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Cadmium accumulation in duckweed relates to pH and oxalate synthesis in Cd shock

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ABSTRACT

Anthropogenic activities increase cadmium (Cd) pollution in aquatic systems. This study investigated how the pH change from 4.3 to 7.3 affected the absorption of Cd by the aquatic plant duckweed (*Lemna turionifera* 5511). Here, the noninvasive microtest technique (NMT), high-performance liquid chromatography (HPLC), and transcriptome analysis were used to study the duckweed's Cd absorption and the oxalic acid metabolism under different pH conditions. The results showed the highest Cd accumulation in duckweed with pH at 6.3. Furthermore, the Cd influx was higher at the root tip of duckweed cultured in a liquid medium at pH 6.3. Notably, Cd stress changed the pH value and H^+ influx in duckweed roots, and significantly upregulated the Na^+/H^+ exchange transporters. Moreover, duckweed was shown to have enhanced oxalate acid secretion and significantly upregulated biosynthesis-related genes under Cd stress. Therefore, these analyses suggest that Na^+/H^+ exchange transporters and oxalic acid might affect Cd accumulation, which could provide new ideas for phytoremediating Cd pollution using duckweed.

Key words: aquatic plant, phytoremediation, stress, organic acids, transcriptome.

INTRODUCTION

Anthropogenic activities, including industrial discharge, mining, and field fertilizer application, increase heavy metal accumulation in soil and water systems (Lee et al. 2005, Lamb et al. 2009). Cadmium (Cd) is a toxin that poses a risk to plants, animals, and even human beings at very low concentrations (above 20 μg/L; Cheng et al. 2017). Cd has been shown to have adverse effects on animal and human health, causing DNA damage and bone disease via the food chain (Gunnar and Nordberg 2004). Moreover, Cd can act as a carcinogen, causing different degrees of kidney and breast cancer (Nawrot et al. 2006). Cd concentrations in groundwater have widespread implications for water supply and agriculture; therefore it is the subject of extensive worldwide attention (Kubier and Pichler 2019). Cd severely affects the biomass accumulation and morphology of plants by decreasing photosynthetic abilities, soluble protein, and sugar synthesis, inhibiting antioxidant enzyme activities, and promoting reactive oxygen species burst, leading to oxidative damage (Smeets et al. 2008, Rizwan et al. 2016, Pramanik et al. 2018). Additionally, Cd can impact polyvalent cations by binding the sites of transporters or proteins, for example, and reducing calcium (Ca) levels in plants (Sandalio 2001, Yang and Poovaiah 2002, Jasinski et al. 2008).

Considering the impacts of Cd, solving the increasing Cd contamination in water is important. Plants can strongly enrich trace elements, including Cd, Cr, N, and P in the soil and water, thereby effectively alleviating heavy metal pollution and eutrophication in water, better known as phytoremediation (Wei et al. 2021). Because it is ecofriendly and uses few resources, phytoremediation is considered an ideal way to remove Cd (Chen et al. 2017). To improve the heavy metal removal efficiency, it is necessary to improve the Cd accumulation in the phytoremediating plants.

pH is critical for plant growth and development and the first to impact Cd accumulation, primarily because changes in pH can cause protein conformation shifts (especially in membrane transporters; Felle 2001). Furthermore, pH affects the ion influx process, because of the changed proton motive (Cheng et al. 2017). pH plays a signaling role during the environmental stress response, especially abiotic stress (Geilfus and Mühling 2014). For example, it was shown that the tomatoes (*Lycopersicon esculentum*) leaf pH increases when exposed to drought stress (Wilkinson et al. 1998). Moreover, previous reports have found a relationship between Cd and pH. Cd uptake and soil pH were found to be inversely related in rice (*Oryza sativa*) (Yanai et al. 2006), as evident from the fact that the pH value slightly increased when the rice (*Oryza sativa*) was treated with Cd, with the H^+ concentration in leaf vascular bundles being negatively correlated to the Cd accumulation (Zhang et al. 2018). When pH was 7, the Cd removal rate of duckweed (*Lemna gibba*) in water could reach 98.1%, and the removal ability was inversely related to pH (Verma and Suthar 2015). Upatham et al. (2002) determined that the maximum adsorption capacity of Cd was reached in duckweed (*Wolffia globosa*) when pH was 7. At pH 1–5, the adsorption of the stalk sponge of *Z. mays* on Cd gradually increased and reached up to 90%, which may be due to the interaction between cellulose and Cd on its surface to form a complex (García-Rosales et al. 2012). It has been reported that at low soil pH (4.1), the addition of Se can effectively reduce the uptake rate of Cd by *U. decumbens*, but promote Cd accumulation in the

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shoot (Borgo et al. 2023). The relationship between Cd uptake and pH value in the soil is well studied, but that relationship in aquatic ecosystems still needs further investigation.

