT4G26010.2	1.040	0.942	1.254	0.825		Peroxidase superfamily protein
AT2G39040.1	0.902	0.746	1.244	0.828	PER24	Peroxidase 24
AT5G47910.1	**2.019**	**0.000**	1.062	0.963	RBOHD	Respiratory burst oxidase homolog protein D
AT1G09090.2	**1.722**	**0.035**	1.328	0.927	RBOHB	Respiratory burst oxidase homolog protein B

The peroxidase and RBOH genes with increased gene expression levels in the Zn0 or Zn0 + Mn75 condition compared with the basal condition were selected and ordered based on fold change values in 'Zn0/basal' or 'Zn0 + Mn75/basal'. *q*-Values <0.05 and the fold changes are shown in bold font. AGI code indicates Arabidopsis Genome Initiative code.

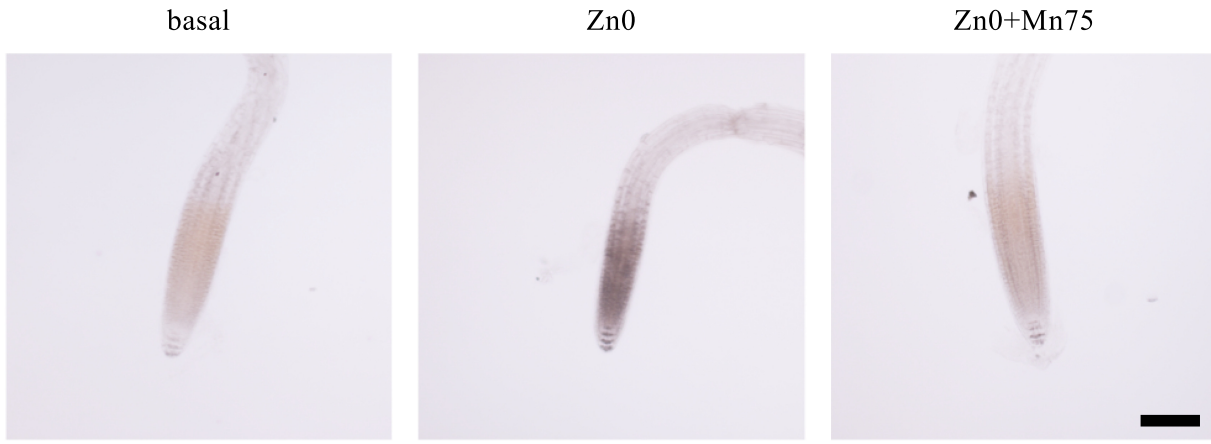

Fig. 8 ROS accumulations in Col-0 roots. ROS accumulation was observed at the root tip of Col-0 grown on basal, Zn0 or Zn0 + Mn75 media for 5 d using DAB staining. The bar shows 100 μm.

Zn-deficiency symptoms in Arabidopsis (**Figs. 1, 2**), although the intracellular Zn content was not altered in the plants (**Fig. 3A, B**). However, this effect was limited to short-term growth, because Zn-deficiency symptoms, such as chlorosis and shorter main axes, were not rescued by additional Mn supplementation after long-term cultivation (**Figs. 4, 5**, Supplementary Tables S2, S3). Measurements of chlorophyll content suggested that photosynthetic activity increased in response to the additional Mn supplementation after short-term growth but decreased after long-term growth (**Fig. 5**). Furthermore, the seeds of Col-0 plants grown on the Zn0 + Mn75 liquid medium had the same low germination rates as those harvested from Col-0 plants grown on the Zn0 medium and the germinated plants showed poor growth (**Fig. 6**, **Supplementary Table S3**). Therefore, we speculate that the supplementation of additional Mn to a Zn-depleted medium enhances the activity of some intracellular mechanisms involved in early plant growth, although Mn does not function as a substitute for Zn.

