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Glutamate alleviates cadmium toxicity in rice via suppressing cadmium uptake and translocation

Meng Jiang[a,b,c], Jie Jiang[a], Shan Li[a,d], Mei Li[a,d], Yuanyuan Tan[a], Shiyong Song[a], Qingyao Shu[a,b,*], Jianzhong Huang[a,c,**]

[a] National Key Laboratory of Rice Biology, Institute of Crop Sciences, Zhejiang University, Hangzhou, 310058, China
[b] Hubei Collaborative Innovation Center for Grain Industry, Yangtze University, Jingzhou, 434025, China
[c] Institute of Nuclear Agricultural Sciences, Zhejiang University, Hangzhou, 310058, China
[d] Analysis Center of Agrobiology and Environmental Sciences, Zhejiang University, Hangzhou, 310058, China

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ABSTRACT

Cadmium (Cd), a naturally occurring heavy metal, is toxic to animals and plants. Minimization of Cd in rice grain is important to human health since rice is the main source of Cd intake for human populations feeding on it as staple food. Glutamate (Glu) is reportedly involved in plant abiotic stress responses, whereas the underlying molecular mechanism remains poorly understood. In this study, we showed that supplement of Glu, but not glutamine, significantly alleviated Cd toxicity in hydroponically grown rice plants. Cd accumulation was reduced by 44.1% and 65.6% in root and shoot of rice plants respectively, after Glu supplementation (3 mM). Glu supplement restored chlorophyll biosynthesis and significantly ameliorated Cd-induced oxidative stress with reduced levels of H_2O_2, $^1O_2$, MDA, and increased activities of major anti-oxidant enzymes, catalase, peroxidase and glutathione S-transferase. Levels of stress-associated free amino acids proline, arginine and γ-aminobutyric acid were also reduced after Glu supplement. We further demonstrated that Glu supplement suppressed the Cd-induced expression of metal transporter genes *OsNramp1, OsNramp5*, *OsIRT1*, *OsIRT2*, *OsHMA2* and *OsHMA3* in roots of Cd-treated plants. Taken together, our results suggest that Glu supplement could alleviate Cd toxicity in rice by suppressing Cd uptake and translocation.

1. Introduction

Cadmium (Cd), a naturally occurring heavy metal, is toxic to plants and animals (Dalcorso et al., 2013). By binding to sulfhydryl amino acids (e.g. cysteine), Cd interferes with the homeostasis of calcium, zinc and iron in cells (DalCorso et al., 2008; Clemens et al., 2013). Accumulation of Cd inhibits plant development and growth with decreased crop production and Cd could eventually enter the human body through food chain (McLaughlin et al., 2006; Satarug et al., 2009; Aziz et al., 2015). Since Cd is ubiquitously present in the environment, it is important to reduce Cd accumulation in plants for better food safety. Cd accumulation in human body will lead to renal dysfunction, osteoporosis, cancer, and cardiovascular disease (Bertin and Averbeck, 2006; Godt et al., 2006; Järup and Akesson, 2009; Satarug et al., 2009). Currently, most populations are exposed to Cd levels exceeding the safety threshold (Nawrot et al., 2006), so it is imperative to block the path of Cd uptake and translocation from polluted soil to harvest plant organs to minimize Cd intake to human body.

Heavy metal uptake in higher plants is mediated by metal transporters responsible for mineral elements such as zinc, manganese, iron, copper and calcium (Axelsen and Palmgren, 2001). Intriguingly, some of the transporters, recognized initially for iron or zinc homeostasis, can facilitate the transport for non-essential metals (Krämer et al., 2007). Several of the Cd transporters have been identified and characterized, including: two members of the Natural Resistance-Associated Macrophage Protein (NRAMP) family, OsNramp1 and OsNramp5, which are responsible for the Cd uptake from soil into roots (Senoura et al., 2011; Ishimaru et al., 2012; Sasaki et al., 2012); two rice Iron-Regulated Transporter 1 (IRT1) homologs, OsIRT1 and OsIRT2, which are also involved in the root uptake of Cd (Ishimaru et al., 2006; Nakanishi et al., 2006); OsHMA2, a rice Heavy Metal ATPase (HMA), which mediates the xylem loading of Cd in roots and its translocation to shoots (Ueno et al., 2010; Takahashi et al., 2012); OsHMA3, which can affect root-to-shoot Cd translocation by sequestering Cd into vacuoles

* Corresponding author at: National Key Laboratory of Rice Biology, Institute of Crop Sciences, Zhejiang University, Hangzhou, 310058, China.
** Corresponding author at: Institute of Nuclear Agricultural Sciences, Zhejiang University, Hangzhou, 310058, China.
E-mail addresses: qyshu@zju.edu.cn (Q. Shu), jzhuang@zju.edu.cn (J. Huang).

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(Miyadate et al., 2011; Ueno et al., 2011); OsLCT1, the only rice Low-affinity Cation Transporter (LCT), which facilitates xylem-to-phloem transfer of Cd in nodes (Uraguchi et al., 2011). However, the precise regulatory pathways influencing the transporter genes involved in uptake and allocation of Cd are poorly understood.

It has been found that plant nutrients, e.g., Si (Zhang et al., 2008), Se (Lin et al., 2012), Fe (Su et al., 2014) and S (Liang et al., 2015), could affect Cd accumulation and alleviate Cd toxicity in diverse higher plants. It is also known that glutamate (Glu) is involved in response to environmental and abiotic stresses in higher plants (Forde, 2014). Ammonium directly absorbed from the soil or derived from nitrate is assimilated into Glu and glutamine (Gln) by glutamate synthase (GOGAT) / glutamine synthetase (GS) cycle (Tabuchi et al., 2007; Seifi et al., 2013). Glu is a precursor of stress-related free amino acids (FAAs) such as Proline (Pro), arginine (Arg) and γ-aminobutyric acid (GABA) (Watkins and Jane, 2006; Forde and Lea, 2007). Nevertheless, it was not known whether Glu plays a role in responses to cadmium and heavy metal stresses.

Proline (Pro) level is thought to be an important indicator of self-protection for higher plants development under abiotic stresses such as heavy metal contamination, salinity, drought and extreme temperatures (Chen et al., 2004; Munns, 2005; Sharma and Dietz, 2006). The Cd-induced Pro accumulation was found to be an important adaptive mechanism for *Silene vulgaris* under Cd stress (Xu et al., 2009; Islam et al., 2009). Likewise, γ-aminobutyric acid (GABA) was found to accumulate in plant under both biotic and abiotic stresses, e.g., pathogen attack, oxygen deficiency, mechanical stimulation, low temperature, heat shock, drought and acidification (Bouché and Fromm, 2004; Bown et al., 2006). Arginine (Arg), as a precursor of polyamines, also plays a vital role in stress responses in higher plants (Slocum, 2005; Alcázar et al., 2006). As a consequence of Cd toxicity, the amount of reactive oxygen species (ROS), such as hydroxyl radical (−OH), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), is elevated (Andresen and Küpper, 2013), causing oxidative stress with lipid peroxidation in membrane of cellular organelles and oxidative degradation of biomolecules such as chloroplast pigments and nucleic acids (Gill and Tuteja, 2010). In response, plants have evolved nonenzymatic scavengers of free radicals, e.g. ascorbate, carotenoids, and glutathione, and enzymatic anti-oxidants, e.g. glutathione S-transferase (GST), peroxidase (POD) and catalase (CAT) (Gallego et al., 2012).

