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# Effects of Zinc Fertilization on Grain Cadmium Accumulation, Gene Expression, and Essential Mineral Partitioning in Rice

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**Abstract:** Cadmium (Cd) is a toxic heavy metal that can cause severe health issues if ingested. Certain varieties of rice can accumulate high levels of the metal in edible tissues thereby transferring the toxin into the food chain. As chemical analogs, interactions between the essential mineral zinc and the toxic heavy metal cadmium play an important role in regulating the transport of both minerals to rice grains. Understanding these interactions is crucial for limiting cadmium and increasing zinc transfer to the food chain. Previous studies have reported conflicting results suggesting synergistic and antagonistic relationships between the minerals. The goal of this work was to identify the effect of external cadmium and zinc on the uptake and translocation of both minerals from roots to grains of rice that differ in grain cadmium concentrations. The results showed that a higher input of external zinc increased cadmium translocation and accumulation to the grain in two of three varieties, while external cadmium does not influence zinc accumulation. Cadmium synergy and antagonism with other essential minerals were also examined and the effects differed between rice lines. Our results showed that the differential expression of the transport proteins OsNramp5, OsHMA2, and OsHMA3 as well as genes involved in the synthesis of glutathione and phytochelatin could have contributed to differences in grain Cd accumulation. These results add to the knowledge of cadmium and zinc partitioning in one of the most consumed plant foods in the world and can assist fortification efforts to establish rice lines that are both safe and nutritious.

**Keywords:** cadmium; zinc; rice; transport; grains



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## 1. Introduction

Cadmium (Cd) is a toxic heavy metal whose industrial uses include batteries, plastic stabilizers, and pigments [1]. It is a contaminant that occurs naturally in some agricultural soils and threatens agricultural productivity, food safety and human health [2,3]. When available, plants can readily accumulate the contaminant in edible tissues and allow it to be transferred through the food chain. *Oryza sativa* has one of the highest propensities of translocating cadmium to the grains when compared to other cereal crops [4–7]. Dietary Cd is currently one of the leading causes of Cd poisoning and is linked to bone mineral density loss, renal disease, and cancers [8–10]. Since grains contribute 27% of human dietary consumption of cadmium, decreasing cadmium in grains will lower dietary exposure [11].

Essential micronutrients such as zinc (Zn) share a similar atomic structure with Cd, and therefore they can interact similarly with plant proteins. Metal specificity is a challenge in breeding low Cd cereals, because the alteration of pathways to decrease Cd transport could inadvertently decrease Zn transport. The uptake of Cd and Zn from soil and their distribution within the plant involve complex and dynamic processes that may be facilitated by common transporters [12]. As with micronutrients, Cd transport and accumulation in the grains is affected by several processes such as Cd uptake into the root, translocation to aboveground tissues, subcellular sequestration, and phloem transport [13–17].

Cadmium uses proteins from key transport families such as the natural resistance-associated macrophage proteins (NRAMP), the Zrt-Irt-like protein family (ZIP), Yellow stripe-like (YSL), and Heavy Metal p1b-type ATPase family (HMA), which are responsible for transporting essential minerals such as zinc, iron (Fe), and manganese (Mn) [4,15,16,18–21]. The transport pathway of Zn and Cd from soil begins at the root cells where the mineral enters the plant through either the symplastic or apoplastic pathways [22,23]. Although the exact mechanisms of Zn transport from soil to rice grain are not completely clear, several tightly regulated proteins from the ZIP, YSL, and HMA families have been identified [24–28]. The root uptake of Zn is mediated by OsZIP1 and OsZIP9, which are expressed in the epidermis of rice roots [29,30]. The transport of Zn to aboveground tissues can be attributed to proteins such as OsZIP4 and OsHMA2, which are localized on vascular bundles in nodes [18,24,31,32]. Wong et al. (2009) have also shown that HMA2 and HMA4 are principal transporters contributing to xylem loading of Cd [33,34]. These large complex protein families are often non-specific and can function in the transport of Cd and other divalent ions when available [26,30,35–40]. Shared cadmium and zinc transport proteins may influence their transport pathways due to interactions between the minerals.

There are a number of transporters specific for Cd or Cd-conjugated thiol ligands [41,42]. The subcellular sequestration of Cd can be achieved by ligand binding to glutathione or phytochelatin and subsequent transport into the vacuole by ABC-type vacuolar transporters homologous to those found in the yeast [43–48]. OsHMA3 is known to play a key role in the root sequestration of Cd, where a single amino acid substitution is associated with high grain Cd [15,32].