Most plants contain Mn levels of 30–500 μg/g dry weight (DW), although plants only require Mn levels of approximately 20–40 μg/g DW to maintain proper biological activity (Millaleo et al. 2010, Shao et al. 2017). We determined that the optimal Mn concentration for recovery from Zn deficiency was 75 μM, as revealed by phenotypic observation (**Fig. 2**). The average Mn content in the plants grown on the Zn0 + Mn75 medium was 627.3 and 1,800.4 μg/g DW in shoots and roots, respectively (**Fig. 3**). Although these concentrations were higher than the typical Mn concentrations, there were no symptoms of excess Mn levels, such as growth suppression, browning and discoloration or decreased lateral root formation, in Col-0 seedlings grown on Zn0 + Mn75 and basal + Mn75 media (**Fig. 2**) (Marschner 1995). Mn is involved in various processes in plants, such as photosynthesis, protein synthesis and hormone activation (Burnell 1988). During photosynthesis, Mn functions in water splitting and oxygen evolution in photosystem II (Schmidt et al. 2016). Mn is also involved in activating >30 enzymes, including Mn SOD (Marschner 1995). Thus, Mn plays an important role in the plant growth. Therefore, it is possible that plant growth recovered under Zn-depleted conditions in response to the additional Mn supplementation because Mn functions in a mechanism related to growth promotion.

Regulation of gene expression by crosstalk between Zn, Mn and N

PCA of gene expression levels based on RNA-seq data showed that the global gene expression pattern in plant roots grown on the Zn0 + Mn75 medium was classified into almost the same cluster as that of plant roots grown on the basal medium but not on the Zn0 medium (**Fig. 7**). These results suggest that many genes function in the same manner under the Zn-depleted condition as under normal environmental conditions in the presence of Mn.

To elucidate the mechanisms underlying the alleviation of Zn-deficiency symptoms by supplementation with Mn, we focused on the expression changes in Zn and Mn transporters. However, the expression of most Zn and Mn transporters was not affected on Zn0 and Zn0 + Mn75 media, except for a few ZIP transporters (**Supplementary Table S6**). Excess or reduced levels of elements in the growth environment affect the concentrations of elements in plant cells (Lee 1972, Maillard et al. 2016). However, the addition of extra Mn had little effect on the intracellular concentrations of other elements (**Supplementary Table S1**). Therefore, growth recovery by Mn was not due to increased Zn concentrations or the increased uptake of other minerals in response to supplementation with Mn.

Furthermore, three nitrate transporters were markedly downregulated on the Zn0 medium and their expression was partly restored by the addition of extra Mn (**Supplementary Table S5**). We previously found that some nitrate transporters were downregulated by Zn deficiency at the protein expression level (Inaba et al. 2015). The concentrations of Zn are significantly decreased in the shoots and roots of Arabidopsis NRT1.1-deficient mutant plants (Pan et al. 2020). Therefore, the downregulation of nitrate transporters may contribute to low Zn accumulation. In addition, Zn uptake in wheat roots is increased by the addition of N, although it is decreased under the Zn-depleted condition (Erenoglu et al. 2011). These results

suggest that there is a crosstalk between Zn and N, although it is still unclear why gene expression of nitrate transporters in roots was downregulated by Zn deficiency.

Commonly responding genes on Zn0 and Zn0 + Mn75 media

The expression of several genes, such as *LON4* (AT3G05790) and *NIT4* (AT5G22300), was altered in the Zn-depleted condition but was not completely restored to the basal level by supplementation with additional Mn (**Supplementary Table S7**). LON4 is an ATP-dependent protease that is localized in chloroplasts and mitochondria in Arabidopsis (Ostersetzer et al. 2007). Upregulated Lon protease contributes to the quality control of mitochondrial proteins in mammalian cells (Wu et al. 2010). Lon proteases degrade oxidized mitochondrial aconitase (Bota and Davies 2002). Indeed, Zn deficiency induces the accumulation of oxidized proteins in plants (Cakmak and Marschner 1993, Pinton et al. 1994, Sharma et al. 2004, Wang and Jin 2005). These results imply that *LON4* might contribute to the degradation of oxidized proteins induced by Zn deficiency. The *NIT4* gene encodes a nitrilase, and overexpression of Arabidopsis *NIT4* in Cassava increases the total amount of amino acids and proteins in the roots (Zidenga et al. 2017). It has also been reported that amino acids leak from Zn-deficient roots (Cakmak 2000). These results suggest that upregulated *NIT4* might compensate for decreased proteins in plant roots.

ROS accumulation was involved in short-term growth recovery from Zn deficiency in response to additional Mn supplementation

Among the altered expressions of many genes affected by Zn deficiency, we examined the expression patterns of various peroxidase and RBOH genes using RNA-seq (**Table 1**). Peroxidases are involved in regulating plant growth through ROS scavenging and production (Mittler et al. 2004, Cosio and Dunand 2009). Arabidopsis contains 73 class III plant peroxidases (Almagro et al. 2009). The expression of *RCI3*, which encodes a class III plant peroxidase, increases in response to K deficiency, and overexpression of *RCI3* increases ROS accumulation in roots (Kim et al. 2010). *PER62* positively regulates plant defense responses accompanied by ROS production (Cosio and Dunand 2009), and *PER34* and *PER71* are involved in ROS production (Daudi et al. 2012, Raggi et al. 2015). ROS produced by RBOHs plays important roles in abiotic and biotic stress responses and plant development (Kaya et al. 2019). RBOHs encode NADPH oxidases and are involved in Zn deficiency-induced ROS production in cotton roots (Cakmak and Marschner 1988).