In this study, we demonstrated that Glu supplement could alleviate toxicity and reduce Cd accumulation in hydroponically grown rice seedlings with reduced oxidative stress and subdued levels of stress-related FAAs. Possible mechanisms of Glu on mitigation of Cd toxicity are discussed.

2. Materials and methods

2.1. Plant materials and treatments

Rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) seeds were treated in 2% (w/v) of aqueous NaClO solution for 20 min, washed with deionized water five times, and then immersed in deionized water for one day at room temperature. After germination at 28 °C for four days, rice seedlings were transplanted into barrels containing standard ½ Murashige-Skoog (MS) liquid medium (Murashige and Skoog, 1962) and grown in a growth chamber with a photoperiod of 14-h light (500 μmol photons m^{-2} s^{-1}, 30 °C) and 10 h darkness (26 °C). For treatments, 14-day-old rice seedlings were planted in ½ MS liquid medium containing 80 μM $CdSO_4$ with supplements of 0, 1, 3 or 6 mM Glu or glutamine (Gln) for one or two weeks. After treatment, the rice plants were sampled for phenotypical, physiological and molecular analyses, which include quantification of total chlorophyll, tetrapyrrole intermediates, dry biomass, shoot height, root length, Cd content, and other physiological and biochemical determinations. The transcriptional expression abundances of Cd transporter genes (*OsIRT1*, *OsIRT2*, *OsNramp1*, *OsNramp5*, *OsHMA2*, *OsHMA3* and *OsLCT1*) were also determined.

2.2. Measurement of chlorophyll and tetrapyrrole intermediates contents

Leaf total chlorophyll content was determined according to the protocol described previously by Fan et al. (2014). Briefly, approximately 0.1 g fresh leaves were pulverized in liquid nitrogen and homogenized with 5 mL of 80% (v/v) acetone. The homogenate was kept for 1 h under darkness before being centrifuged at 12,000 × *g* for 5 min. Then absorbance of supernatant was measured at 645 and 663 nm and chlorophyll content calculated.

The tetrapyrrole intermediates contents were determined according to Li et al. (2017). The heme content was measured as previously described (Peter and Grimm, 2009), and the 5-aminolevulinic acid (ALA) content was determined according to Czarnecki et al. (2012). The contents of protoporphyrin IX (Proto), Mg-protoporphyrin IX (Mg-Proto) and protochlorophyllide *a* (Pchlide) were measured following the protocols of the respective assay kits (Jiangsu Jingmei Biotechnology Co., Ltd., Yancheng, China), which are based on double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Jiang et al., 2019).

2.3. Measurement of dry biomass and Cd content

Cd content in rice seedlings was measured according to Ding et al. (2018). Briefly, Cd absorbed on root surface was removed by immersing roots in 20 mM of disodium ethylenediamine tetra-acetic acid (Na_2-EDTA) for 30 min and followed by rinsing thrice with deionized water. Plant samples were then dried for 2 h at 105 °C followed by two days at 60 °C. After achieving a constant weight, the whole plant samples was weighed as dry biomass. Approximately 200 mg of dry plant material was treated with 6 mL nitric acid (HNO_3) at 140 °C with a Multiwave (CEM MARS 6, USA) until completely digested. Concentrations of Cd were determined by an atomic absorption spectrometer AA-7000 (Shimadzu, Tokyo, Japan).

2.4. Measurement of free amino acids contents

FAAs (Glu, Pro, Arg and GABA) contents were performed according to Martinelli et al. (2007). Approximately 1 g of dry plant material was snap frozen in liquid nitrogen and ground in 2% (w/v) aqueous solution of sulfosalicylic acid. The homogenate was shaken at room temperature for 1 h at 200 × *g*, then centrifuged at 12,000 × *g* for 10 min. After passing through a 0.22 μm membrane Millex-LG (Waters, USA), the supernatant was used for FAAs analysis by a free amino acid automatic instrument L-8900 (Hitachi, Japan).

2.5. Measurement of hydrogen peroxide (H_2O_2) content

Hydrogen peroxide (H_2O_2) was quantified according to Zhang et al. (2014) using a hydrogen peroxide assay kit (Solarbio, Beijing, China). Briefly, approximately 10 mg plant tissues were pulverized in liquid nitrogen and homogenized with 500 μL acetone. After centrifugation at 8000 × *g* for 10 min at 4 °C, aliquot supernatant was mixed with equal volume of hydrogen peroxide detection reagent by vortexing and incubated at room temperature for 5 min. Then the absorbance was determined at 415 nm, and H_2O_2 content was calculated from the standard curve.

2.6. Measurement of singlet oxygen ($^1O_2$) content

Singlet oxygen ($^1O_2$) content was measured by use of the $^1O_2$ sensor green fluorescent probe (Hideg et al., 2002). Frozen rice root and shoot tissues were pulverized in liquid nitrogen and mixed with 1 mL of pre-chilled lysis buffer (GENMED Scientifics Inc., United States). The

mixture was centrifuged at 5000 × *g* at 4 °C for 10 min. The supernatant was then mixed with a solution consisting of 0.02% (v/v) methanol and 10 mM $^1O_2$ sensor green probe (GENMED Scientifics Inc.). The fluorescence at 485 nm (excitation) and 520 nm (emission) were determined, respectively, using a 359S fluorescence spectrophotometer (Lengguang Tech., China).

2.7. Measurement of malondialdehyde (MDA)

Malondialdehyde (MDA) content was measured according to the protocol as previously described (Tang et al., 2013). About 100 mg of plant tissues were homogenized with 10 mL of 10% trichloroacetic acid (TCA) and the homogenate was centrifuged at 10,000 × *g* for 20 min. The supernatant was mixed with equal volume of thiobarbituric acid, incubated at 95 °C for 30 min, and quickly cooled on ice. After centrifugation at 10,000 × *g* for 20 min, the absorbances at 450, 532, and 600 nm were measured.

2.8. Anti-oxidant enzyme activity assays

The activity of anti-oxidant enzymes, including glutathione S-transferase (GST), peroxidase (POD) and catalase (CAT) were measured following the protocols of the respective assay kits (Solarbio, Beijing, China) as described previously (Aebi, 1984; Hossain et al., 2010; Duan et al., 2012). The anti-oxidant enzyme activities were defined as follows: One unit of the GST activity will conjugate 1 μmol of 1-chloro-2,4-dinitrobenzene with reduced glutathione per minute; One unit of POD activity will cause a 0.01 absorbance increase at 470 nm per minute. One unit of CAT activity will cause 1 nmol of H_2O_2 to be degraded per minute.