Organic acids can affect pH, which could influence the Cd chemical forms (Zhang et al. 2018). The interaction of organic acids with Cd was crucial for Cd accumulation in the plant–water system, because of the influence on both Cd uptake and transport. Organic acids played a critical role in Cd uptake by determining the environment Cd availability (Cieśliński et al. 1998). Moreover, heavy-metal ions form noncationic metal chelates by bonding with an organic acid or amino acid, mainly because that heavy-metal ion transport was limited in xylem vessels having high cation-exchange capacity (Song et al. 2017). Therefore, to improve the Cd accumulation ability of aquatic plants, studying the plant's organic acid secretion during Cd stress in plant–water systems is imperative.

Oxalate, the simplest dicarboxylic acid, is produced in most organisms. It has been reported that oxalate participates in heavy metal detoxification in plants, including Cd, Pb, Cu, and Sr, by forming different oxalate crystals (Franceschi and Schueren 1986, Mazen and Maghraby 1997, Yang 2000, Choi et al. 2001). As a counterion to bind inorganic ions, oxalate promotes cationic equilibrium and forms calcium oxalate crystals, which is vital for regulating the Ca concentration in plant organs and tissue. Besides, in amaranth (*Gomphrena claussenii*), Cd has been reported to associate with oxalate in calcium oxalate crystals and compete with calcium for translocation to stems in the Cd bioindicator, indicating the possible role of oxalate during Cd accumulation (Paula et al. 2018). Therefore, it is important to investigate the oxalate released by aquatic plants during Cd treatment.

Duckweed (*Lemnaceae*) is an aquatic plant characterized by rapid propagation, easy cultivation, and global distribution (Dong et al. 2018). Duckweed is a hyperaccumulator for Cd metal and a moderate accumulator for Cr metal (Chaudhary and Sharma 2019). Greenhouse studies have demonstrated that duckweed can remove Cd from urban water to a large extent (90%) via adsorption (Bokhari et al. 2019). Exploring better conditions to optimize Cd accumulation in duckweed is important for its phytoremediation of Cd.

Thus, we examined the Cd^{2+} concentration changes in duckweed (*Lemna turionifera* 5511) to evaluate how pH changes affect the accumulation of Cd. Furthermore, we also investigated the organic acid changes in water, especially the oxalate change under Cd stress and explored the gene expression associated with proton transport during Cd treatment.

MATERIALS AND METHODS

The duckweed was initially collected from the Fengchan River in the Xiqing District in Tianjin, China (117°12′E, 39°06′N). The duckweed was transferred to the culture medium in a sterile environment for the propagation of new leaves. Subsequently, it was identified as *Lemna turionifera* (Yang et al. 2017) by polymerase chain reaction (PCR). According to Wang and Kandeler (1994), sterile culture was conducted in the medium that had a pH of 5.8 and was steam sterilized at 121 C for 20 min in a steam pressure sterilizer (SQ510C, Yamato, Japan) before use. The composition of the liquid medium is 0.27 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.24 mM $MgSO_4 \cdot 7H_2O$, 0.24 mM KH_2PO_4, 0.03 μM $CaCl_2 \cdot 2H_2O$, 0.24 mM $Mg(NO_3)_2 \cdot 6H_2O$, 0.6 mM KNO_3, 29.9 μM KCl, 3.67 μM $Na_2MoO_4 \cdot 2H_2O$, 41.8 μM H_3BO_3, 1.64 μM $ZnNa_2EDTA \cdot 4H_2O$, 17.8 μM $K_2H_2EDTA \cdot 2H_2O$, 33.8 μM $FeNH_4EDTA$, 8.19 μM $MnCl_2 \cdot 4H_2O$, 2.99 μM $CoSO_4 \cdot 7H_2O$, and 11.1 μM $Na_2EDTA \cdot 2H_2O$. The culture conditions were 16 h light/8 h dark and 26 C/20 C day/night and a light intensity of 95 μmol m^{-2} s^{-1}. Duckweeds treated with or without 50 μM $CdCl_2$ for 24 h were adjusted with 0.1 mol hydrogen chloride (HCl) to maintain the pH at 4.3, 5.3, 5.8, 6.3, 7.3. Each treatment group contained about 15 to 20 duckweed groups, a group contains two leaves.

Leadmium™ green dye[1] (specific pair of Cd^{2+}/Pb^{2+} staining) was used to detect the Cd content in duckweed. The working solution was prepared by mixing 50 μl dimethyl sulfoxide (DMSO) with 50 μg Leadmium green dye, followed by diluting it 10 times with 0.85% normal saline. The rhizoids after Cd treatment were incubated in the working solution for 0.5 h in the dark and then washed by three times with 0.01 M phosphate-buffered saline (PBS, pH 7.2 to 7.4) to remove the dye. Finally, the stained roots were immersed in PBS, then placed on a slide, observed, and photographed with a fluorescence microscope[2] with a wavelength of 488 nm. Imagine J[3] was used to measure the fluorescence intensity of Cd^{2+} at the rhizoid tip 200 μm. And the ratio of fluorescence intensity under different pH value was calculated by dividing the fluorescence intensity values compared with that at pH 4.3 (the relative fluorescence intensity under pH 4.3 was 1), which means the relative fluorescence intensity.

