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Research article

Mature trichome is the earliest sequestration site of Cd ions in *Arabidopsis thaliana* leaves

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A B S T R A C T

Sequestration of heavy metals by plants in non-glandular leaf trichomes is important for survival in toxic soils and has the potential for environmental remediation. Although heavy metals are particularly toxic to many plants during development, the integration of sequestration into the developmental timecourse is unknown. We tested the hypothesis that plants preferentially sequester heavy metals into mature trichomes by investigating the timecourse of Cd^{2+} ions into the leaves of the model plant *Arabidopsis thaliana*. Results supported the hypothesis and surprisingly showed no Cd^{2+} ions accumulated in earlier trichome development stages and that sequestration and release by mature trichomes were periodic and dynamic. Studies in mutants suggested that these dynamics were governed by the trichome's secondary cell wall, which matures late in development. Results suggest a developmentally timed pathway for excluding heavy metal toxins and the existence of mechanisms for controlled release that may relate to proposed functions of mature trichomes in plants.

1. Introduction

Large-scale phosphatic fertilizer, metal mining, exhaust fumes, and atmospheric deposition caused heavy metal pollution in the agricultural environment, such as in soil and underground water. Heavy metal ions in the farming environment can be transferred to the human food chain and pose severe public health problems (Hu et al., 2016; Yang et al., 2019; Zhang et al., 2015). For example, ingestion of cadmium (Cd) contaminated food (e.g., crustaceans, organ meats, leafy vegetables, rice from certain areas of Japan and China) or water (either from old Zn/Cd sealed water pipes or industrial pollution) can lead to cancer and organ system toxicity such as skeletal, urinary, reproductive, cardiovascular, central and peripheral nervous, and respiratory systems (Bernhoft, 2013). Cd ions concentration greater than 5 μM in soil is high pollution (Wagner, 1993). Reports on China's Soil Pollution Survey indicated that Cd ion was the primary pollutant in southern China (China's Soil Pollution Surveying). Cd ions are toxic to both plants and animals and have no known physiological function in any organism. In plants, Cd ions reduce the activity of enzymes such as superoxide dismutase, catalase and nitrate reductase, and furthermore cause plant growth retardation,

chlorosis, and necrosis of leaves (Gouia et al., 2000; Baryla et al., 2001; Irfan et al., 2014).

Plants developed several strategies, such as forming metal chelates/complexes, excretion or extrusion, and sequestration metal ions, to restrict the translocation and accumulation of heavy metal ions absorbed in plant cells. Therefore, the process of heavy metal ions translocation and accumulation in the plant cell is highly spatial and temporal dynamics. In *Arabidopsis thaliana* leaves, epidermal cells, mesophyll cells, and trichomes can be the spatial storage sites of Cd, Zn, and Ni ions (Sarret et al., 2009; Küpper et al., 2000). Those cells exhibit different capacities to sequester different metal ions. In *Arabidopsis helleri*, a hyperaccumulator plant, the epidermal cells contain lower Zn and Cd ions than mesophyll cells. The trichomes have the largest concentrations of Zn and Cd ions (Küpper et al., 2000). The spatial distribution of heavy metal ions in roots and leaves has been investigated in several plants (Belleghem et al., 2007). However, the temporal distribution of heavy metal ions in the leaf, especially in the early development stage, is relatively poorly understood.

Arabidopsis trichomes begin to form on the leaves in leaf early development, at a time when the developing protoderm is still dividing rapidly (Larkin et al., 1996). The first trichome form at the distal end of

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the immature leaf. As trichomes mature at the leaf tip, new trichomes arise gradually toward the base. Trichome initiation is found only in regions where epidermal cell division is occurring (Marks, 1997). Trichomes in the mature leaf are distributed relatively uniformly. Observing the Cd ions distribution with trichome development can shed light on the temporal distribution of heavy metal ions in the early stages of leaf development.

In this study, we investigated the temporal distribution of Cd ions in *A. thaliana* leaves. We identified that the mature trichome is the earliest sequestering site of Cd ions in immature and young expanding leaves. No Cd ions were sequestered in other trichome development stages, and the accumulation state of Cd ions in mature trichomes is highly dynamic.