2.9. Gene expression analysis

Total RNA of fresh tissues was extracted by use of RNeasy Plant RNA Mini Kit (Qiagen, Hilden, Germany) and cDNA was prepared by reverse transcription from 1 μg of total RNA using GoScript™Reverse Transcription System (Promega). Quantitative real-time PCR (qRT-PCR) was performed using a SYBR Green GoTaq® qPCRMaster Mix (Promega, WI, USA). The rice ACTIN gene (*Os11g0163100*) was used as an internal reference and relative expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primers for qRT-PCR are listed in Table S1.

2.10. Statistical analysis

All statistical analyses were performed using the students' *t*-test. The data is presented as mean with standard error (SE) based on three or six replications. The significance of differences between group means was calculated following Bonferroni Post-tests, and the means were compared by ANOVA.

3. Results

3.1. Supplement of Glu significantly alleviated Cd toxicity in rice

To determine the effect of Glu supplement on Cd toxicity, rice seedlings were grown hydroponically in culture medium containing either Glu (1, 3, or 6 mM), Cd (80 μM) or both. Treatment of Cd (80 μM) resulted in toxicity in rice plants (Figs. 1A, S1). After Cd treatment, plants displayed visible chlorosis in young leaves (Fig. 1B) and the content of total chlorophyll was reduced by 64.8% after one-week Cd exposure (Figs. 1C, S2), compared to non-treated control (CK). The dry biomass and plant height after one-week Cd exposure were only about 21.0% and 26.3% those of CK, respectively (Figs. 1D, S2).

While rice seedling's growth was best in the medium supplemented with 6 mM Glu, both of 3 mM and 6 mM Glu supplements could significantly alleviate Cd toxicity in seedlings with one- or two-week Cd exposure (Figs. 1, S1, S2). We selected 3 mM Glu for further analyses. In the presence of 3 mM Glu in culture medium, the chlorosis symptoms in young leaves was greatly relieved and the total chlorophyll content restored to 87.9% that of CK, 2.49-fold greater than in plants treated with Cd alone (Figs. 1, S1, S2). Likewise, the dry biomass of plants reached 82.9% that of CK, 1.21-fold greater than that only treated with Cd (Figs. 1, S1, S2). Supplement with 3 mM Gln was found to be ineffective in mitigating Cd toxicity (Fig. S3).

3.2. Supplement of Glu reduced Cd accumulation under Cd stress

Cd treatment significantly increased Cd concentrations in both root and shoot. In seedlings grown on Cd-containing medium for one week, Cd concentration reached 262.6 and 83.6 mg/kg, respectively, in root and shoot, which were 35.2- and 61.4-fold greater than those in the absence of Cd (Fig. 2A). After Cd treatment, the shoot/root ratio of Cd accumulation was increased to 1.7-fold (Fig. S4), whereas the contents of Glu were reduced by 44.1% in root and by 36.1% in shoot compared to those of CK (Fig. 2B).

Glu supplement significantly reduced Cd accumulation by 44.1% and 65.6%, respectively, in root and shoot compared to plants exposed to Cd treatment alone (Fig. 2A). Addition of Glu also significantly reduced the shoot/root ratio of Cd accumulation by 38.6% compared to that only treated with Cd after one week of Cd exposure (Fig. S4). Furthermore, Glu concentration reached 19.6 and 12.5 μmol/g, respectively, in root and shoot, which were 3.8 and 3.1-fold greater than that of seedlings with only Cd treatment (Fig. 2B). Compared with CK, Glu supplement could not significantly reduce the Cd accumulation in root and shoot, and the shoot/root ratio of Cd (Figs. 2, S4). These results showed that exogenous Glu reduced Cd uptake into the roots and root-to-shoot Cd translocation.

3.3. Glu supplement partially restored tetrapyrrole biosynthesis under Cd stress

To understand the biochemical basis of reduced Cd-induced chlorosis, we determined the levels of five tetrapyrrole intermediates of the tetrapyrrole biosynthesis (Figs. 3 and 4, S5). In plants grown on Cd spiked medium, the concentration of the intermediates in shared steps of Mg- and Fe-branch (ALA, Proto), as well as those of Mg-branch (Mg-Proto and Pchlide) were significantly reduced, i.e. by 18.2%, 22.5%, 35.4% and 58.0%, respectively, compared with those of CK (Fig. 3), which might well explain the reduction of total chlorophyll content (-64.8%) (Fig. 1B, C). In contrast, heme content was significantly elevated by 35.4% as compared to CK (Fig. 3).

Addition of Glu strongly ameliorated the Cd-induced chlorosis symptoms due to increased chlorophyll content (Figs. 1, S1). The contents of ALA, Proto, Mg-Proto and Pchlide of tetrapyrrole biosynthesis in seedlings exposed to Cd treatment were restored to similar levels of CK in the presence of Glu supplement, and were 1.3–2.0-fold higher than those without Glu (Fig. 3). Inconsistent with the increase of these intermediates, the heme level was reduced by 21.3% in plants grown in Glu supplemented medium compared with Cd treatment alone (Fig. 3). Compared with CK, Glu supplement significantly induced the ALA, Proto, Mg-Proto and Pchlide, but had no effect of heme content (Fig. 3).

To understand why the level of intermediates of tetrapyrrole biosynthesis were differentially changed under Cd stress and Glu supplement, we further determined the transcription of 30 genes involved in tetrapyrrole biosynthesis. The abundance of all transcripts, except for those of *OsGUN6-1* and *OsGUN6-2* in Fe-branch was significantly reduced by 38.7–89.7% in response to Cd treatment (Fig. 4). Glu supplement completely or partially restored the transcription of all these genes except *OsGUN6-1* and *OsGUN6-2*, which were decreased by 74.7% and 55.4%, respectively. Compared with CK, Glu supplement significantly induced over-expression of all genes, except for *OsGUN6-1*