The noninvasive microtest technique (NMT) at the Younger USA NMT Service Centre (Xuyue, Beijing) was used to determine the transmembrane ion fluxes of Cd^{2+} and H^+ in duckweed. The duckweed in pH 5.3/5.8/6.3 medium was treated with 50 μM $CdCl_2$ for 5 min. The roots were balanced for 10 min in the equilibrium solution (0.05 mM $CdCl_2$, 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.3 mM MES, pH 5.3/5.8/6.3) of the Cd treatment group to achieve a stable physiological state. The transmembrane fluxes of Cd^{2+} and H^+ within 10 min at 100 μm from the rhizoid tip were determined by NMT, and the H^+ fluxes without $CdCl_2$ in the medium pH of 5.3/6.3 were also determined.

Rhizoid pH was stained by the pH-sensitive dye BCECF-AM[4], as mentioned previously (Bassil et al. 2011). The duckweed was incubated at a working concentration of 10 mM BCECF-AM. After dyeing for 30 min in the dark, the duckweed was washed with PBS by three times to remove the dye. Dye fluorescence images were collected with excitation wavelengths of 408 and 488 nm. Image J was used to analyze the fluorescence intensity on the region 0.27 mm away from the rhizoid tip, after the background correction. The standard pH curve was established by using the fluorescence ratio value of 488/408, and the pH value was calculated from the standard curve. The scale image generated by the molecular apparatus and the color contrast of the color bar was used to determine the pH value. The fluorescence intensity was measured by Image J software.

High-pressure liquid chromatography (HPLC) was used for measuring the oxalate content of duckweed at pH 5.8, with the culture medium filtered with a 0.45-μm needle being separated by the reversed-phase C18 column (250 mm × 4.6 mm). The

