Overexpression of a novel microRNA IamiR-4-3p from water spinach (*Ipomoea aquatica* Forsk.) increased Cd toxicity and accumulation in Arabidopsis thaliana

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Abstract

The function of lamiR-4-3p was investigated by using wild type (WT), transfected with empty vector pCambia1302 (CK) and lamiR-4-3p transgenic Arabidopsis in this study. The expression level of *GST3* was reduced by 20% in the transgenic Arabidopsis (*p35S*::*miR-4-3p* Arabidopsis) when compared to WT, and both of its shoot and root were shorter than WT and CK. After 3 d Cd treatment, root Cd concentrations of *p35S*::*miR-4-3p* Arabidopsis was significantly higher than WT and CK, while no significant difference was found in shoot Cd concentrations. MDA and H_2O_2 concentrations were positively correlated with the Cd concentrations in Arabidopsis. Interestingly, even though there was no significant difference among the shoot Cd concentrations, shoot MDA and H_2O_2 of *p35S*::*miR-4-3p* Arabidopsis were higher than those of WT and CK, and shoot T-AOC exhibited a opposite trend. These results are clearly related to the lowered expression of *GST3* by the overexpression of miR-4-3p in *p35S*::*miR-4-3p* Arabidopsis. It is suggested that the function of lamiR-4-3p is able to diminish the expression level of *GST3*, and is responsible to the growth dwarf, higher Cd uptake and oxidative damage but not the Cd translocation from root to shoot in Arabidopsis.

1. Introduction

Cadmium (Cd) is a common toxicity heavy metal, which tends to deposit in human kidney tubules, guts, bones and other organs via the food chain, posing a severe threat to human health (Ishikawa et al. 2012; Rafati Rahimzadeh et al. 2017). Breeding and selecting pollution-safe cultivars (PSCs) is an effective way to alleviate Cd accumulation in crops and reduce the Cd accumulation risks in human bodies (Yu et al. 2006). Water spinach (*Ipomoea aquatica* Forsk.) is an important leafy vegetable in south China. In our previous studies, a a typical Cd-PSC with low-shoot-Cd (QLQ) and a high-shoot-Cd accumulation cultivar (T308) had been screened from 30 water spinach cultivars (Wang et al. 2009; Xin et al. 2010). Furthermore, the different Cd accumulation abilities of QLQ and T308 were largely determined by the different transcriptomic profiles of Cd absorption and detoxification (Huang et al. 2016). The regulatory mechanism for the transcriptomic performance remains unknown and needs further stepped into discovery. Therefore, it is of great value to investigate the low-shoot-Cd accumulation mechanisms of water spinach by comparing the different molecular regulation level of Cd absorption and detoxification between QLQ and T308.

Glutathione transferases (GSTs) are reported to be a family of multifunctional enzymes that involve in various biotic and abiotic stresses amelioration including heavy metal stresses (Dixit et al. 2011). The biotic and abiotic stresses would bring about the boosted production of reactive oxygen species (ROS), resulting in DNA degradation and lipids damages to plants (Jalmi and Sinha 2015). Cd is known to induce ROS and could trigger oxidation damage to plant. One of the important functions of GSTs is to eliminate the oxidative damage by conjugating the generated ROS products with glutathione (GSH) in plants (Nianiou-Obeidat et al. 2017). Moreover, GSTs can also function in abiotic stress defense by improving the biosynthesis of sulfur-containing secondary metabolites, such as phenolics, glucosinolates and flavonoids (Dixit et al. 2011). In most cases, the increased expressions of *GSTs* could enhance Cd

tolerance in organisms, by promoting the formation of low-toxic glutathione (GSH)-Cd complex (Song et al. 2019). However, whether the GSH biosynthesis would influence the Cd accumulation in plant remains unclear.

A novel miRNA (IamiR-04m-3p: GATCACAGTGTATTATGGTGTCC) expressed differently between QLQ and T308 under the Cd treatment was found in our previous study. The expression level of IamiR-4-3p decreased significantly in T308 but kept stable in QLQ under Cd stress (Shen et al. 2017), indicating that IamiR-4-3p might play critical role in the cultivar-dependent Cd responsive regulation. *GST3* was identified as one of the dominant targets of IamiR-4-3p according to psRNATarget server and the target area located at the 3' untranslated region (3' UTR) of *GST3* indicated that IamiR-4-3p may played an important role in regulating the *GST3* expression levels. Therefore, this research has important significance to uncover the molecular mechanisms of IamiR-4-3p in the Cd accumulation ability of water spinach.

In the present study, we developed transgenic *Arabidopsis thaliana* (Arabidopsis) expressing the IamiR-4-3p from water spinach to investigate the function of IamiR-4-3p in Cd accumulation. It is hypothesis that: 1) IamiR-4-3p targeted in the *GST3* expression levels in transgenic Arabidopsis. 2) Oxidative damage from Cd toxicity was elevated by IamiR-4-3p in the *p35S:: ia-miR-4-3p* Arabidopsis, which should attribute to the retarded generation of GSH-Cd complex catalyzed by *GST3*. 3) IamiR-4-3p displayed significant role in the higher Cd uptake but not the Cd translocation from root to shoot in the transgenic Arabidopsis. It is expected that this study would be helpful to elucidate the critical role of IamiR-4-3p on Cd accumulation through GST expression in water spinach and provide effective molecular understandings for the breeding of low Cd cultivars.