2. Materials and methods

2.1. Arabidopsis growth

A. thaliana wild type and exo70H4-1 mutant seeds were placed in a 1.5 mL centrifuge tube then disinfected with 75% alcohol for 4 min, and 100% alcohol for 1 min. Placed the sterilized seeds in MS medium containing 0, 0.3 μM, 5 μM, and 40 μM CdCl$_2$, and vernalized for 3 days at 4 °C. Then, they were transferred into the culture room (22 °C, 16 h of light, 8 h of darkness) to grow.

2.2. Histological analysis

A. thaliana plants grown in MS medium containing different concentrations of Cd ions were stained with dithizone according to the method developed by Seregin (Seregin and Ivanov, 1997). 30 mg dithizone dissolved in 60 mL acetone and 20 mL distilled water, and 600 μL acetic acid was added to the solution to make the reaction more specific. Soaked plants in 50 ml dithizone solution for 24 h and then rinsed with distilled water three times.

A. thaliana plants exposed to Cu for 5 days to 3 weeks were fixed with acid-methanol for 3 h, then stained with 0.1% (w/v) dithiooxamide solution in 70% (v/v) ethanol for overnight.

2.3. Trichome transcriptome analysis

The expression profile analysis of early stage and mature trichomes was obtained from the ArrayExpress database (accession number E-MEXP-1038) (Kryvych et al., 2008). The GO term enrichment was analyzed on the Gene Ontology website (http://geneontology.org/).

3. Results and discussion

3.1. Mature trichomes are the earliest sequestration site of Cd ions in young expanding leaves of A. thaliana

Several studies demonstrated that *Arabidopsis* trichomes present the highest metal concentrations in leaves (Salt et al., 1995; Isaure et al., 2006). However, in those studies, no temporal distribution of metal ions was investigated. Since the plant cells absorbed metal ions at the germination stage, not at the seedling stage, we examined Cd ions localization in young expanding leaves of *A. thaliana* from the germination stage to 2 weeks. In young expanding leaves, the red Cd-dithizone complexes only appeared in trichomes (Figure 1a, c, and e). In old leaves, the Cd-dithizone complexes were observed in the trichomes, guard cell walls, epidermal cells, and mesophyll cells of plants exposed to 5 μM or 40 μM CdCl$_2$. For *A. thaliana* leaves exposed to 0.3 μM CdCl$_2$, Cd-dithizone complexes mainly presented in trichomes (Figure 1e, f). These results indicated that trichomes in young expanding leaves are the first Cd ion sequestration site, and do not just present the highest metal

concentrations in leaves. The Cd-dithizone complexes also present in the guard cell walls (Fig. S1), epidermal cells (Figure 1b) and mesophyll (Figure 1d) cells. Cd ions accumulated in epidermal cells were earlier than in mesophyll cells.

3.2. Cd ions also accumulated firstly in the mature trichome, even in immature leaves

Arabidopsis trichome development was divided into 6 stages: cell enlargement, tubular outgrowth, branch initiation, extension and papillae formation and cell wall thickening (Marks et al., 2009). These six stages occurred at the same time only in immature leaves. Trichomes in young expanding and old leaves were all mature cells. To confirm whether Cd ions accumulated in unmatured trichomes, we investigated Cd ions distribution in immature leaves. The red Cd-dithizone complex deposits only present in mature trichomes (Figure 2a, b). No Cd-dithizone complexes show in other trichome development stages (Figure 2c, d, e). This result indicated that heavy metal Cd ions also accumulate in mature trichomes firstly, even in immature leaves.