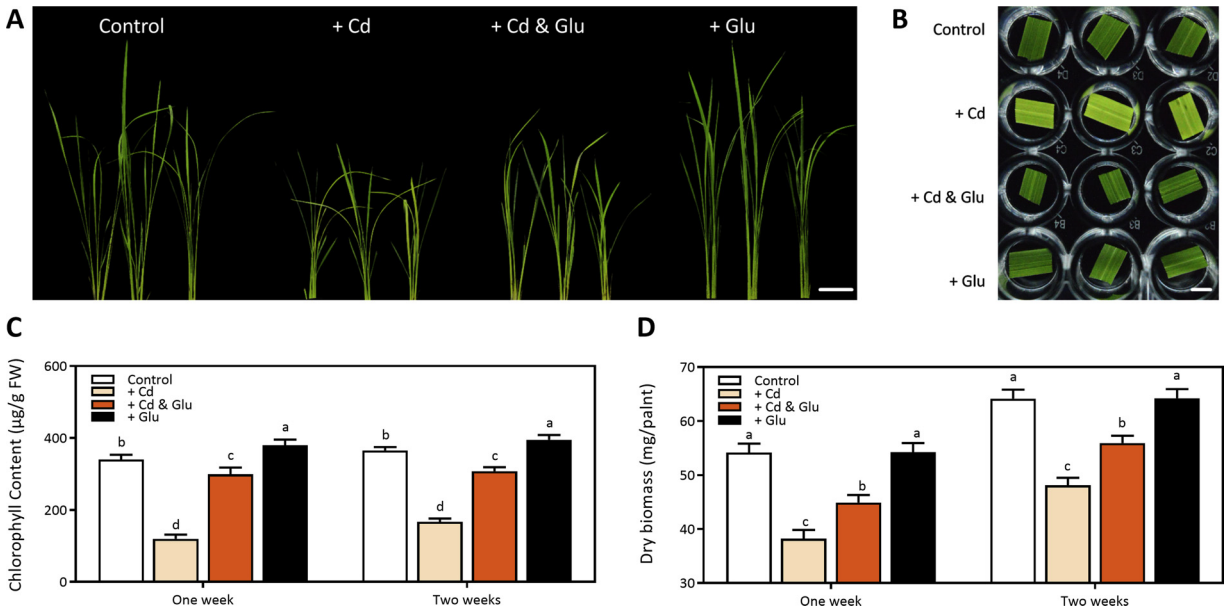

Fig. 1. Effect of cadmium (Cd) and glutamate (Glu) supplementation on the growth of rice seedlings. Rice seedlings were first grown in ½ MS liquid medium for two weeks after germination, then transferred to medium supplemented with either Cd (80 μM), Glu (3 mM), or both. Seedlings were phenotyped two weeks after treatment (A, B). A, bar = 5 cm. B, bar = 5 mm. Chlorophyll content in leaves (C) and dry biomass of whole plant (D) were determined one week and two weeks after treatment with six replicates. The different letters above error bars show the significant differences at a probability of $P < 0.05$. Treatments: Control: 0 μM $CdSO_4$, 0 mM Glu; + Cd: 80 μM $CdSO_4$, 0 mM Glu; + Cd & Glu: 80 μM $CdSO_4$, 3 mM Glu; + Glu: 0 μM $CdSO_4$, 3 mM Glu.

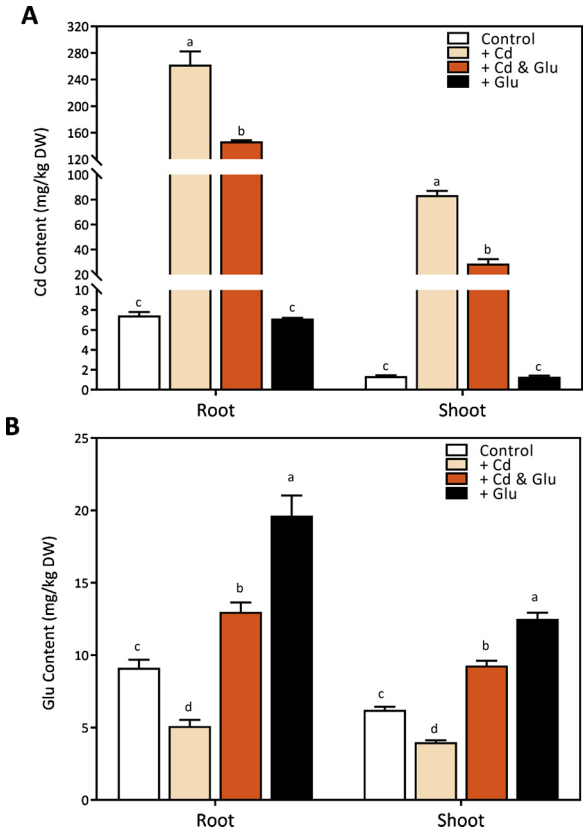

Fig. 2. Cadmium (Cd) and glutamate (Glu) accumulation in rice seedlings. Rice seedlings were grown in ½ MS medium with different treatments of Cd and Glu for one week. All analyses were performed with three replicates. The different letters show the significant difference at a probability of $P < 0.05$. For treatments see Fig. 1.

and *OsGUN6-2* (Fig. 4). These findings showed that supplement of Glu partially restored tetrapyrrole biosynthesis in seedlings under Cd stress.

3.4. Supplement of Glu ameliorated Cd-generated oxidative stress

To investigate how supplement of Glu ameliorated Cd toxicity, we determined the concentrations of stress-related FAAs (Pro, Arg and GABA), ROS (H_2O_2, $^1O_2$) and MDA, and anti-oxidant enzyme activities (CAT, POD and GST) in Cd-stressed plants (Figs. 5–7).

Firstly, we observed that the concentrations of stress-related FAAs were significantly increased after Cd treatment, i.e. by 2.3–3.2-fold in root, and by 2.0–3.0-fold in shoot, respectively, relative to CK after one week of Cd exposure (Fig. 5). Glu supplement significantly reduced the level of Pro, Arg and GABA, by 36.7–46.2% in root, and by 28.4–42.5% in shoot, respectively, compared to one-week Cd exposure (Fig. 5). Compared with CK, Glu supplement had no effect of stress-related FAAs contents (Fig. 5).

Secondly, we observed that Cd treatment led to obvious oxidative stress as reflected by induced overproduction of ROS in both non-radical (molecular) form H_2O_2 and free radical form $^1O_2$ (Fig. 6), which are highly reactive and toxic and cause significant damage to cell structures. One week of Cd exposure significantly induced accumulations of H_2O_2, $^1O_2$ and MDA by 42.2–88.8% in the root and by 53.5–96.6% in the shoot, respectively (Fig. 6). Glu supplement significantly reduced H_2O_2, $^1O_2$, and MDA contents by 15.2–27.3% in root, and by 19.9–28.8% in shoot, respectively, compared to single Cd treatment (Fig. 6). Compared to CK, contents of H_2O_2 and $^1O_2$ in seedlings were decreased, by 17.5% and 16.3% in root, and by 20.3% and 28.1% in shoot, after Glu supplementation (Fig. 6). While, MDA content in Glu supplement seedlings was similar to CK. (Fig. 6).