2. Materials And Methods

2.1 Plant materials and growth condition

Water spinach (T308) and *Arabidopsis thaliana* (ecotype Columbia) were used in the present study. Seeds of water spinach and *Arabidopsis thaliana* were surface disinfected in the presence of 0.7% NaClO for 5 minutes, then thoroughly washed with deionized water for 3 times. After germination, the seedlings were cultivated in pots filled with growth media and watered with half-strength Hoagland nutrient solution every 3 days. T308 was cultivated in greenhouse with the light cycle of 12h light / 12h dark, 30 ℃ every day. After 4 weeks, T308 roots were harvested for DNA extraction. *Arabidopsis thaliana* grew under suitable conditions in a plant incubator, which was used for plant transformation and Cd treatment (de Felippes, Ott, and Weigel 2011).

2.2 Vector constructs and plant transformation

The genomic DNA of water spinach was extracted by CTAB method(Schiebelhut et al. 2017) and then tested by nanodrop 2000c (Thermo scientific, USA). The IamiR-4-3p precursor sequence was amplified from genomic DNA by PCR using gene-specific primer pairs designed with NcoI and Spe site (Table S1, supporting information). The amplified fragments were digested and cloned into the binary vector,

pCambia 1302, containing a CaMV 35S promoter, kanamycin and hygromycin resistance gene. The construct was introduced into *E. coli* DH5α and confirmed by DNA sequencing. The confirmed constructs was named as *p35S::lamiR-4-3p* as shown in Figure S1 (supporting information). Also, the empty vector pCambia1302 without any modification was utilized as control (CK) in this research. The *p35S::lamiR-4-3p* and CK vector were introduced into *Agrobacterium tumefaciens* LBA4404 (Shanghai Weidi Biotechnology, China). Arabidopsis transformation was performed by the floral dip method (Clough and Bent 1998). T2 homozygous lines were used in all experiments presented in the current study. All transgenic plants were selected by 40mg/L hygromycin in 1/2 MS medium, then confirmed by PCR analysis of lamiR-4-3p and green fluorescent protein (*GFP*) with leaf genomic DNA as templates (Table S1).

2.3 RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was separately isolated from shoot and root samples with the aid of the RNA Easyspin Isolation System (Aidlab, China). RNA concentration and quality were tested by nanodrop 2000c (Thermo scientific, USA). miRNA cDNA Synthesis Kit (ABM, Canada) and EvaGreen miRNA qPCR MasterMix (ABM, Canada) were used for the quantitative of lamiR-4-3p. PrimeScript™ RT regent kit (Takara, Japan) and YBR GreenII PCR Master Mix (Takara, Japan) were used for the quantitative of *GST3*, the target gene of lamiR-4-3p. All the primers for lamiR-4-3p, *GST3* and their reference gene U6 and actin for qRT-PCR were shown in Table S2. The reactions were performed on LightCycler® 480 Real-Time PCR System (Roche, Germany). Each qRT-PCR analysis was done in triplicate and with three biological replicates. Results were analyzed with the integrated LightCycler® 480 service software. Expression levels of the tested genes were determined by CT values and calculated by the $\Delta\Delta$Ct method (Schmittgen and Livak 2008).

2.4 Cd concentration measurement

Six-week-old WT, CK and transgenic *Arabidopsis thaliana* seedlings were treated with half strength Hoagland solution containing 5 mg/L Cd for 3 days, and 3 replicas were made for WT, CK and transgenic *Arabidopsis thaliana*. Shoot and root samples were harvested separately, deactivated enzyme at 105°C for 30 min, and then dried at 70°C to constant weight. The dried samples were digested (HNO_3:H_2O_2, 5:1) and then measured by an atomic absorption spectroscopy (Hitachi Z-5300, Japan). To control the precision of the analytical procedures, a certified reference material (CRM) of plant (GBW-07603, provided by the National Research Center for CRM, China) with a Cd concentration of 0.38 mg/kg was employed.

2.5 MDA, H_2O_2 concentrations and T-AOC measurement

Shoot and root MDA, H_2O_2 concentrations and T-AOC in WT, CK and transgenic *Arabidopsis thaliana* were tested by using MDA, H_2O_2 and T-AOC assay kits (Suzhou comin biotechnology, China), respectively. Three repeats were conducted for each treatment.

2.6 Statistical analysis

SPSS 23.0 and GraphPad Prism 8 were used for statistical analyses and figures development. One-way ANOVA with the least significant difference (LSD) test was performed to evaluate the significance of

treatment effects. Values were considered to be statistically significantly different when $p < 0.05$.