3.3. Cd ions accumulation state in mature trichomes is dynamic

Within leaves, genes involved in cysteine biosynthesis, O-acetylserine(thio)lyase, serine acetyltransferase, glutamylcysteine synthetase, and glutathione synthetase, were highly expressed in trichomes (Gutierrez-Alcala et al., 2000). Since these enzymes involved in metal detoxification and trichome accumulated more toxic metal ions than surrounding cells, the metal detoxification function of trichome has been proposed for many years (Gutierrez-Alcala et al., 2000; Salt et al., 1995). However, the mechanism of detoxification remains unclear. Observation of the accumulation states of metal ions provides clues to the trichome detoxification mechanism. Metal ion-rich ring structures in trichomes have also been reported for several elements, such as Zn (Sarret et al., 2009), Cu, Mn and Si (Kulich et al., 2018). However, we found that this ring structure is not the initial accumlated state of Cd ions in the trichome. When Cd ions were translocated to trichome, they uniformly scatter in trichome intracellular region (Figure 3a), then transform to tiny granular deposits (Figure 3b), large granular deposits (Figure 3c) located in the trichome stalk, then distributed in the trichome cell wall (Figure 3d), finally form metal ion-rich ring structures (Figure 3e). We also found that the red Cd-dithizone complexes may disappear in some trichomes in older leaves (data not shown). Our observations indicated that the metal ion-rich ring structure in the trichome base was the later accumulation status and the state of Cd ions in trichomes is highly dynamic.

3.4. Cd ions accumulated in cell wall mutant trichomes is less than that in wild type trichomes

During trichome maturation, the cell wall can thicken to approximately 5 μm, and there are several mutations that alter the final stages of trichome development (Hülskamp, 2004). Kulich reported that Cu ions cannot accumulate in the mature trichomes of exo70H4-1, the mutant lacking the secondary cell wall (Kulich et al., 2015). We tested whether this mutant also influences Cd ion sequestration in trichomes. Cd ions accumulation in the trichomes of the exo70H4-1 mutant and wild type is shown in Figure 4. For the 9-day-old plants, no Cd ions were detected in the exo70H4-1 mutant, while in the wild type, Cd ions were distributed throughout the trichome (as shown in Figure 4a, b). For the 15-day-old plants, there were few small Cd-dithizone complexes in the trichome of exo70H4-1 (as show in Figure 4c,D). The color intensity of the Cd-dithizone complexes indicated that the content of Cd ions in the trichomes of exo70H4-1 was much less than that in the trichomes of wild type.
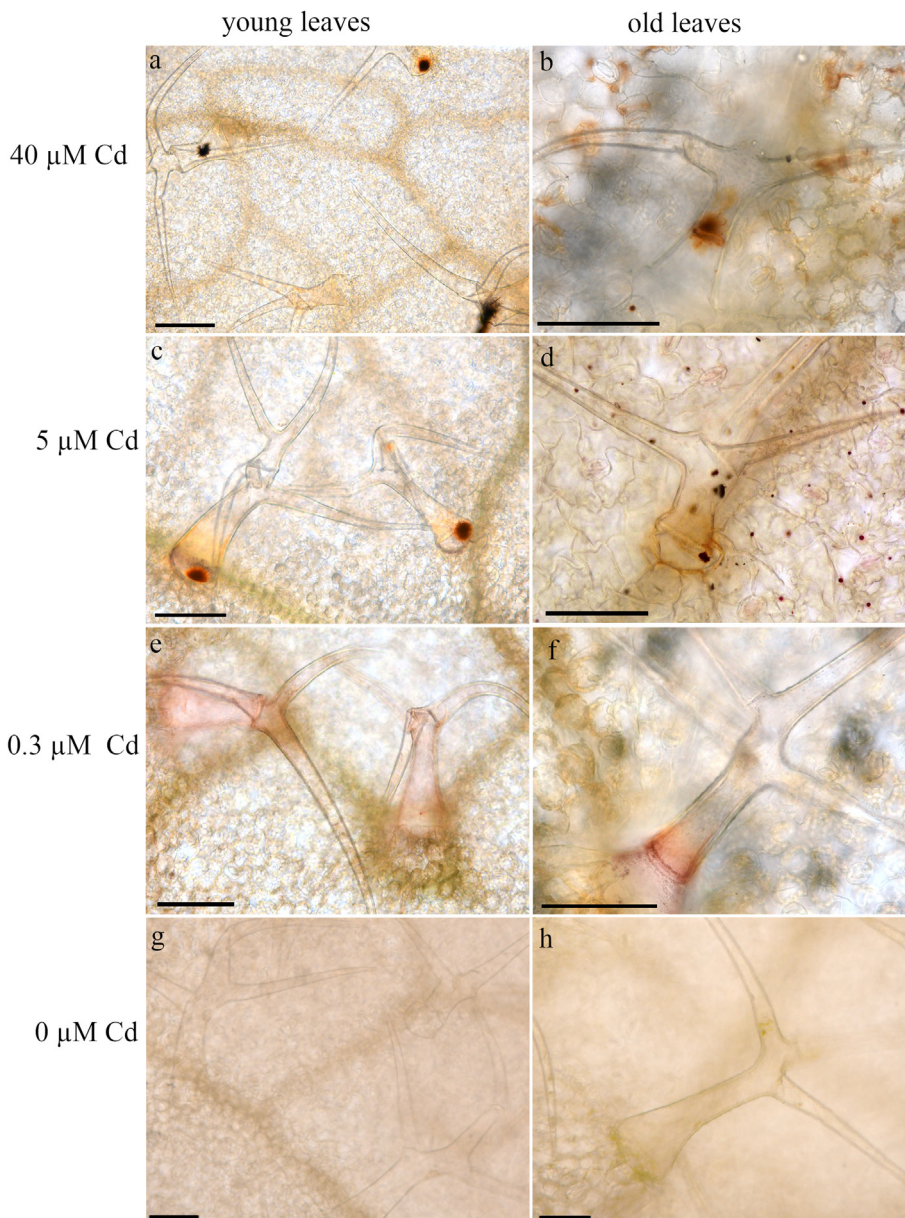
young leaves   old leaves