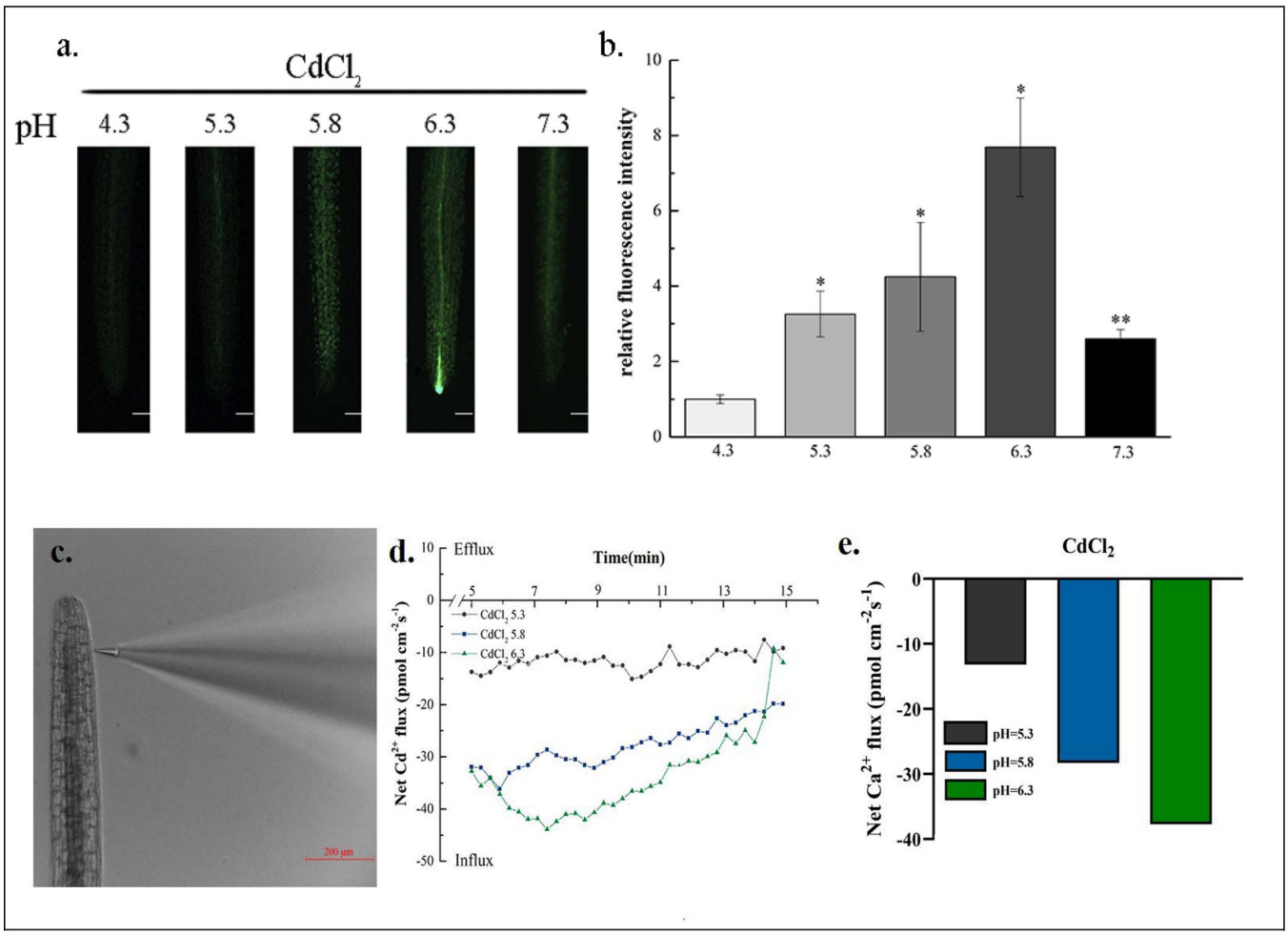

Figure 1. (a) Signal changes of Cd^{2+} in rhizoid of duckweed after 50 μM $CdCl_2$ treatment at pH 4.3/5.3/5.8/6.3 for 24 h, with Leadmium™ Green AM rhizoid being used for staining (scale bar = 100 μm). (b) The ratio of different pH values' fluorescence intensity to pH 4.3 at 200 μm from the rhizoid tip. (c) Noninvasive microtest technique (NMT)–determined Cd^{2+} and H^+ fluxes at 100 μm from the rhizoid tip. (d) NMT of the Cd^{2+} fluxes in the duckweed rhizoid was determined at pH 5.3, 5.8, and 6.3 after 5 min treatment with 50 μM $CdCl_2$. Asterisks represent significant differences when compared with net of the Cd^{2+} fluxes at pH 5.8 according to the independent-sample *t* test (*$P < 0.05$, **$P < 0.01$).

column temperature was 30 C, the flow rate was 1.0 ml/min, and the injection volume was 20 μl. The mobile phase was 3% CH_3OH-0.05 mol/L Na_2HPO_4 (pH 2.50) and the detection wavelength was set at 215 nm.

The gene sequencing and expression analysis of duckweed at pH 5.8 were conducted by Novogene (Chaoyang, Beijing). A total amount of 1.5 μg RNA per sample was used for transcriptome sequencing. Gene functional annotation was selected from the following authoritative databases: GO (Gene Ontology), Swiss-Prot (a manually annotated and reviewed protein sequence database), KOG/COG (clusters of orthologous groups of proteins), Pfam (protein family), Nt (NCBI nonredundant nucleotide sequences), KO (KEGG ortholog database) and Nr (NCBI nonredundant protein sequences). RNA-Seq by expectation maximization (RSEM; Dewey and Bo 2011) was used to assess the gene expression level in each sample.

All experiments were replicated independently three times. The data were analyzed through the independent-sample *t* test using the SPSS software (IBM SPSS Statistics, Version 24), and the graphs were drawn using Origin 9.0 (Origin Lab, USA).

RESULTS AND DISCUSSION

To investigate the effect of different pH on Cd accumulation, we measured the Cd level in duckweed at pH 4.3, 5.3, 5.8, 6.3, and 7.3. The relative fluorescent intensity obtained by staining with the Leadmium green dye showed the Cd^{2+} accumulation in the root (Figures 1a and 1b). The study showed that with the increase of pH value from 4.3 to 6.3, the Cd^{2+} fluorescent intensity gradually increased under 50 μM $CdCl_2$ stress. Furthermore, the highest Cd^{2+} accumulation was measured in the root under pH 6.3. This suggested that pH changes can affect the Cd^{2+} enrichment of duckweed.

NMT has been conducted to detect Cd^{2+} flux at the root tip of duckweed under pH of 5.3, 5.8, and 6.3. After 5 min of Cd treatment, average net Cd^{2+} influx of 13.735, 31.904, 32.755 pmol cm^{-2} s^{-1} were detected at the pH level of 5.3, 5.8, or 6.3, respectively (Figure 1c). Notably, compared to the pH 5.8, the Cd^{2+} influx of rhizoid at pH 5.3 was significantly lower, and the Cd^{2+} influx of rhizoid at pH 6.3 was remarkably higher. These results suggested that the pH of 6.3 was favored Cd absorption during aquatic bioremediation. In