3. Results

3.1 Transcript analysis of lamiR-4-3p and *GST3* in transgenic plants

To ensure the reliability of the transformation of Arabidopsis, the PCR of pre-lamiR-4-3p (gene of interest) and *GFP* (marker gene) was conducted by using the DNA of leaves samples from each plant. As shown in Figure S2, *GFP* was detected in both CK and *p35S*::*lamiR-4-3p* Arabidopsis plants, and pre-lamiR-4-3p could only be detected in *p35S::lamiR-4-3p* plants. Meanwhile, neither pre-lamiR-4-3p nor *GFP* could be detected in WT plants. The results indicated that both *p35S*::*miR-4-3p* and CK vectors had been successfully transfected into Arabidopsis. The results of qRT-PCR showed that *p35S::miR-4-3p* plants exerted significantly higher expression levels in lamiR-4-3p, when compared with WT and CK plants. While, the *GST3* expression level in *p35S::miR-4-3p* plants was only 80% of those in WT and CK plants (Figure 1). The GST3 and lamiR-4-3p sequences between water spinach and Arabidopsis shared only 50.15% of sequence similarity according to sequence alignment analysis (Figure S3). Also, there were 3 mismatches detected between lamiR-4-3p and *GST3* of water spinach, and 5 mismatches detected between lamiR-4-3p and *GST3* of Arabidopsis at their 3'UTR, respectively (Figure 2).

3.2 Effects of lamiR-4-3p on Arabidopsis growth

When compared with WT plants, CK and *p35S::miR-4-3p* plants did not show evident morphological difference (Figure 3). However, when compared the growth status of these *Arabidopsis* plants, we noticed that sizes of *p35S::miR-4-3p* plants were smaller than those of WT and CK. The height of WT, CK and *p35S::miR-4-3p* plants were 15.6, 15.3 and 11.5 cm, respectively, and the plant height of *p35S::miR-4-3p* plants was significantly lower than those of WT and CK ($p < 0.05$). Also the average root length of *p35S::miR-4-3p* plants was 4.9 cm, which was significantly shorter than those of WT and CK ($p < 0.05$). These findings suggested that overexpression of lamiR-4-3p inhibited the growth of Arabidopsis.

3.3 Cd concentrations in Arabidopsis

In order to investigate the effect of lamiR-4-3p overexpression on Cd accumulation ability in Arabidopsis, Cd contents of shoots and roots of WT, CK and *p35S::miR-4-3p* Arabidopsis plants was measured at 0 h and day 3 after Cd exposure (5 mg/L $CdCl_2$), respectively. As displayed in Table 1, the results showed that there was no significant difference ($p > 0.05$) in both the shoot and root Cd concentrations among WT, CK and *p35S::miR-4-3p* Arabidopsis at 0 h. After 3 days of Cd treatment, Cd concentration of the shoot in *p35S::miR-4-3p* was still similar to those of WT and CK Arabidopsis. However, Cd concentration of the root in *p35S::miR-4-3p* was 1.13 and 1.10 times of those in WT and CK Arabidopsis respectively at day 3, and the differences between *p35S::miR-4-3p* and WT as well as CK were significant ($p < 0.05$), indicating that *p35S::miR-4-3p* plants accumulated more Cd in its root.

3.4 MDA concentrations in Arabidopsis

When treated with Cd at 0 h, the shoot MDA concentrations in WT, CK and *p35S::miR-4-3p* plants were 5.8, 5.5 and 5.4 nmol g^{-1} (Figure 4), and the differences were no significant ($p < 0.05$). The root MDA concentrations in WT, CK and *p35S::miR-4-3p* were 8.4, 8.2 and 9.1 nmol g^{-1}, respectively, and the differences were also without significance ($p < 0.05$). However, after 3 days of Cd treatment, shoot MDA concentrations in WT, CK and *p35S::miR-4-3p* plants were 6.6, 6.7 and 9.2 nmol g^{-1}, and root MDA concentrations of those plants were 10.8, 10.7 and 13.5 nmol g^{-1}, respectively. Both the shoot and root MDA concentrations in *p35S::miR-4-3p* plants were significantly higher than those of WT and CK plants ($p<0.05$). These results showed that the *p35S::miR-4-3p* plants suffered more lipid peroxidation damage when treated with Cd.

3.5 H_2O_2 concentrations in Arabidopsis

As shown in Figure 5, there was no significant difference of both shoot and root H_2O_2 concentrations in WT, CK and *p35S::miR-4-3p* at 0 h of Cd treatment ($p>0.05$). When treated with Cd for 3 days, the shoot H_2O_2 concentrations of WT, CK and *p35S::miR-4-3p* plants were 21.6, 23.8 and 27.7μmol g^{-1}, and the root H_2O_2 concentrations of WT, CK and *p35S::miR-4-3p* plants were 32.5, 34.4 and 39.8μmol g^{-1} at day 3. The H_2O_2 concentrations of both the shoot and root in *p35S::miR-4-3p* plants under the 3 day Cd treatment were significantly higher than WT and CK ($p<0.05$), which also indicated that *p35S::miR-4-3p* plant was subjected to a higher oxidative stress.

3.6 T-AOC concentrations in Arabidopsis

As shown in Figure 6, before Cd treatment (0 h), the T-AOC contents of both shoot and root in WT, CK and *p35S::miR-4-3p* Arabidopsis were about 15 U/mg, and there was no significant difference was found ($p>0.05$). The shoot and root T-AOC contents in WT, CK and *p35S::miR-4-3p* plants were significantly increased after 3 days of Cd treatment. Shoot T-AOC contents in WT, CK and *p35S::miR-4-3p* plants were 30.6, 31.0 and 25.8 U/mg, respectively, and root T-AOC contents in these plants were 40.0, 41.5 and 31.35 U/mg, respectively, after 3-day Cd exposure. Both the shoot and root T-AOC contents in *p35S::miR-4-3p* plant were significantly lower than those of WT and CK plants ($p<0.05$). indicating that the degree of ROS damage in *p35S::miR-4-3p* Arabidopsis was elevated.