At last, we observed that one-week of Cd exposure significantly decreased activities of all three tested anti-oxidant enzymes (CAT, POD and GST), by 43.2%, 60.7% and 42.1% in root and 61.8%, 58.9% and 39.1% in shoot, respectively, relative to CK (Fig. 7). Amendment of Glu significantly increased the CAT activity by 33.6% and 89.8%, POD activity by 86.2% and 83.8%, and GST activity by 26.6% and 25.6%, respectively, in the root and shoot of Cd-treated plants compared to Cd treatment alone (Fig. 7). Compared with CK, Glu supplement had no

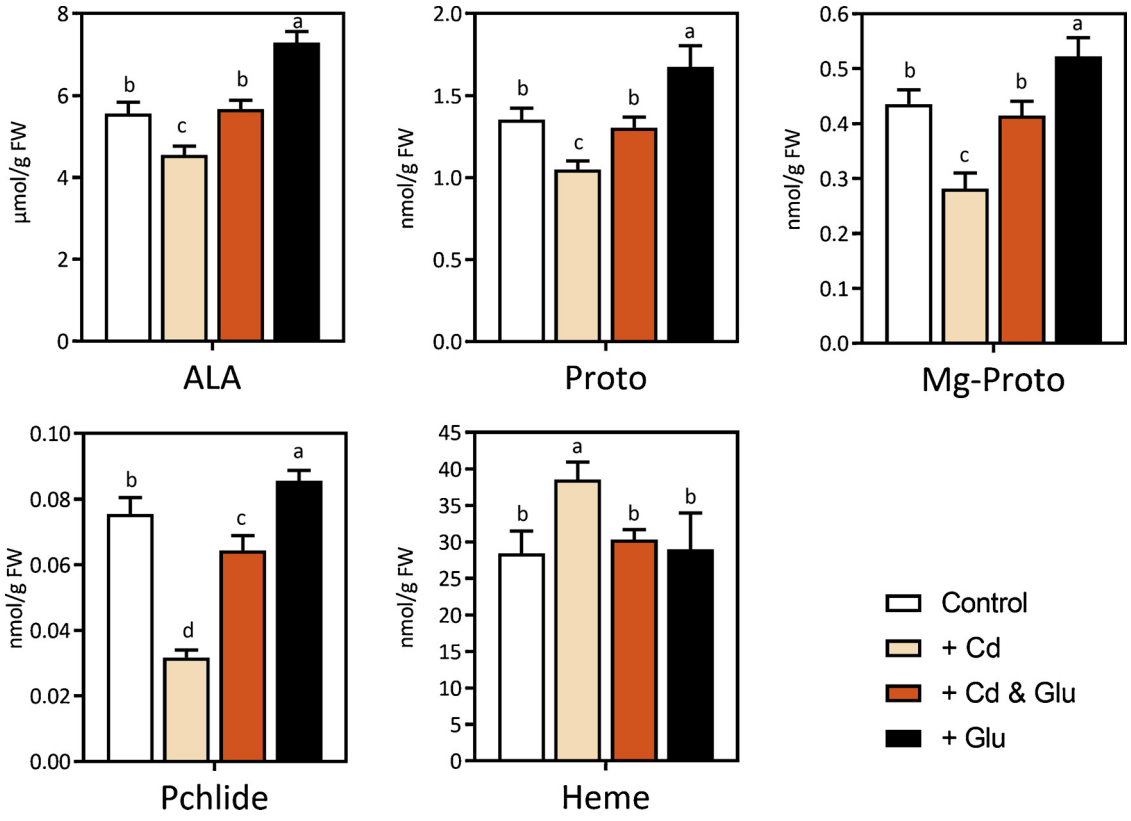

Fig. 3. Steady-state levels of tetrapyrrole intermediates in rice shoot. Rice seedlings were grown in ½ MS medium with different treatments of Cd and Glu for one week. All analyses were performed with three replicates. The different letters show the significant difference at a probability of $P < 0.05$. See Fig. 1 for treatments.

effect of all three tested anti-oxidant enzymes content (Fig. 7). Activities of antioxidant enzymes were partially restored by Glu supplement to levels as in the absence of Cd.

These results showed that Glu supplement reduced accumulation of ROS and relieved oxidative stress with reduced levels of stress-related FAAs and increased activities of antioxidant enzymes in rice under Cd stress.

3.5. Supplement of Glu down-regulated Cd uptake transporter genes in roots under Cd stress

To understand the molecular mechanism of how Glu regulates Cd

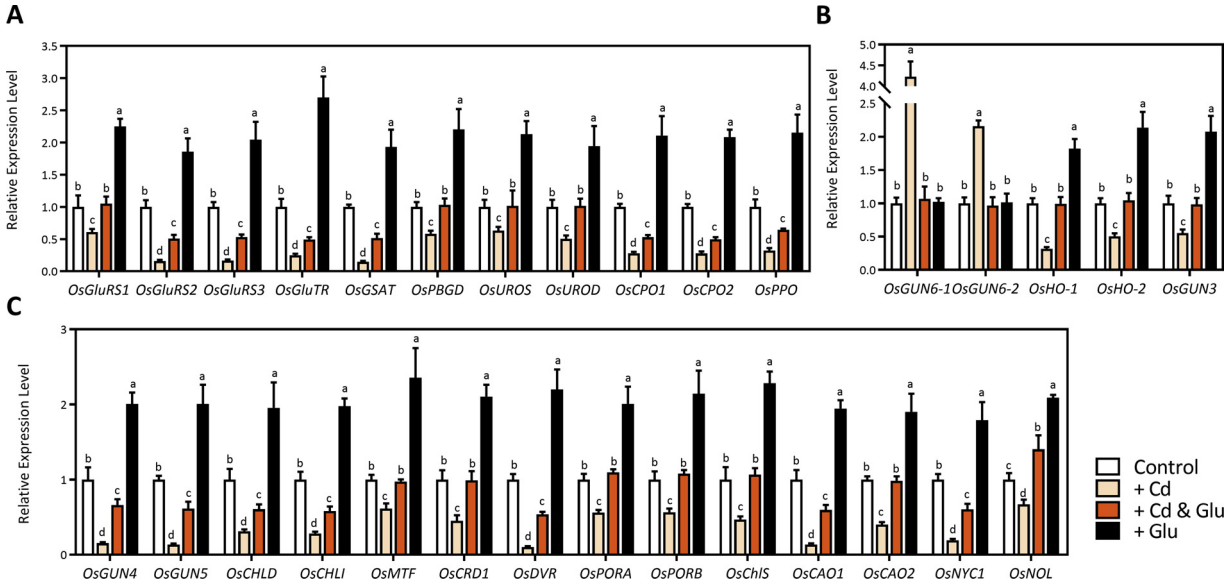

Fig. 4. Relative expression levels of tetrapyrrole biosynthesis-associated genes in rice shoots. Rice seedlings were grown in ½ MS medium with different treatments of Cd and Glu for one week. (A) Expression levels of genes encoding enzymes for the shared steps of the tetrapyrrole biosynthesis pathway. (B) Expression levels of genes from the Mg branch. (C) Expression levels of genes from the Fe branch. The expression levels were first normalized to the internal control gene *OsACTIN* and reported relative to each gene's expression level of control (assigned a value of 1). All analyses were performed with three replicates. The different letters show the significant difference at a probability of $P < 0.05$. See Fig. 1 for treatments.