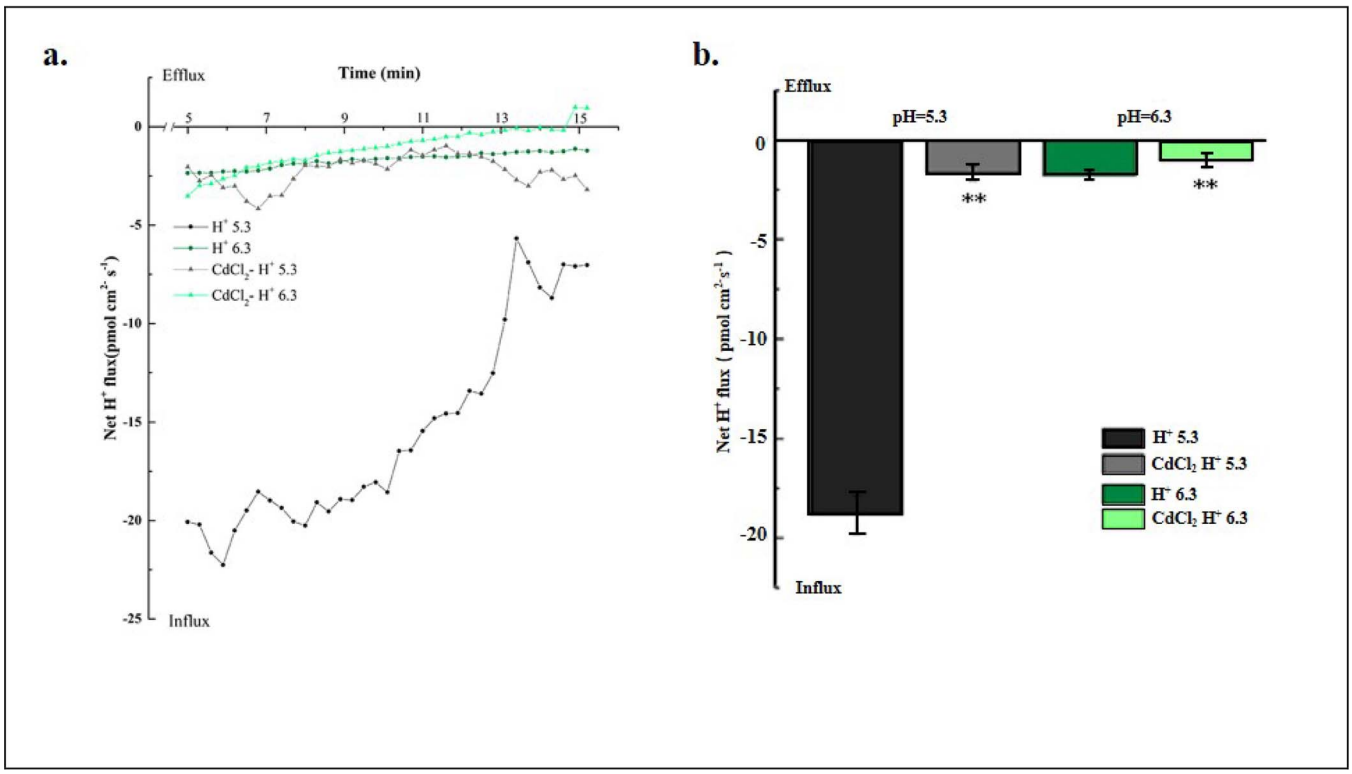

Figure 2. Noninvasive microtest technique (NMT) assay was performed for H^+ fluxes at pH 5.3 and 6.3 in the duckweed rhizoid, with or without 50 µM $CdCl_2$ for 24 h. Asterisks represent the significant differences when compared with net of the H^+ fluxes without Cd treatment at pH 5.3 or 6.3 according to an independent-sample *t* test (*$P < 0.05$, **$P < 0.01$).

previous studies, the pH level in soil for the highest Cd accumulation was different. In grafted muskmelon (*Cucumis melo* L.), the shoots and roots showed the highest Cd content at pH 5.5 and the lowest Cd content at pH 8.0 regardless of the Cd concentration (Zhang et al. 2019). In bamboo charcoal, the Cd adsorption capacity increased as the pH level increased, and the optimum pH for Cd accumulation was 8.0 (Wang et al. 2010). Bashir et al. (2017) found that using biochar to increase soil pH could enhance Cd immobilization, suggesting that because of different plant species. In this study, we suggested that the pH be adjusted to pH 6.3 to optimize Cd accumulation during phytoremediation in aquatic ecosystems.

To further explore the relationship between Cd^{2+} and H^+, we detected the change of the transmembrane flux of Cd^{2+} and H^+ (Figure 2). Without Cd, the H^+ influx peaked at a pH of 5.3 and decreased with time, with a peak of approximately 22 pmol cm^{-2} s^{-1}. However, at a pH of 6.3, the H^+ influx stabilized within a lower-level range of 0 to 2.5 pmol cm^{-2} s^{-1}. Interestingly, following Cd treatment, H^+ influx decreased at a pH of 5.3 and 6.3, with 5.3 being the most significant. These manifested that H^+ and Cd^{2+} might have a reverse transport effect. The aqueous solution pH can affect the change of positive and negative charges on the biochar (BC) surface, and the negative charges can control the absorption of Cd by BC through the combination of electrostatic adsorption and Cd cation (Ahmed et al. 2021). In plants, pH played an important role in response to environmental stress. Under drought stress, apoplastic pH was increased by enhancing H^+ efflux in soybean (*Glycine max* L.) under drought stress (Mak et al. 2014). Also, in tomatoes (*Lycopersicon esculentum*), the xylem pH alkalization was observed from pH 5 to pH 8 under drought (Wilkinson 1999). In rice (*Oryza sativa*), the H^+ efflux was significantly enhanced under Cd stress, and the decrease of H^+ resulted in enhanced Cd remobilization from shoots to grain (Zhang et al. 2018). Our present study suggested that the immediate H^+ response played a role in duckweed during Cd stress.