4. Discussion

4.1 The GSTs expression level was regulated by lamiR-4-3p in Arabidopsis

As a new miRNA, we found that lamiR-4-3p was able to regulate *GST3* expression level in water spinach under Cd treatment. The lamiR-4-3p expression level of T308 was lower than QLQ, and accordingly higher expression level of *GST3* was observed in T308 (Shen et al. 2017; Huang et al. 2016). As higher abundance of GSTs has been found in responding to Cd stress (Nianiou-Obeidat et al. 2017; Cao et al.

2017; Zhao et al. 2019), implying the role of IamiR-4-3p in regulating expression of GST3 should be also related to the Cd stress response in water spinach. The qRT-PCR results indicated that IamiR-4-3p was able to down regulate the *GST3*'s expression level in Arabidopsis, but the performance was weaker than some other miRNAs investigated previously. Lin et al found that the miRNA160 was capable of down regulating the expression levels its targets, *ARF10*, *ARF16* and *ARF17*, by 65% in Arabidopsis (Lin et al. 2018). (Li et al. 2013) discovered that OsmiRNA396c was able to down regulate the expression level of *OsGRF4* by 80%. Also, the function study of miRNA in cotton suggested that ghr-miR414c had the capacity to down regulate the expression level of *GhFSD1* by 75% (Wang et al. 2019). In the present study, IamiR-4-3p was able to down regulate the expression level of *GST3* by only 20% in Arabidopsis, which was lower than the previous reports. This low regulation efficiency might probably be ascribed to the low sequence similarity (50.15%) of *GST3* between water spinach and Arabidopsis, leading to the defects in regulation ability of IamiR-4-3p in Arabidopsis. The target preference of IamiR-4-3p might be another responsible conjecture for the low regulation efficiency in GST3. Multiple target genes have been found for IamiR-4-3p in water spinach, such as RNA-capping enzyme-like and TMV-associated RING finger protein, etc (Shen et al. 2017). The target preferences were also observed between other miRNAs and their target genes. For example, the miRNA827 preferred to regulate the expression of nitrogen limitation adaptation (*NLA*) in Arabidopsis and phosphate transporter 5(*PHT5*) in *Oryza sativa* (Lin et al. 2018). Similarly, miRNA21 exerted target preference for different regulatory capacities which was 75% down regulation in acidic nuclear phosphoprotein and NADP but only 35% down regulation in cold shock domain-containing protein and high-density lipoprotein-binding protein1 (Schramedei et al. 2011).

Therefore, it is likely that IamiR-4-3p had the preference to regulate the expression of the other target genes other than *GST3* in Arabidopsis. Also, the IamiR-4-3p overexpressed Arabidopsis (six-week-old) presented with growth retardation, which was indicated by the 20% and 15% shorter shoot and root than WT as well as CK. As Arabidopsis were not treated with Cd during the first six weeks, the overexpression of IamiR-4-3p still influenced the growth of Arabidopsis, which stood a good chance that IamiR-4-3p should have target preference, and the other target genes maybe related to the regulation of growth and development. For a better understanding of the function of IamiR-4-3p, therefore, the IamiR-4-3p overexpressed water spinach should be constructed, and the target preference of IamiR-4-3p should be further studied.

4.2 Oxidative damage from Cd toxicity was elevated in *p35S::IamiR-4-3p* Arabidopsis

Numerous studies have shown that Cd toxicity causes the formation of ROS in plants, which would result in the accumulations of H_2O_2 and O^{2-} and cause oxidative damage to plant organisms (Zhang et al. 2017; Xu et al. 2018). Therefore, the H_2O_2 accumulation induced by Cd is one of the very important factors resulting in lipid peroxidation damage and increase of MDA in plants. In the present study, the MDA and H_2O_2 increments in the Cd exposed Arabidopsis especially in the *p35S::miR-4-3p* transgenic plant were consistent with the studies in many other plants, such as rice, tomato, tobacco, ect (Guo et al. 2018; Cai et al. 2019) .

T-AOC is an important antioxidant for plants under heavy metal stresses, and the lower the content of T-AOC in plants, the more oxidative damage will plants suffer (Sytar et al. 2013). In the present study, we also found that there was a significant negative correlation between T-AOC and Cd concentration in plants, which was consistent with the research of (Zhan et al. 2018), who found that a significant negative correlation (-0.856, $p < 0.01$) existed between T-AOC and Cd contents in maize. Therefore, the lamiR-4-3p overexpression reduced the antioxidation ability in Arabidopsis, which should be one of the reasons of the increases of MDA and H_2O_2 contents in the *p35S::miR-4-3p* plant.