In the current study, Zn deficiency significantly induced the expression of class III PER genes, including *PER10*, *PER59*, *PER53*, *PER54*, *PER39*, *PER22*, *PER66*, *PER30* and *PER23*, as well as *RBOHD* and *RBOHB*, which were suppressed by the addition of extra Mn (**Table 1**). To understand the reason for the induction of these PER gene expressions by Zn deficiency, the functions of these PERs on ROS homeostasis need to be elucidated. ROS, such as superoxide ($O_2\bullet^-$) and H_2O_2, accumulate in root cells and are important for regulating root growth (Dunand and Penel 2007). Zn deficiency induces ROS production in plants (Cakmak and Marschner 1988, Pinton et al. 1994, Höller et al. 2014). Similarly, here, ROS levels in Arabidopsis root tips increased under Zn0 compared with basal conditions and decreased in response to Mn application (**Fig. 8**). While excess Mn causes rapid ROS accumulation in plants, leading to leaf chlorosis and necrosis (Le Bot et al. 1990), the application of 75 μM Mn did not enhance Zn deficiency-induced ROS accumulation. These observations imply that PER and RBOH positively regulate Zn deficiency-induced ROS production, and 75 μM Mn treatment inhibits ROS production during short-term growth.

Zn plays a role as a cofactor of Cu/Zn-SOD. Two Cu/Zn-SODs, *CSD1* (AT1G08830) and *CSD2* (AT2G28190), and one copper chaperone for SOD, *CCD* (AT1G12520), are downregulated by Zn depletion (Wintz et al. 2003, Sakamoto et al. 2011). These three genes were also downregulated on the Zn0 medium and recovered comparably to those on the basal medium by supplementation with Mn (**Supplementary Table S5**). This result is consistent with excess Mn upregulating and activating Cu/Zn-SOD in plant shoots (Zhao et al. 2012, Ribera-Fonseca et al. 2013). Therefore, these results suggest that SOD activity that was decreased upon Zn depletion was restored by Mn supplementation, leading to reduced ROS accumulation (**Fig. 8**).

In Arabidopsis 26S proteasome subunit mutants *rpt2a* and *rpt5a*, Mn concentration is decreased compared with that in Col-0 and the mutants are more sensitive to Zn deficiency owing to the elevation of oxidative stress (Sakamoto et al. 2011). It has been reported that Mn can protect against oxidative stress in several organisms (Archibald and Fridovich 1981, Daly et al. 2004, Reddi et al. 2009). In addition, the *sod1* mutant yeast is sensitive to Zn-deficiency rescue by supplementation with excess Mn (Sanchez et al. 2005). Furthermore, Mn supplementation accelerates the development of *Caenorhabditis elegans* and improves the lifespan of the short-lived mutant (Lin et al. 2006). These results suggest that the supplementation of additional Mn to Zn-depleted conditions might contribute to the tolerance of Zn-deficient symptoms in Arabidopsis.

Together, our findings indicate that Arabidopsis accumulates ROS and thereby reduces biomass under undesirable growth conditions. Mn might rescue the inhibited growth of plants under Zn deficiency by altering ROS homeostasis. Future studies should examine the relationships between PERs and RBOHs in Zn deficiency-induced ROS accumulation.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana accession Col-0 was used for all experiments in this study. They were grown on the MGRL medium (referred to basal), the MGRL-based Zn-depleted medium (referred to Zn0) and the Zn0 media including different elements, such as 8.6 μM $FeSO_4 \cdot 7H_2O$, 10.3 μM $MnSO_4 \cdot 5H_2O$, 7 mM KNO_3 or 1.5 mM K_2SO_4, 24 nM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ or $MoCl_5$. We determined these elemental concentrations by subtracting that of the Zn0 medium from that of the MS-Zn0 medium in our previous report (Inaba et al. 2015). The elemental contents in MGRL and MS media are summarized in **Supplementary Table S8**. To determine the elements for plant growth recover, 100 μM Fe, 100 μM Mn,