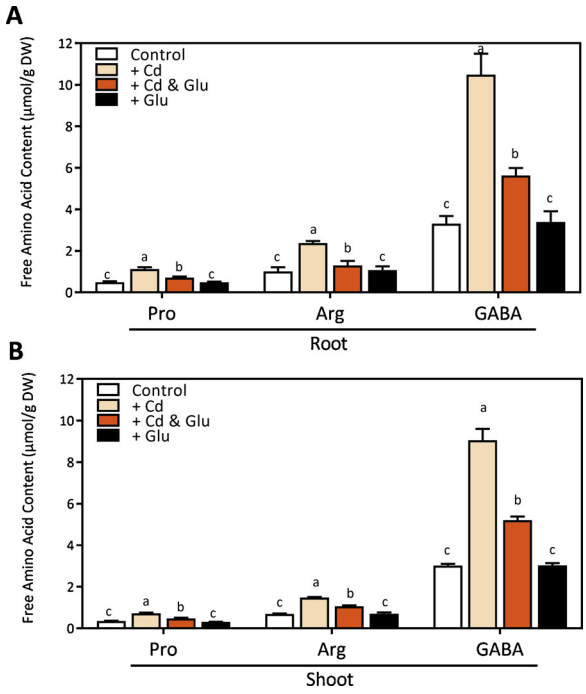

Fig. 5. Accumulation of stress response-related free amino acids (Pro, Arg and GABA) in rice seedlings. Rice seedlings were grown in ½ MS medium with different treatments of Cd and Glu for one week. Levels of Pro, Arg and GABA in root (A) and shoot (B) were determined after one-week treatment. All analyses were performed with three replicates. Error bars represent standard error. The different letters show the significant difference at a probability of $P < 0.05$. See Fig. 1 for treatments.

uptake, translocation and allocation, expression analysis was performed for genes associated with Cd transport functioning in different parts of rice plant. Exposure to Cd greatly increased the abundance of *OsNramp1*, *OsNramp5*, *OsIRT1*, *OsIRT2*, *OsHMA2* and *OsHMA3* in roots, but not *OsLCT1*, which remained at CK level (Fig. 8). In contrast, only the abundance of *OsNramp1* and *OsNramp5* among these genes was significantly elevated in shoots by Cd treatment (Fig. 8).

Exogenous Glu significantly decreased transcription of the six genes (*OsNramp1*, *OsNramp5*, *OsIRT1*, *OsIRT2*, *OsHMA2* and *OsHMA3*) in Cd-treated roots, with their transcript abundance reduced by 23.3–34.2%, compared to those only treated with Cd (Fig. 8). Again, expression of *OsLCT1* was not responsive to Glu addition. Supplement of Glu, however, did not completely bring down the transcription of these six transporter genes in root to levels as in CK. Interestingly, we found that *OsNramp1* and *OsNramp5* were the genes with higher relative expression levels among the seven Cd transporter genes in rice shoot (Fig. S6). These results demonstrated that Glu supplement suppressed Cd-induced expression of Cd uptake transporter genes in rice roots under Cd stress.

3.6. Two-week effects of Glu on Cd stress

Beneficial effect of Glu supplement was also observed in plants treated for two weeks. The effect of Glu on relieving Cd stress in two-week Cd treatment was similar to those observed for one-week. Supplement of Glu reduced Cd accumulation under Cd stress (Fig. S7) and significantly alleviated Cd-induced chlorosis by partially restoring chlorophyll biosynthesis (Fig. S8). Glu supplement significantly ameliorated Cd-generated oxidative stress, e.g. reduced level of Pro, Arg, GABA, H_2O_2, $^1O_2$ and MDA (Figs. S9, S10), and increased activities of major anti-oxidant enzymes (CAT, POD and GST) (Fig. S11). Glu supplement suppressed Cd-induced over-expression of Cd uptake transporter genes in rice roots under Cd stress.

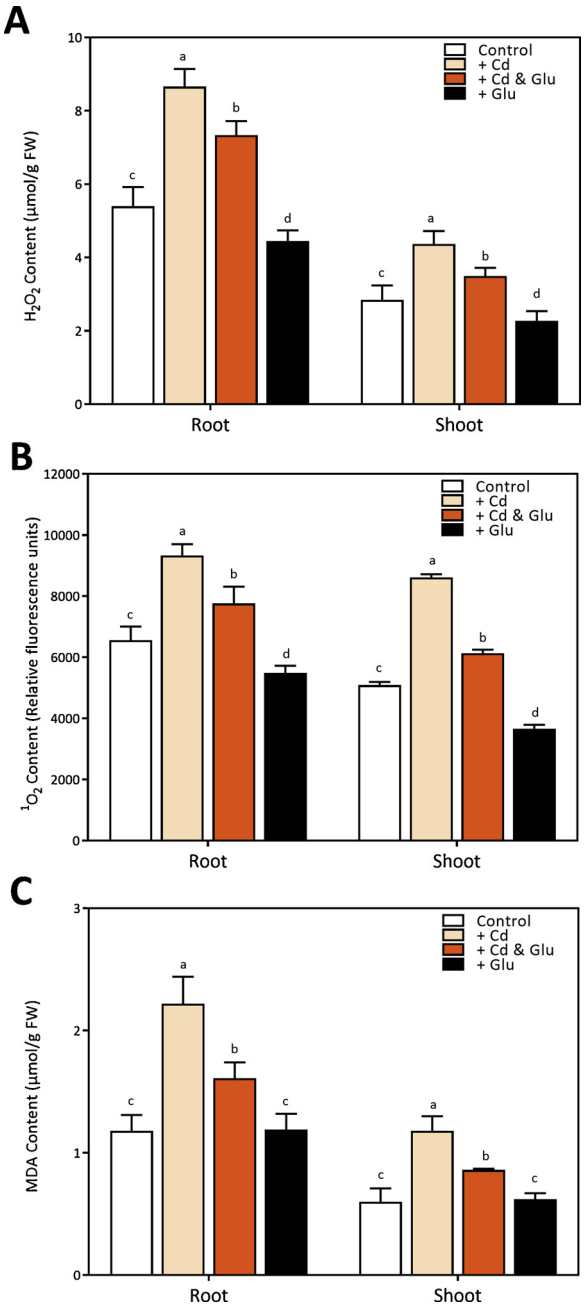

Fig. 6. Accumulation of H_2O_2, $^1O_2$ and MDA in rice seedlings. Levels of H_2O_2 (A), $^1O_2$ (B) and MDA (C) in root and shoot were determined after one-week treatment. All analyses were performed with three replicates. See Fig. 1 for treatments.