As is well known, GSTs was considered to involve in the plant protective mechanism under various stresses, by regulating the reversible S-glutathionylation of protein thiol residues (Mieyal and Chock 2012). The overexpression of *GST* gene can significantly enhance salt, drought and heavy metals resistance of tomato tobacco and rice (Csiszár et al. 2014; Dixit et al. 2011; Kumar et al. 2013). As for water spinach, Wang et al found that the level of Cd in the NaCl-extractable form, which is related to the content of Cd-binding complex, was higher in T308 than in QLQ, indicating that the Cd chelated with proteins or peptides in T308 should be more than that in QLQ(Wang and Ren 2014; Zhang et al. 2010). In our other study, it was found that T308 possessed a higher sulfur metabolic protein and GST3 expression level, which was helpful to reduce toxicity of Cd and improve Cd tolerance by generating GSH-Cd complex (Huang et al. 2016). Because the overexpression of lamiR-m04-3p leaded to a 20% decrease in the expression level of *GST3* in Arabidopsis, the detoxification capacity through the production of GSH-Cd complex catalyzed by GST3 would be impeded, resulting in a series of oxidative damage caused by Cd in the lamiR-m04-3p overexpressed Arabidopsis. Therefore, the presence of Cd can easily trigger ROS reaction and oxidative damage when lamiR-m04-3p was overexpressed, which explained why shoot of lamiR-m04-3p overexpressed Arabidopsis suffered a more severe oxidative damage than WT and CK Arabidopsis, although their Cd accumulation levels were almost the same.

4.3 lamiR-4-3p affects Cd uptake rather than Cd transloction in Arabidopsis

In the present study, an unusual observation has to be explained, that the shoot Cd concentrations among WT, CK and *p35S::miR-4-3p* plants were not significant, although the root Cd concentration of *p35S::miR-4-3p* plant was pretty higher than those of WT and CK after 3 days of Cd treatment. The results indicated that lamiR-4-3p overexpression exerted no effect on Cd translocation from root to shoot, but it increased Cd uptake in the root of Arabidopsis. It might be attributed to the higher Cd-induced oxidative damage in *p35S::miR-4-3p* Arabidopsis as above-mentioned. According to Han et al and Javed et al, the oxidative damage would increase the cell membrane permeability and release of organic acids of root, which could promote the Cd uptake in the root (Han et al. 2006; Javed et al. 2017). Besides, multiple target genes of lamiR-4-3p have been found in water spinach, and one of which is AWPM19-like protein (Shen et al. 2017). The function of AWPM19-like protein is to mediate abscisic acid (ABA) influx through the plasma membrane. Low levels of ABA in plants would result in minimized deposition of apoplastic barriers and allowed maximization of Cd uptake (Tao et al. 2019). It is thus suggested that the lamiR-4-3p overexpression should also reduce the generation of AWPM19-like protein and the influx of ABA in

Arabidopsis, which could explain why Cd uptake was increased in the root of the *p35S::miR-4-3p* Arabidopsis. Accordingly, we proposed that the higher oxidative damage and the down-regulation of AWPM19-like protein would cause the higher Cd uptake in the root of *p35S::miR-4-3p* Arabidopsis. As for the shoot Cd concentration of *p35S::miR-4-3p* Arabidopsis similar to those of WT and CK, it might be attributed to the down regulation of the level of *GST3* by IamiR-4-3p. According to He et al, GST was able to promote the produce of GSH-Cd complex, which could increase the mobility of Cd from root to shoot (He et al. 2018). It is considered that the Cd translocation from root to shoot in *p35S::miR-4-3p* Arabidopsis with lower *GST3* expression would be influenced by the lower generation of GSH-Cd complex, which deterred the transportation of Cd from root to shoot. Therefore, even though the root Cd concentration was higher in *p35S::miR-4-3p* Arabidopsis, its shoot Cd concentration showed no significant difference with WT and CK. Similarly, Cd accumulation in leaves of *Vicia faba* L. was positively correlated with the contents of PCs-Cd and GSH-Cd compound, it seemed that sulfur-containing compound facilitated Cd uptake in cell walls of leaves without retaining Cd in roots (Wu, Sagervanshi, and Mühling 2018). Matraszek-Gawron et al also stated that PCs and GSH could act as long-distance carriers of Cd in *Triticum aestivum* L(Matraszek-Gawron and Hawrylak-Nowak 2019). These results could also help to explain the restrained accumulation of Cd in shoots of *p35S::miR-4-3p* Arabidopsis, which involved in the deterred transportation of Cd with the inhibition of generation of GSH-Cd complexes by ia-miR-4-3p. For another, the function of other target genes of IamiR-4-3p in water spinach, such as RNA-capping enzyme-like and TMV-associated RING finger protein, etc (Shen et al. 2017) is still not clear, and their impacts on the Cd translocation and transportation in *p35S::miR-4-3p* Arabidopsis as well as water spinach are necessary to be further investigated.

5. Conclusion

It is concluded that IamiR-4-3p is responsible to the oxidative damage and higher Cd uptake but not the Cd translocation from root to shoot, and to the regulation of AWPM19-like protein and GST3 in Arabidopsis. Oxidative damage from Cd toxicity was elevated in *p35S:: ia-miR-4-3p* Arabidopsis attributing to the retarded generation of GSH-Cd complex catalyzed by *GST3*. These findings should be helpful to elucidate the critical role of IamiR-4-3p on Cd accumulation and transportation in water spinach and provide effective molecular understandings for the breeding of low-Cd cultivars. Other functions of IamiR-4-3p on Cd accumulation and detoxification are valuable to be further verified.

Declarations

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Contributions

Conception and design of the study were accomplished by Chun-Tao He *, Chuang Shen and Zhong-Yi Yang *; Data collection and analysis was performed by Chuang Shen, Ying-Ying Huang and Jun-Liang Xin; first draft was written by Chuang Shen and Ying-Ying Huang. Data interpretation and manuscript polishing was carried out by Zhong-Yi Yang *. All the authors approved the final manuscript.