Studies on Cd–Zn interactions in rice are limited and remain inconclusive. Some studies have shown that Zn fertilization can ameliorate physiological stresses induced by Cd [49,50]. Zinc supplied to roots or leaves as a foliar spray reduces Cd accumulation in roots and grains [50–53]. However, other studies have shown that Cd translocation to shoots and accumulation in grains are actually increased as a result of Zn fertilization [54–56]. Cadmium and zinc interactions can influence the response of genes in different tissues, resulting in high and low grain Cd accumulators [28]. Tian et al. (2022) have shown that significant differences in the translocation rates of essential minerals under Cd stress were due to competitive interactions between minerals for HMA proteins [57]. Stress response genes, such as γ-glutamyl cysteine ligase (GSH ligase), glutathione synthase (GSHS), and phytochelatin synthase (PCS1), are involved in the biosynthesis of non-protein peptide chelators that bind Cd and Zn, and can also influence cellular transport [19,58–61]. Because the specifics of these interactions are unknown, there is a need to analyze whole plant mineral transport and gene expression patterns to elucidate the mechanisms responsible for low grain-Cd rice phenotypes. In this study, we sought to understand the dynamics of Cd transport and response to the increased supply of Zn, especially during the grain filling stage. The gene expression of select transport proteins, OsNRAMP5, OsHMA2, and OsHMA3, and stress response genes, GSH ligase, glutathione synthase, and phytochelatin synthase, were compared between the three lines of rice that differed in grain cadmium concentration so that we could investigate the contributions of these genes to the observed phenotypes. Furthermore, we analyzed other essential minerals to elucidate the overall effects of Cd and Zn availability on the transport of essential macro- and micronutrients. A comprehensive understanding of Cd–Zn interactions and the effect of Zn fertilization on Cd accumulation is important to understand the pathway of both Cd and Zn from root to shoot to seed, especially if Zn fertilization is used as a method to reduce grain Cd or increase grain Zn concentration in rice.

## 2. Materials and Methods

### *2.1. Sample Selection*

Rice accessions (*Oryza sativa*) were obtained from the USDA-ARS, Dale Bumpers National Rice Research Center (Stuttgart, Arkansas, Genetic Stocks Oryza Collection-GSOR). Based on the grain Cd concentrations published in Pinson et al. (2015) [62], we selected

three lines, PI 310546 (herein referred to as line 546), PI 311667 (herein referred to as line 667), and PI 301428 (herein referred to as line 428). Rice lines were grown in hydroponics (details below) containing a full nutrient solution and 1 mM Cd to ensure they could successfully reach reproductive maturity and accumulate Cd in grains. When grown hydroponically, rice lines 546, 667, and 428 accumulated low, medium, and high concentrations of Cd, respectively. As such, these three rice lines were chosen for further experimentation.

### *2.2. Plant Growth and Treatment*

Seeds were surface sterilized with 1% hypochlorite for 10 min, imbibed overnight MilliQ $H_2O$ (Millipore, Billerica, MA, USA), and germinated in moistened filter paper lined Petri dishes for 5 days before being transplanted to hydroponics. A total of 96 germinated seedlings per line were transferred to 4 L pots (2 plants per pot/3 pots total for every line $\times$ treatment $\times$ reproductive stage permutation) (Supplementary Table S1). Each pot represented one of three experimental replicates. Hydroponic solution consisted of a modified Johnson nutrient solution with the following nutrients: 2 mM $KNO_3$, 1 mM $Ca(NO_3)_2$, 1 mM $KH_2PO_4$, 1 mM $MgSO_4$, 25 μM $CaCl_2$, 25 μM $H_3BO_3$, 0.5 μM $H_2MoO_4$, 0.1 μM $NiSO_4$, 2 μM $MnSO_4$, 0.5 μM $CuSO_4$, iron supplied as 20 μM Fe(III) HEDTA (N-(2-hydroxymethy) ethylenediaminetriacetic acid), and 2 mM 2-(N-morpholino) ethane-sulfonic acid (MES; Sigma Chemical, St. Louis, MO, USA) to buffer the solution to pH 6.0. Solutions were aerated continuously and replaced every week. The plants were grown in the Lehman College greenhouse with natural lighting plus supplemental metal halide lamps (100W) allowing for a 16 h day and 8 h night photoperiod, 25 $\pm$ 3 °C day/23 $\pm$ 3 °C night.

A completely randomized factorial design was used to examine Cd and Zn partitioning in three lines (line 546, line 667, and line 428) at different stages of development. When plants grew to the 4 to 5 leaf stage, a fresh solution was given where the plants were assigned to a treatment consisting of different Cd and Zn combinations: 0 μM Cd + 2 μM Zn (c0z2), 1 μM Cd + 2 μM Zn (c1z2), 0 μM Cd + 10 μM Zn (c0z10), or 1 μM Cd + 10 μM Zn (c1z10), respectively. Full solution changes were performed weekly to ensure roots were always submerged in growing solution and mineral availability was consistent over time. Cadmium was supplied as cadmium sulfate for the cadmium treatments and plants were harvested at 1 of 4 predetermined reproductive stages where "vegetative (Veg)" represents 30 days of growth, "Anthesis (Ant)" represents the flowering stage, which was determined to be the first day of heading, and 2 (2WAA) and 4 (4WAA) weeks after anthesis. At harvest, plant material was separated into roots, stems, lower leaves, flag leaves, peduncles, rachis, and grains when available (Supplementary Table S1). Roots were rinsed with deionized water before further processing. Grains were further separated from husks, but low husk weights prevented the tissue from being used in follow-up experiments. Plants from one pot were treated as one biological replicate. A total of three biological replicates were collected and analyzed for each line x treatment x harvest permutation (Supplementary Table S1). All plant material was roughly separated into equal parts, where half of the material was collected for mineral partitioning and the other half was flash frozen in liquid nitrogen to be used for gene expression analyses.