4. Discussion

It is well known that accumulation of Cd in plants can induce the oxidative stress (Yuan et al., 2013; Chen et al., 2019) and reduce chlorophyll content (Kim et al., 2014). It has also been shown that Cd enhanced the expression of Cd transporter genes (Uraguchi et al., 2011; Kim et al., 2014; Farooq et al., 2016; Chen et al., 2019). In the present study, we confirmed these findings in rice seedlings exposed to Cd stress. We found that Glu supplement can alleviate the Cd toxicity by down-regulation of Cd transport genes responsible for Cd uptake and translocation. The reduction in Cd accumulation by Glu supplement improved Cd-induced oxidative stress by increasing anti-oxidant enzyme activities and decreasing ROS level in rice plants. In addition, chlorophyll biosynthesis was restored by Glu in rice plants exposed to

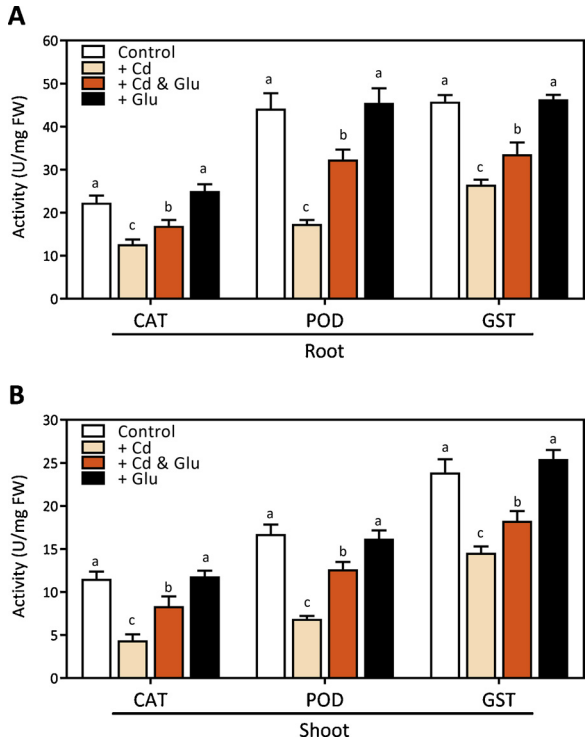

Fig. 7. Activities of CAT, POD and GST in rice seedlings. Activities of CAT, POD and GST were assayed in root (A) and shoot (B) after one-week treatment. All analyses were performed with three replicates. See Fig. 1 for treatments.

Cd. To our knowledge, this is the first study that demonstrated supplement of Glu could alleviate Cd toxicity in rice with partially restored plant growth.

Antioxidant enzyme activities are well known to be inducible by oxidative stress generated by Cd toxicity. However, the response varies with plant species and exhibits genotype-, development-, tissue- and dose-dependence (Hassan et al., 2005). For example, under low Cd exposures (0.1–5.0 μM), CAT activity showed very limited variation while POD activity varied substantially in rice plants (Hassan et al., 2005). Under higher Cd exposures, POD activity was increased substantially at both 100 μM and 500 μM; while CAT activity was elevated at 100 μM but declined at 500 μM in rice seedlings (Shah et al., 2001). However, Chen et al. (2019) reported that both CAT and POD activities were significantly reduced after exposure to 100 μM Cd in rice roots, while the former was significantly increased and the latter significantly reduced in rice shoots. In current study, activities of CAT, POD and GST were all reduced after exposure to 80 μM Cd (Fig. 7), which is similar to the results of Chen et al. (2019). The reduced antioxidant enzyme activities may have led to oxidative burst in rice seedlings exposed to Cd as indicated by the accumulation of ROS, such as H_2O_2 (Fig. 6A) and $^1O_2$ (Fig. 6B) in Cd-stressed plants, and consequently resulted in elevated lipid peroxidation (Fig. 6C). The oxidative stress seemed to be significantly mitigated by Glu supplement (Figs. 6 and 7), suggesting that enhancement of anti-oxidant defense capacity is a major mechanism for Glu-mediated alleviation of Cd toxicity in rice plants.

Regulation and manipulation of metal transporters is an important mechanism of alleviating heavy metal toxicity in plants (Ueno et al., 2010; Liu et al., 2013; Adrees et al., 2015). Although there are no specific transporters for Cd, the metal transporters *OsNramp1*, *OsNramp5*, *OsIRT1*, *OsIRT2*, *OsHMA2*, *OsHMA3* and *OsLCT1* have been thought to be associated with the transport of Cd in addition to transport of other divalent metal ions such as Fe^{2+}, Zn^{2+}, Mn^{2+} (Ishimaru et al., 2006; Nakanishi et al., 2006; Ueno et al., 2010; Miyadate et al., 2011; Senoura et al., 2011; Ueno et al., 2011; Uraguchi et al., 2011; Ishimaru et al., 2012; Sasaki et al., 2012; Takahashi et al., 2012). It was reported that Cd exposure increased expression levels of *OsNramp1*, *OsNramp5*, *OsHMA2* and *OsHMA3* in rice roots (Chen et al., 2019). Consistent with this, Cd stress resulted in elevated expression of six Cd transporters, *OsNramp1*, *OsNramp5*, *OsIRT1*, *OsIRT2*, *OsHMA2* and *OsHMA3* in rice roots.

The higher expression level of metal transporters *OsNramp1*, *OsNramp5*, *OsIRT1*and *OsIRT2*, involved in Cd uptake and *OsHMA2*, involved in root-to-shoot translocation in root is expected to result in increased accumulation of Cd in plant (Clemens and Ma, 2016). The observed 35- to 60-fold increases of Cd, respectively, in rice root and shoot (Fig. 2) would cause significant oxidative stress and damage to rice plants, *e.g.* chlorosis, retarded plant growth as reflected in greatly reduced biomass and lengths of shoot and root (Fig. 1). *OsHMA3* expression was observed to increase under Cd stress, consistent with Chen et al. (2019) and Kim et al. (2014), but different from Shao et al. (2017). Since OsHMA3 is involved mainly in Cd vacuolar sequestration in root, over-expression of it would retain more Cd in root, relieving Cd toxicity (Ueno et al., 2010; Miyadate et al., 2011). Inconsistency between different studies might be due to Cd concentrations used in experiments. Shao et al. (2017) used a low Cd, up to 5 μM and a short duration (24 h) while we used much higher one (80 μM) for one to two weeks. OsLCT1 and OsHMA2 are involved in intervascular transfer in rice shoot (Clemens and Ma, 2016). There was no significant effect of Cd on the expression of *OsLCT1* and *OsHMA2* in rice shoot (Fig. 8).

Upon Glu addition, the expression of six Cd transporters, *OsNramp1*, *OsNramp5*, *OsIRT1*, *OsIRT2*, *OsHMA2* and *OsHMA3* in roots were significant declined under Cd stress (Fig. 8), indicating that Glu suppressed the Cd-induced expression of these genes. Again, Glu did not affect the expression of *OsLCT1* and *OsHMA2* in the shoot. Glu reversed the Cd-enhanced expression of transporter genes responsible for Cd uptake and translocation, which might be the principal cause for the Glu-mediated alleviation of Cd toxicity. With less Cd accumulation in plant, there were less oxidative stress and damage in plants, as reflected by restored chlorophyll biosynthesis and reduced amounts of ROS, MDA and increased activities of antioxidant enzymes. It is yet unknown how Glu can regulate the expression of metal transporters.