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Ethics declarations

Competing interests: There is no conflict of interest among the authors.

Ethical approval: Not applicable.

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Data availability

The data of this study are available on request from the corresponding author.

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Tables

Table 1. Cd concentrations in shoot and root of different types of *Arabidopsis thaliana*

	time	WT	CK	IamiR-4-3p
Shoot (mg/kg)	0h	2.52±0.10 Ba	2.06±0.22 Ba	2.41±0.29 Ba
	3d	56.05±3.08 Aa	49.33±3.93 Aa	56.57±4.84 Aa
Root (mg/kg)	0h	8.86±0.32 Ba	9.17±0.30 Ba	8.51±0.17 Ba
	3d	504.22±10.25 Ab	522.32±10.21 Ab	574.79±8.92 Aa

Notes: Different lower-case letters stand for significant differences among the three types of *Arabidopsis thaliana* within the same Cd treatment time(p<0.05); Different capital letters stand for significant differences between the two different Cd treatment time within each of the *Arabidopsis thaliana* types ($p < 0.05$).

Figures

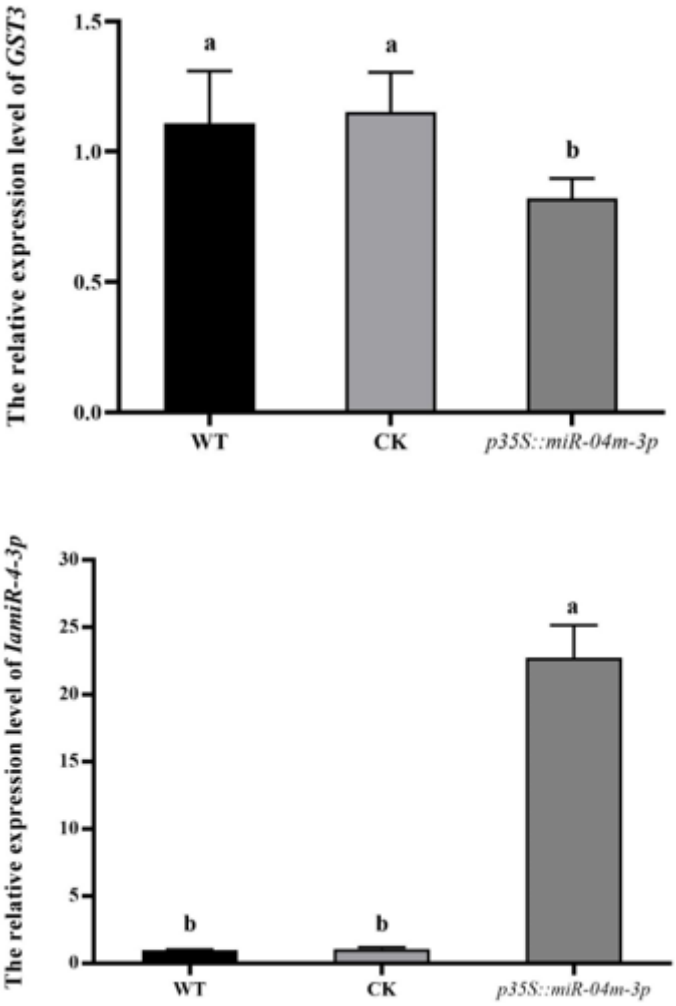

Figure 1

The expression level of GST3 (A) and Ia-miR-4-3p (B) in six-week-old WT, CK and p35S::miR-4-3p Arabidopsis. Note:error bars of gene expression levels represent the standard deviation among three replicates. Statistical significance was defined at $p < 0.05$ determined using analysis of variance (ANOVA). Different letters indicate significant differences between WT, CK and p35S::miR-4-3p Arabidopsis.

At-*GST3*	680	A	G	A	C	A	A	C	A	T	G	A	T	A	C	T	C	T	T	T	G	A	T	C	702
Ia-*GST3*	680	G	G	A	C	A	A	C	A	T	A	C	T	A	C	A	C	T	G	T	A	A	T	C	702
		|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	
IamiR-4-3p	23	C	C	T	G	T	G	G	T	A	T	T	A	T	G	T	G	A	C	A	C	T	A	G	1

Figure 2

Sequence comparison between IamiR-4-3p and GST3 3' UTR of Arabidopsis and water spinach. Note: At-GST3 stands for the GST3 sequence of Arabidopsis and Ip-GST3 stands for the GST3 sequence of water spinach. The letter with red color means the mismatches between Arabidopsis and IamiR-04-3p, and the letters with blue color means the mismatches between water spinach and IamiR-04-3p.