Figure 1. Cd ion accumulation in young and old *Arabidopsis* leaves. *Arabidopsis* plants grown in MS plate with or without addition of 40 μM (a, b), 5 μM (c, d), 0.3 μM (e, f), and 0 μM (g, h) CdCl$_2$. In young expanding leaves, the red Cd-dithizone complexes only show in trichomes (a, c, e); as the leaves aged, Cd-dithizone complexes also present in mesophyll cells (b) and epidermal cells (d). The red Cd-dithizone complexes do not present in the leaves of control plants without exposure to CdCl$_2$ (g, h). (Bar = 100 μm).

3.5. High metal ion response activity occurred in mature trichomes

Trichome-specific omics data was investigated ten years ago (Kryvych et al, 2008, 2011; Lieckfeldt et al., 2008; Marks et al., 2009), as *Arabidopsis* trichomes are unicellular and easily collected. The expression profile analysis of early staged and mature trichomes can capture the mechanism of heavy metal ions accumulated in mature trichomes. We analyzed two sets of data on transcript profiling of trichomes in the ArrayExpress database (Kryvych et al., 2008). One involves mature trichomes, and the other relates to trichome initial cells. Transcript profiling data sets show that 509 genes were expressed higher in mature trichomes than in trichome initial cells (Kryvych et al., 2008). We performed Gene Ontology (GO) term enrichment analysis of these 509 genes on the website http://www.geneontology.org with the built-in Fisher's exact test function. Considering the biological process ontology, the GO term 'response to metal ion' was significantly overrepresented in mature trichomes (Figure 5). Table S1 summarizes the genes related to 'response to metal ion' and expressed highly in mature trichomes. GO term analysis

suggested that the metal ion response was activated in mature trichomes not in the initial cells.