14,000 μM K and 0.7 μM Mo were added into the Zn0 medium. Also, to determine the optimized concentration of Mn, 50, 75, 100, 125 and 150 μM Mn were added into the Zn0 medium or 75 μM Mn was added into the basal medium. To verify the effects on the plant growth by the different purity of Mn, 99% or 99.9% reagents were used (Wako, Japan). The surface of seeds was sterilized to avoid contamination. Seeds were sown and incubated at 4°C in the dark for 3 d to promote the germination of seeds. Plants for short-term phenotypic observations were grown for 10 d on each growth medium at 22°C under long-day conditions (16-h light/8-h dark cycle). For the long-term phenotypic observation, 10-day-old plants grown on each medium were transplanted on rock wool and further grown for 4 weeks by hydroponics.

Measurement of chlorophyll content

Chlorophyll contents in Col-0 shoot grown on basal, Zn0 and Zn0 + Mn75 media vertically for 14 d or long-term growth with hydroponics were determined. Approximately 100 mg of FW samples were homogenized with 500 μl of ice-cold 80% acetone. The chlorophyll content was measured as described by Porra et al. (1989). Three replicates were prepared from three independent experiments at different times.

Observation of seed germination rate and shape

The germination rate of Col-0 seeds grown with rich nutrient (HYPONeX, Japan) or grown on basal, Zn0 and Zn0 + Mn75 liquid media by hydroponics was examined by growing on basal, Zn0 and Zn0 + Mn75 media as shown above. Also, 15 seeds grown with each liquid medium or control growth condition were placed on a glass slide and observed their shape under a stereoscopic microscope (Moniken 2 BA210E1080, Shimadzu Rika, Japan).

Elemental analysis

Col-0 was grown on basal, Zn0 or Zn0 + Mn75 media for 10 d. For the elemental analysis, the shoots and roots were collected approximately 300 mg FW and dried at 60°C for 3 d. Ten parts per million of the 99% and 99.9% $MnSO_4$ solution in water was prepared. The dried shoots and roots (>10 mg DW) were digested in ultrapure HNO_3 using a microwave digester (START D; Milestone General). The 99% and 99.9% $MnSO_4$ solution was diluted at a given concentration with 5% HNO_3. The digestate and diluted samples were analyzed by inductively coupled plasma mass spectroscopy (Agilent 7500cx; Agilent Technologies, CA). For total N contents, the shoots and roots were collected approximately 1,000 mg FW and dried at 60°C for 3 d. Forty to fifty milligrams of DW samples were measured using an elemental analysis system (Vario MAX cube; Elementar Japan Co., Ltd.). For these elemental analyses, three replicates were prepared from three independent experiments at different times.

RNA-seq analysis

The total RNA was isolated from roots of Col-0 grown on basal, Zn0 and Zn0 + Mn75 media for 10 d using an RNeasy plant mini kit (QIAGEN, Germany), according to the manufacturer's instruction. The quality of the total RNA was checked using an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies). The samples [RNA Integrity Number ≧ 7.0, rRNA ratio (25S/18S) ≧ 1.0] of three biological replicates were analyzed by RNA-seq (Novaseq, Illumina, SAN). Sequencing was conducted by Macrogen Japan to produce 20 million 300-bp paired-end reads per sample on average. The fastq files were processed by the quality trimming software fastp (Chen et al. 2018) followed by mapping to Arabidopsis transcriptome (Araport11) and quantification by kallisto (Bray et al. 2016). The quantified data were normalized by the sleuth package in the R environment (version 3.5.1). Statistical analyses including PCA and DEGs were conducted by the packages of sleuth and shiny in the R. Statistical significance of the DEGs was assessed by the Wald test with the *q*-value using the Benjamini–Hochberg's multiple comparison correction (Bray et al. 2016). The raw RNA-seq data were deposited in the Gene Expression Omnibus database at the National Center for Biotechnology Information (accession number PRJNA622958: under depositing).

Analysis of ROS accumulation

Col-0 grown on basal, Zn0 and Zn0 + Mn75 media for 5 d was used for DAB staining. Seedlings were incubated in 1 mg/ml DAB (D5637; Sigma-Aldrich) in 10 mM Na_2HPO_4, 0.05% (v/v) Tween 20, for 1 h under dark at room temperature. The seedlings were cleaned in 60% (v/v) ethanol, 20% (v/v) acetic acid and 20% (v/v) glycerol and the root tips were observed under the Axiovert 200 M microscope (Zeiss, Germany).

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

No conflicts of interest declared.

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