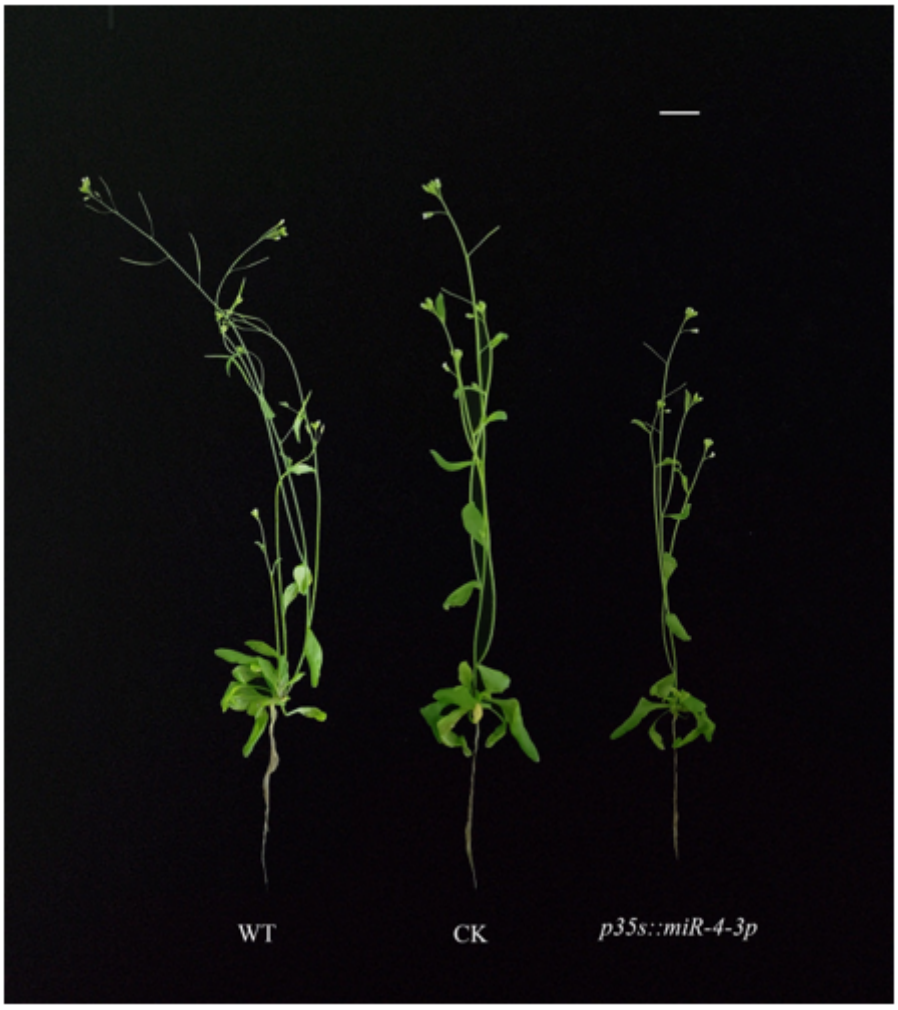

Figure 3

The growth status of six-week-old WT, CK and p35S::miR-4-3p Arabidopsis. Scale bar 1 cm.

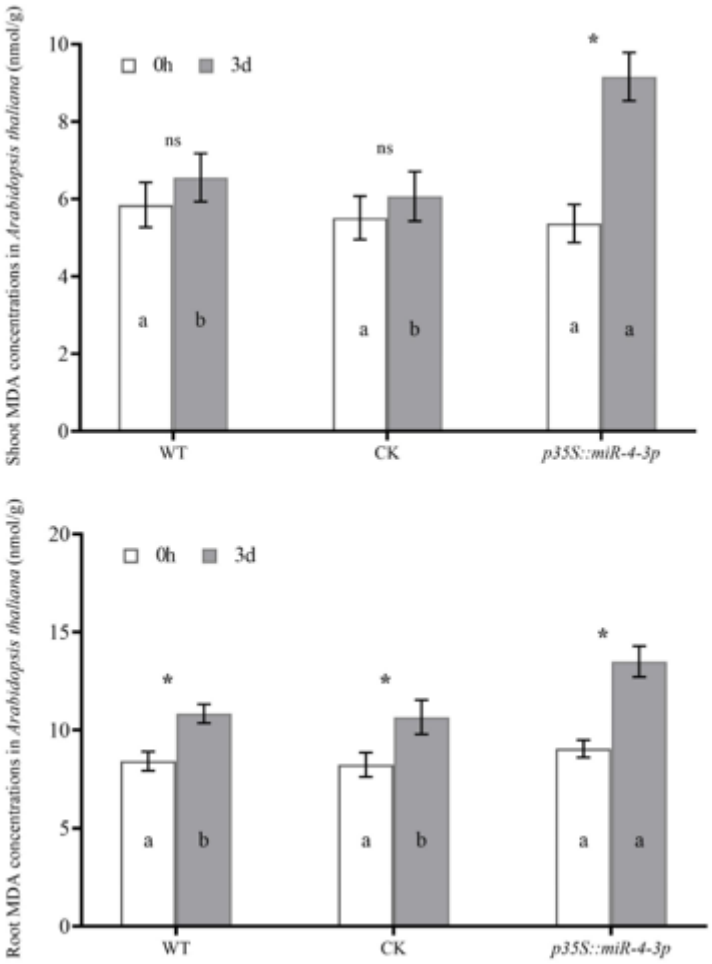

Figure 4

Effects of Cd treatment on MDA concentration in shoot (A) and root (B) of Arabidopsis thaliana at 0h and 3d. Different letters within the same cultivar indicate significant differences at $p < 0.05$ between different Cd treatment time; ns and * indicate differences between the cultivars within the same treatment is not significant at $p > 0.05$ level and significant at $p < 0.01$ level, respectively.