4. Discussion

The earliest sequestration site of Cd ions in immature and young expanding leaves indicated that mature trichomes may play an essential role in the early stage of leaf development. With the trichome getting maturation, the metal ion response activated in the mature trichome. The sequestration of Cd ions in mature trichomes will protect the rapidly dividing cell from metal toxicity. Genetic analyses have shown that phytochelatins, ABC transporters (Brunetti et al., 2015) (Bovet et al., 2007), and cyclic nucleotide-gated channels (Moon et al., 2019) involved in Cd ions uptake and transport. How these genes cooperate to get Cd ions into the mature trichome is unknown. There is still much to learn about the molecular mechanistic aspects of Cd ions uptake and distribution during leaf and trichome development.
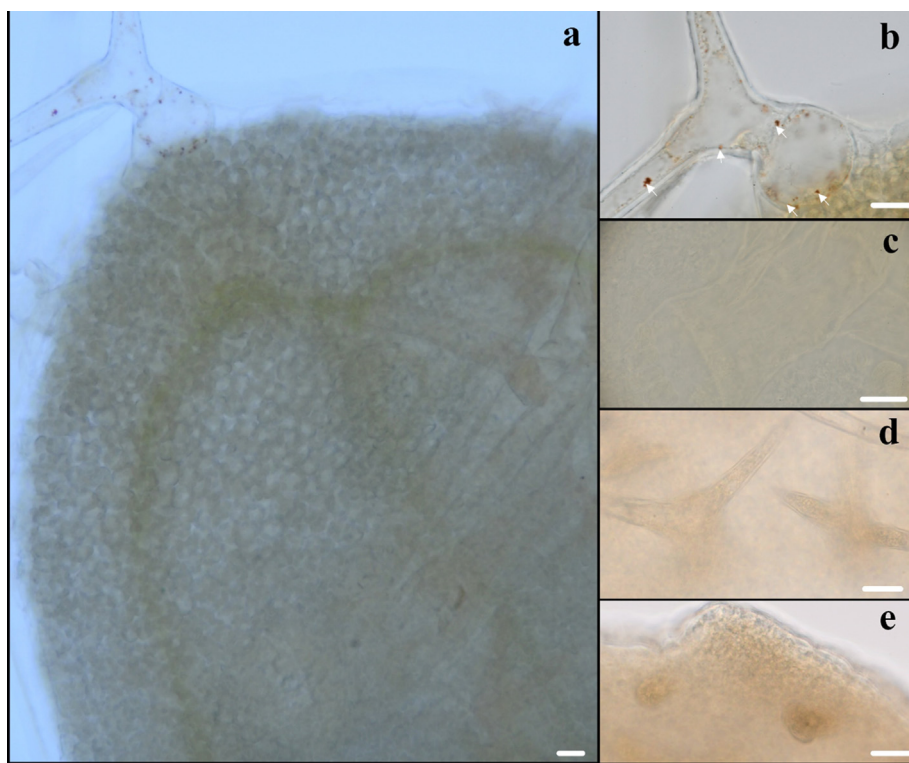
Figure 2. Cd ions distribution in immature leaves of *A. thaliana* seedlings. Five days old *Arabidopsis* seedlings grown in MS plates with or without adding CdCl$_2$ were stained with dithizone. The red Cd-dithizone complex deposits (indicated as white arrow) present in mature trichomes of immature leaves (a). No Cd-dithizone complexes show in the early trichome development stages. Close-up images of mature (b) and the other development stages of the trichome, including papillae formation (c), branch extension (d), and tubular outgrowth (e). (a), (b) and (c) were imaged from the same immature leaf. (d) and (e) were imaged from another immature leaf. (Bar = 100 μm).

Based on the dynamic accumulation states of Cd ions in the trichome, we think that the trichome is not just detoxification. When caterpillars or aphids crawl on plant leaves, they inevitably touch plant hairs-physically defensive structures (Louis et al., 2012; Peiffer et al., 2009; Weinhold and Baldwin, 2011). Some herbivores crawling on plant leaves alter trichome stability (Zhou et al., 2017; Liu et al., 2016) and wound trichomes (data not sown). Can the Cd ions sequestered in trichomes leak out from wounded trichomes and thus serve a defense function? From an ecological point of view, Boyd proposed the elemental defense hypothesis, which postulated that high internal metal concentrations can protect plants from herbivores (Boyd, 2007). Defense by hyperaccumulated Ni, Zn, and Se has been shown for leaf/root chewing herbivores and pathogens (Stolpe et al., 2017).

Cu ion-based fungicides (Bordeaux mixture) have been widely used against grapevine diseases. Can Cu ions also accumulate in mature trichomes first? Therefore, we detected the temporal distribution of Cu ions in *A. thaliana* leaves. As shown in Figure 6, Cu ions first accumulated in leaf trichomes and then in mesophyll and epidermal cells. Considering the primary sequence of Cd and Cu ions in leaves and the position and volume of trichomes, we propose that *Arabidopsis* trichomes not only serve as reservoirs for metal detoxification but also can utilize heavy metals as a defense.