The effect of Cd on root pH was detected in different pH (5.3, 5.8, and 6.3) environments (Figure 3). At external pH of 5.3, the root pH changes to 5.4 after Cd impact. However,

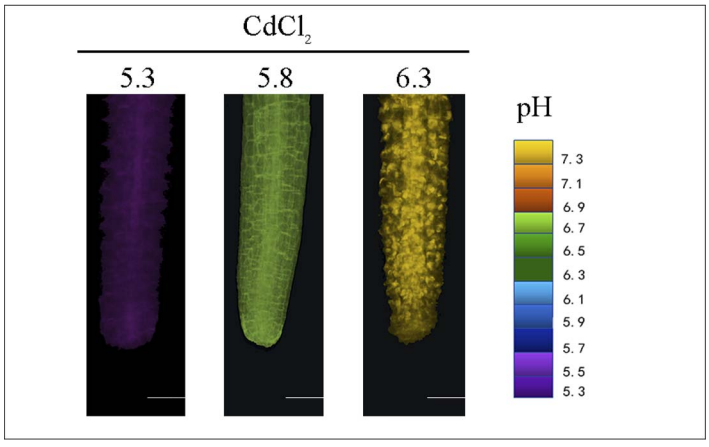

Figure 3. pH value of rhizoid of duckweed treated with 50 µM $CdCl_2$ for 24 h (scale bar = 50 µm). The color of the roots represents the changed pH value.

Table 1. Changes in Na^+/H^+ exchange-related gene expression levels.

Description	Gene ID	WT readcount	WT-Cd readcount	log2FoldChange	P value
Na^+/H^+ exchange 6 isoform X2	Cluster-7365.3405	35.3071908	621.6415593	0.81346	1.21E-09
Na^+/H^+ exchange family	Cluster-7365.19951	0	6.912965	5.1254	0.043203
Na^+/H^+ exchange protein	Cluster-7365.99555	29.44123	17.96929	−0.70881	0.037375
Metal ion binding	Cluster-7365.36097	95.36097	50.13687	−2.1278	6.81E-17

when the external pH was 5.8 or 6.3, the root pH changed to 6.6 or 7.4 after Cd impact. Contrastingly, Cd has the strongest effect on root pH when the external pH was 6.3. This was consistent with our observation that duckweed absorbed the highest Cd when pH was 6.3. These suggested that Cd modified the pH of the root and had a great influence on the environment. This result was consistent with that of rice (*Oryza sativa*) treated with Cd (Zhang et al. 2018).

Na^+/H^+ antiporters (NHX) regulated the pH of plant cells (Yamaguchi et al. 2001) and promoted plant growth (Bassil et al. 2011). Our previous study found that NHX1 effectively improved the resistance of duckweed under Cd stress (Yang et al. 2019). Here, we analyzed H^+ transport channels at the transcriptome level. The expression of genes related to Na^+/H^+ exchange was analyzed as shown in Table 1. The expression of Na^+/H^+ exchange increased significantly with Cd treatment, which might lead to the pH value modification under Cd stress.

Organic acids, small molecules in plants, could be secreted by plant roots and form Cd chelates (Zenk 1996). Organic acid secreted by roots was important to aluminum (Al) tolerance in plants. Chelation is involved in phytoextraction during both metal uptake and accumulation (Xu et al. 2012, Kumar et al. 2014, Vítková et al. 2015, Tao et al. 2016). The oxalate content secreted by duckweed under Cd stress was significantly higher than that in the control medium, with an increase of 7.8% (Figure 4). Increased organic acid might be connected with Cd stress response. pH could be affected by the organic acids (Zhang et al. 2018), and the interaction of organic acids with Cd was important to Cd accumulation in the plant–soil system (Cieśliński et al. 1998). These also explained why Cd stress promoted the synthesis of oxalic acid and the absorption of Cd.