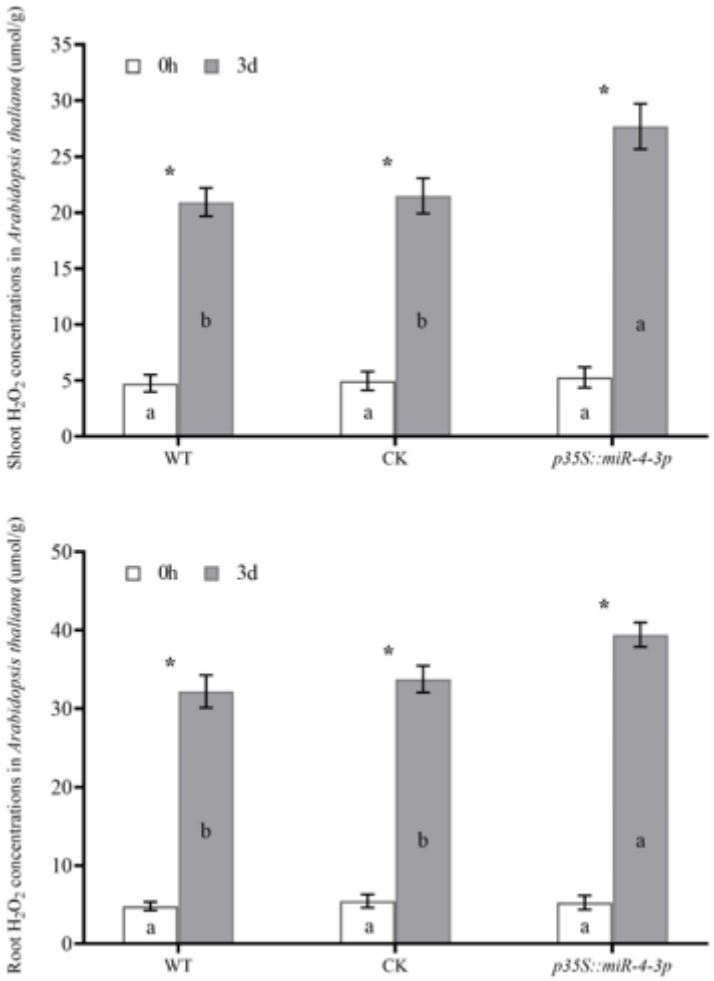

Figure 5

Effects of Cd treatment on H2O2 concentration in shoot (A) and root (B) of Arabidopsis thaliana at 0h and 3d. Different letters within the same cultivar indicate significant differences at $p < 0.05$ between different Cd treatment time; ns and * indicate differences between the cultivars within the same treatment is not significant at $p > 0.05$ level and significant at $p < 0.01$ level, respectively.

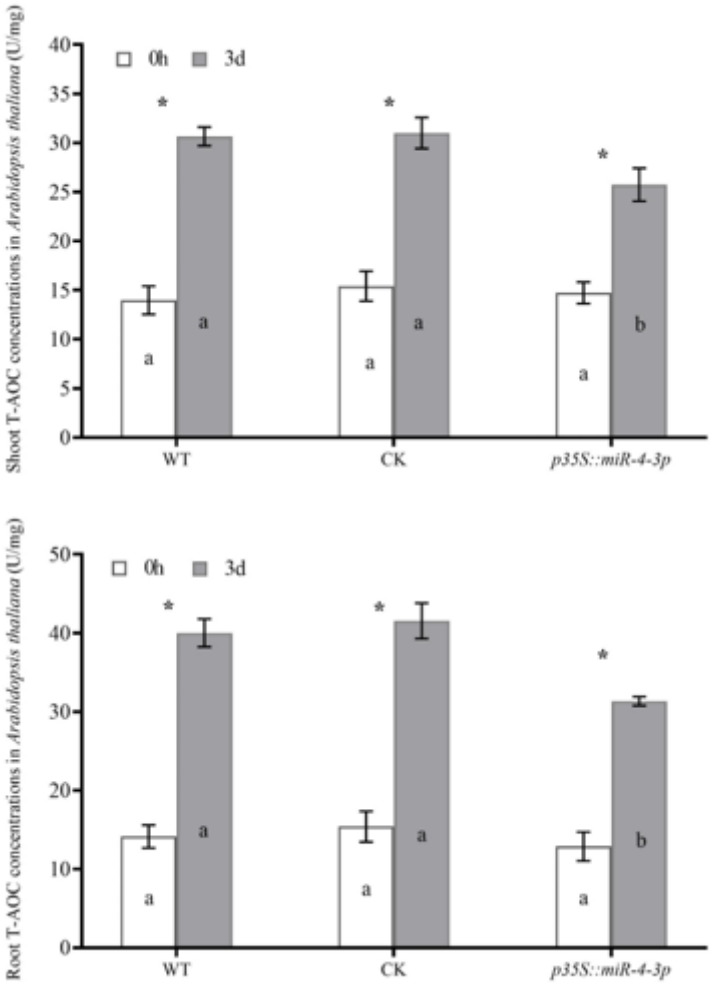

Figure 6

Effects of Cd treatment on T-AOC concentration in shoot (A) and root (B) of Arabidopsis thaliana at 0h and 3d. Different letters within the same cultivar indicate significant differences at $p < 0.05$ between different Cd treatment time; ns and * indicate differences between the cultivars within the same treatment is not significant at $p>0.05$ level and significant at $p < 0.01$ level, respectively.

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