Glu orchestrates multiple biological pathways by acting either as nutrient, metabolite, energy-yielding substrate, structural determinant in proteins, or even signaling molecule (Watkins and Jane, 2006; Forde and Lea, 2007; Seifi et al., 2013). Treatment of Glu could activate the transcription of a large variety of defense-related rice genes (Kan et al., 2017). Yang et al. (2017) found in tomato fruit that Glu suppressed the incidence of disease caused by *Alternaria alternata* and suggested that the Glu-induced disease resistance might be associated with salicylic acid (SA) signaling pathway. Glutamate has been proposed to be applied in rice plants as forliar fertilizer to increase plant resistance against abiotic stress, drought (Wu, 2017). A large set of stress-responsive genes including metal transporters were found to be differentially methylated and transcriptionally activated in rice seedlings exposed to Cd (Feng et al., 2016). Whether Glu regulates the expression of metal transporters in plants via epigenetic regulation pathway awaits further study.

A common plant response to environmental stress is the accumulation of amino acids. Under Cd stress, Pro and GABA were increased, Arg decreased, while Glu remained unchanged in tomato leaves (Chaffei et al., 2004). In roots, strong decrease in Glu was accompanied by increased Pro and Arg, and decreased GABA (Chaffei et al., 2004). In the present study, changes in Glu, Pro, GABA and Arg were similar in both shoot and root: Glu was decreased while all the other three amino acids increased when submitted to Cd treatment. Glu is the starting substrate for the biosynthesis of stress-related FAAs, Pro, Arg and GABA. The strong increase of GABA in Cd-treated plants might cause a deficiency of Glu, resulting in its short supply for other essential processes such as biosyntheses of chlorophyll, glutathione and phytochelatins, which are required for Cd tolerance. Such a shortage of Glu in Cd-stressed plants can be mitigated by Glu supplement: higher Glu

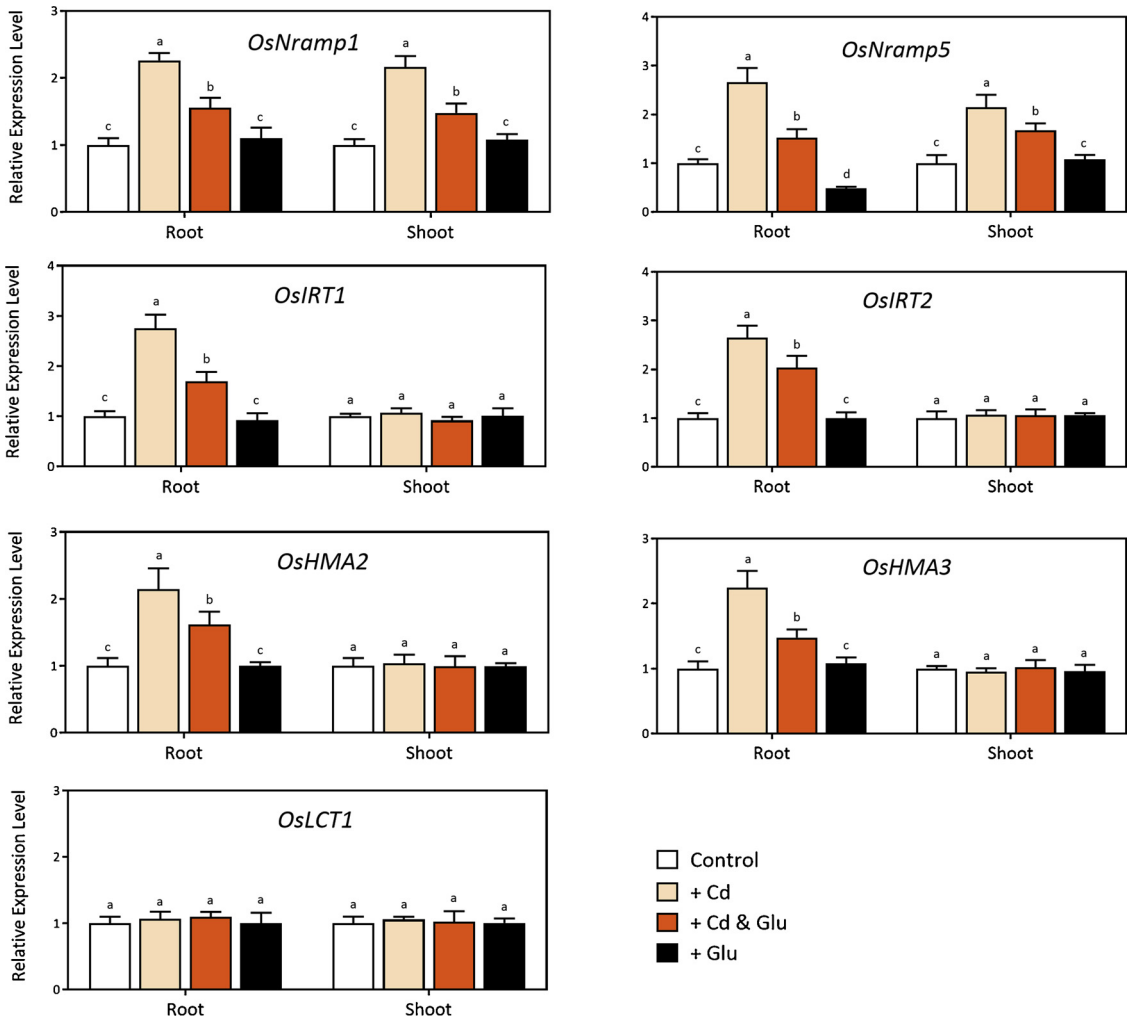

Fig. 8. Relative expression levels of the Cd-transporter genes in rice seedlings. Gene expression were analyzed by qRT-PCR with root and shoot tissues from seedlings grown in ½ MS medium after one-week treatment. The expression levels were first normalized to the internal control gene *OsACTIN* and reported relative to each gene's expression level of control (assigned a value of 1). All analyses were performed with three replicates. The different letters show the significant difference at a probability of $P < 0.05$. See Fig. 1 for treatments.

content and much lower GABA level (Fig. 5).

In summary, we demonstrated that Glu can improve the tolerance of rice plant against Cd stress. Possible mechanisms may include: decreased root-to-shoot translocation and uptake of Cd, improved chlorophyll biosynthesis, enhanced anti-oxidant systems. Our results also imply that Glu application or other measures that can promote its accumulation in plant may be used to reduce Cd accumulation in crops exposed to Cd.

5. Conclusion

Glu alleviated toxicity and reduced Cd accumulation by down-regulation of Cd transporter genes, resulting in reduced uptake and translocation of Cd in rice plant. Glu also enhanced the anti-oxidant enzymes activities, thus ameliorating Cd-induced oxidative stress. Furthermore, restoration of chlorophyll biosynthesis in rice leaves was observed to alleviate Cd toxicity. Glu-mediated mitigation of Cd toxicity may represent a common resistance mechanism in plant response to heavy metal stress. The use of glutamate as Cd mitigators is worthwhile to be exploited in rice production in Cd contaminated areas.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.121319.

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