The expression of gene related to organic acid synthesis has been measured with Cd treatment for 24 h. The process of oxalate biosynthesis and catabolism has been studied (Figure 5). There are three pathways for oxalate biosynthesis in plants. Two pathways were connected with the glyoxylate (GLOX) cycle, and one was related to the tricarboxylic acid (TCA) cycle. Oxaloacetate is a precursor for the oxalate synthesis in the TCA cycle and GLOX cycles. In the GLOX cycle, both GLOX and oxaloacetate were important, and were directly catalyzed to oxalate. The expression of glycolate oxidase that catalyzes GLOX was decreased. In the TCA cycle, the expression of malate dehydrogenase, citrate synthase, aconitase, isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, succinate dehydrogenase, and fumarase has been

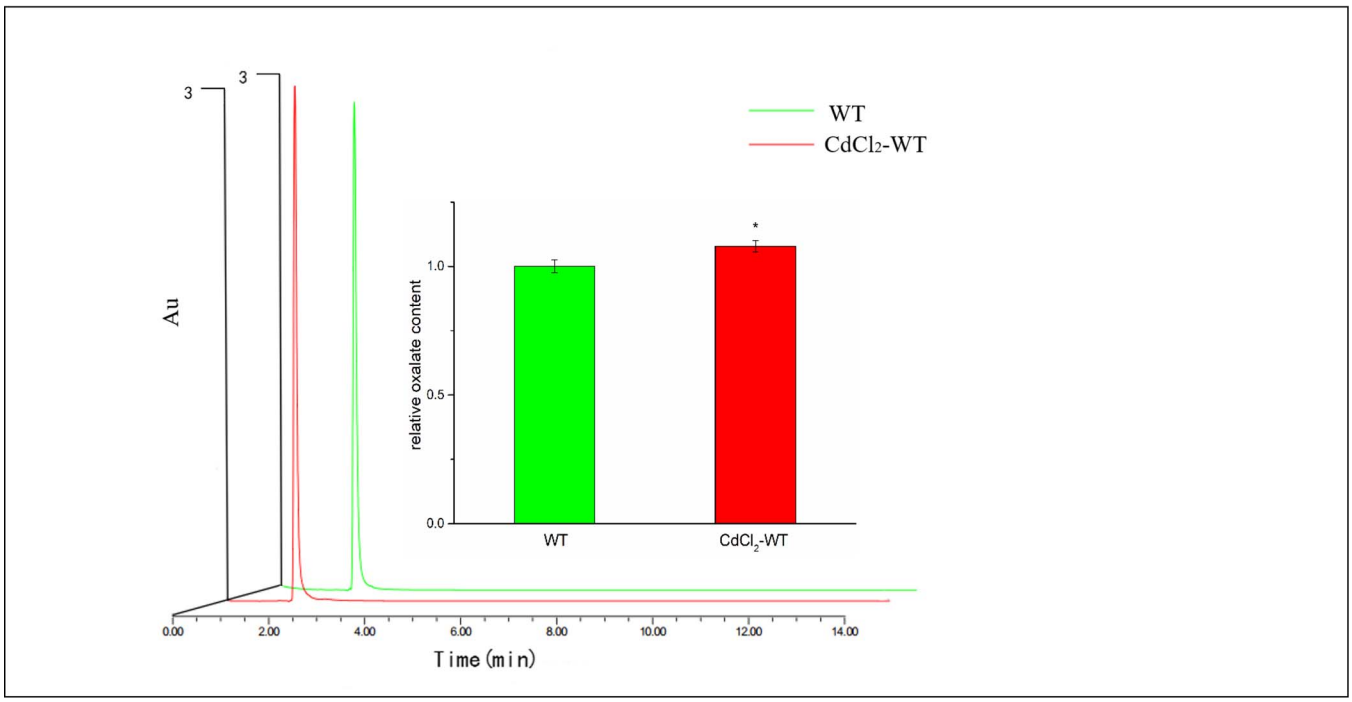

Figure 4. High-performance liquid chromatography of the culture filtrate for duckweed. The oxalate content in duckweed medium cultured with or without 50 μM $CdCl_2$ for 24 h was determined. Asterisks represent significant differences according to the independent-sample *t* test (*$P < 0.05$, **$P < 0.01$).