Excretion of metal ions is one of the strategies for plant tolerance to heavy metals stress. Glandular trichomes of tobacco (*Nicotiana tabacum L*) and the salt glands of *Armeria maritima*, *Silene vulgaris* actively exclude heavy metal Cd and Zn ions (Choi et al., 2001; Sarret et al., 2006). It is unknown whether non-glandular trichomes exclude heavy metal ions. Based on the color intensity of Cd-dithizone complexes, we infer that the Cd ions sequestered in the trichome gradually decrease (may even diminish) with leaves getting old. Where do the reduced toxic Cd ions go? Li et al. found that foliar-applied Zn ions can get in the non-glandular trichomes of sunflower and soybean within 15 min (Li et al., 2018; Li et al., 2019). They also suggested that an absorption pathway may exist in trichomes to facilitate heavy metal ions to pass through the cell wall into the intracellular compartment. We think the toxic Cd ions might be excluded trichome through the same pathway.

Ion diffusion and redistribution may occur during the histochemical procedure. Since dithizone has a high affinity for Cd ions, and acetone
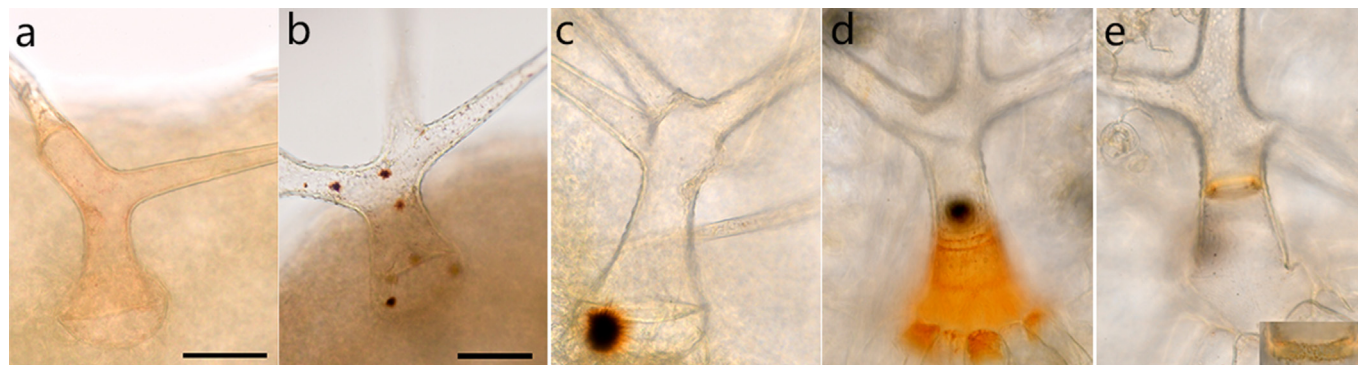