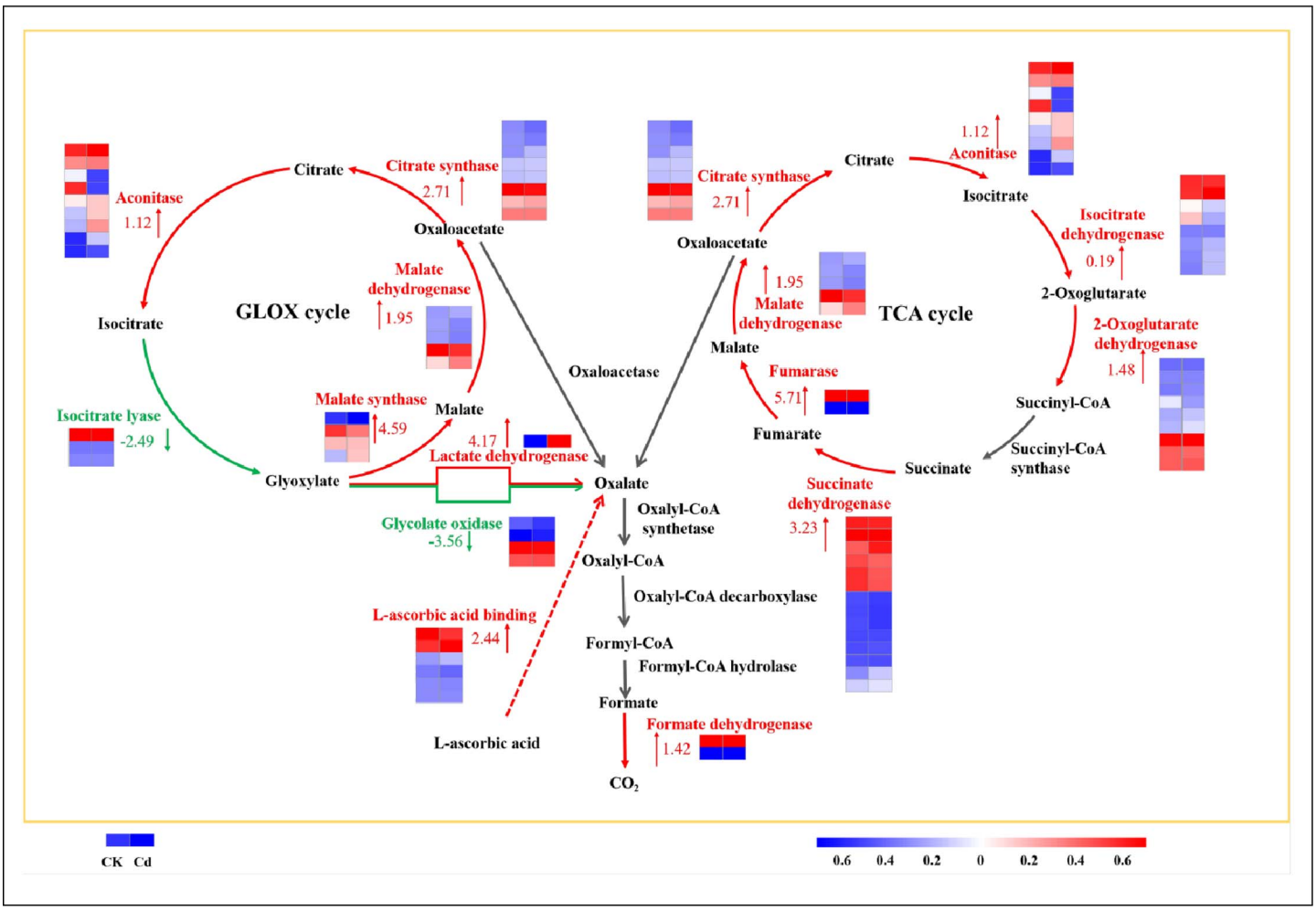

Figure 5. Response of oxalate metabolism under cadmium stress in duckweed. Several oxalate metabolism and related enzymes in response to cadmium stress are presented in the graph. Arrows indicate the direction of processes, with red indicating up and green indicating down. The unigenes involved in enzyme synthesis were selected and listed in the corresponding module. CK and Cd represent duckweed treated with 50 μM $CdCl_2$ for 0 and 24 h, respectively. The color in this figure legend ranges from red to blue, which means $\log_{10}$(FPKM + 1) from big to small. Red means high expression; blue means low expression. The number under the enzymes means log2-fold change compared to the CK.

improved by 1.95, 2.71, 1.12, 0.19, 1.48, 3.23, and 5.71 log2 fold. This may be the reason for the increased oxalate content. The catabolic pathway of oxalate is via four enzymes that convert oxalate into CO_2. The final step is catalyzed by formate dehydrogenase, and its expression was improved by 1.42 log2 fold. L-ascorbic acid was also related to oxalate synthesis; the exact metabolic pathway remains unclear (Yang et al. 2018). In this study, the expression of L-ascorbic acid binding relative protein has been improved. Almost all of the enzymes in these pathways have been improved under 24 h Cd stress. These results suggested that oxalate played a role during Cd stress and suggest a possible mechanism: Cd stress → oxalate synthesis → pH change → Cd uptake. Although our current results provided baseline insights that pH and oxalate could play a signal role during short-term Cd stress, further research should be conducted to evaluate the role of pH and oxalate during long-term exposure to Cd contamination.

Cd accumulation in duckweed relates to pH level, and oxalate synthesis has been presented in this study. Our results are potentially important for the phytoremediation in Cd-polluted water. The underlying molecular mechanisms of organic acid during Cd stress also provided new insights into enhancing Cd accumulation in higher aquatic plants for phytoremediation.

SOURCES OF MATERIALS

[1]Leadmium™ green dye, specific dyes for Cd^{2+} and Pb^{2+}, Thermo Fisher Scientific (China) Co., Ltd., Building 3&6&7, 27 Xinjinqiao Road, Pudong New Area, Shanghai.

[2]Leica DFC450C, DM5000, upright fluorescence microscope, Berlin, Germany.

[3]Image analysis software, National Institutes of Health, Bethesda, MD, USA.

[4]BCECF-AM, pH-sensitive dye, MedChemExpress LLC Company, Building 3, Lane 1999, Zhangheng Road, Pudong New Area, Shanghai.

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