Figure 3. Cd ions accumulation states in trichomes in different aged leaves. (a) Trichomes in immature leaves, (b)in young expanding leaves, (c) and in fourth pair leaves of 15-day-old plants. (d) and (e) were taken in the first pair leaves of 13- and 17-day-old plants, respectively. (a), (b), and (c) were imaged from the same plant. (a), (c), (d), and (e) use the same scale bar. (Bar = 100 μm).
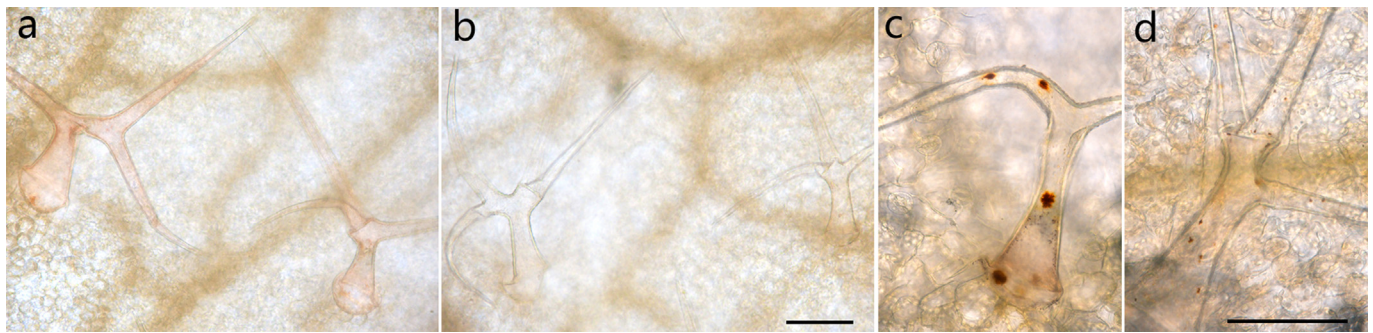
Figure 4. Cd ions distribution in WT and *exo70H4-1* cell wall mutants. 9 and 15 days old *Arabidopsis* seedlings grown in MS plates with adding 5 μM CdCl₂ were stained with dithizone. For 9-day-old WT (a) and mutant (b) plants, the red Cd-dithizone complexes show clearly in WT plants, while no Cd-dithizone complexes observed in mutants. For 15-day-old WT (c) and mutant (d) plants, the size of Cd-dithizone complexes in WT are much bigger than in *exo70H4-1* cell wall mutant. Arrow heads point to the red Cd-dithizone complexes. (Bar = 100 μm).
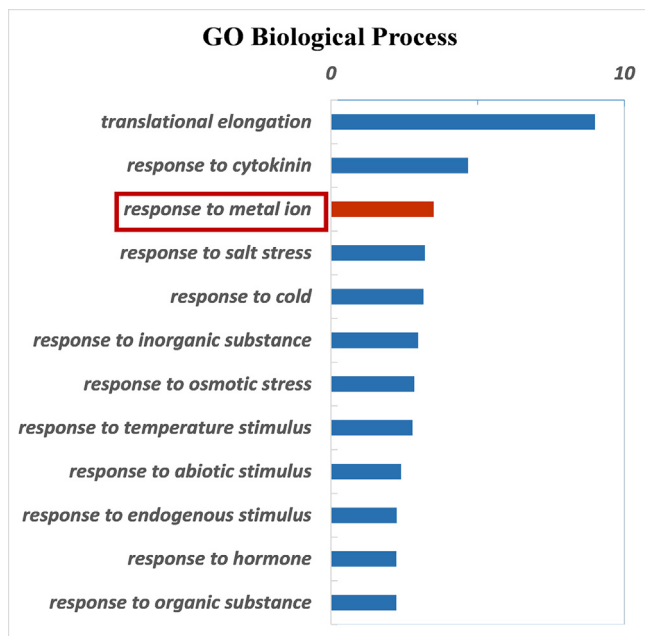

Figure 5. GO term analysis of genes that are highly expressed in mature trichomes indicates that high metal ion response activity occurred in mature trichomes.

can limit ion diffusion. The histochemical reactions developed by Sergin and Ivanov can determine the pattern of Cd ions (Seregin and Ivanov, 1997). The previous study showed that this method yield similar results to those obtained by electron microscopy, X-ray fluorescence, and radioautography. Moreover, we observed the Cd ion-rich ring structure (shown in Figure 4f and Fig. S2), previously reported by SEM-EDXA and X-ray fluorescence methods (Küpper et al., 2000; Isaure et al., 2006). Therefore, we think the results in our paper are convincing.

5. Conclusion

We demonstrated that mature trichomes are the earliest sequestration site of Cd ions in young expanding and immature leaves. The Cd ions that accumulated in trichomes were initially dispersed inside the trichome, then transformed into granular deposits, and may eventually be chelated on the base of the trichome cell wall. Cd ions exhibited different accumulation preferences in the epidermal and mesophyll cells of *A. thaliana* leaves. The accumulation of Cd ions in the trichome was altered in the cell wall mutant. The temporal distribution of heavy metals in trichomes provides clues regarding metal detoxification at the single cell level.

Declarations

Author contribution statement

Wenqiang Gao: Performed the experiments; Analyzed and interpreted the data.
Chao Guo, Jingjing Hu: Performed the experiments.
Jingao Dong: Contributed reagents, materials, analysis tools or data.
Li Hong Zhou: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Figure 6. Cu ions distribution in young and old *Arabidopsis* leaves. In young expanding leaves, Cu ions accumulate in trichomes firstly (a). As the leaves aged, Cu ions also accumulated in mesophyll cells (b) and epidermal cells (c, d). (Bar = 100 μm).

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2021.e07501.

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