### *2.3. Tissue Digestion and Mineral Concentration Determination*

Tissue for mineral partitioning analysis was collected and oven dried at 60 °C to constant mass and homogenized using a Wiley mill with a 0.2 mm screen (Thomas Scientific, Swedesboro, NJ, USA). For each tissue, ~0.25 g was digested using 3 mL of concentrated nitric acid at 100 °C in a BD50 Digestion Block (Seal Analytical, Kitchener, OT, Canada). Once cooled, 4 mL of 30% hydrogen peroxide was added and temperatures were periodically ramped up to 250 °C over the course of 6 h to dissolve all organic material. Digests were resuspended in 10 mL of 1% nitric acid and filtered using a 0.22 mm pre-syringe filter. The acids used were trace metal-grade (Fisher Scientific, Pittsburg, PA, USA) and the water used was deionized using the MilliQ system. Cadmium sulfate was used as the positive control and blanks were used for every digestion run. Samples were analyzed for nine

essential minerals (Ca, Mg, P, S, K, Cu, Fe, Zn, Mn) and Cd by inductively coupled plasma optical emission spectroscopy (ICP-OES; iCAP 7000; Thermo Electron North America LLC, Madison, WI, USA), calibrated with certified standards to detect mineral concentrations as described previously [63]. Negative controls (blanks) were processed and analyzed to monitor for background levels of minerals in the digestions and resuspension solutions. Background mineral levels were subtracted from sample values before concentrations were calculated. The reported averages and standard error are derived from concentrations from three biological replicates (three pots of six plants).

### *2.4. Gene Expression*

Tissue specific gene expression was analyzed to assess the contributions of transport proteins in Zn and Cd transport in different varieties of rice. All harvested tissue for gene expression analysis was instantly flash frozen using liquid nitrogen and crushed using a pre-chilled mortar and pestle. About 0.5 g of tissue was collected and combined with 600 mL of Trizol reagent (Invitrogen. Waltham, MA, USA). After 10 min, 125 mL of chloroform was added, tubes were mixed thoroughly, and chilled on ice for 10 min. Samples were then centrifuged for 20 min at 11,000 RPM. The supernatant was carefully separated and put through the RNeasy Plant RNA Extraction kit (Qiagen. Germantown, MD, USA), where RNA was diluted in 30 mL RNA Free H2O. RNA concentration and quality were analyzed using a Nano drop ND-2000 (Thermo Fisher Scientific, Waltham, MA, USA). DNA impurities were degraded using the RQ1 RNAse-Free DNAse kit (Promega. Madison, WI, USA). RNA (1 mg) was then used for first-strand synthesis RT-PCR with random primers using the GoScript Reverse Transcription kit (Promega. Madison, WI, USA). Primers designed using Integrated DNA Technologies primer design tools were then used (Supplementary Table S2) with SYBR green master mix (Promega. Madison, WI, USA) to perform quantitative real-time polymerase chain reaction. The transport genes OsNramp5, OsHMA2, and OsHMA3 and heavy metal chelator biosynthesis genes phytochelatin synthase (PCS1), γ-glutamyl cysteine ligase (GSH ligase), and glutathione synthase (GSHS) were assessed due to their importance in both zinc and cadmium transport in rice [4,58,59,64,65]. Reactions of two technical replicates and three biological replicates were carried out in a CFX Connect Real Time PCR System (BioRad, Hercules, CA, USA) in 15 mL reactions. Results were calculated as relative gene expression using the delta-delta Ct method with values normalized to the Ubiquitin housekeeping gene [66]. The reference gene was run on each plate to ensure stability. Reported values are average expression and standard error from $n = 3$ biological replicates.

### *2.5. Statistics*

Analysis of variance and Pearson's correlation coefficients were performed and determined using GraphPad Prism version 9.1 (GraphPad. San Diego, CA, USA). Two-way analysis of variance (ANOVA) multiple comparisons test with a Tukey correction were used to analyze statistical differences in mean tissue cadmium and mineral concentrations or relative gene expression between lines at a particular treatment and harvest or between treatments in a particular line and harvest (biological replicates $n = 3$). Significance of $p \leq 0.05$ are annotated on graphs and tables. Pearson's correlation coefficients were also calculated between Cd concentration and essential mineral concentrations. Significant correlations $p < 0.05$ are annotated on tables.

## 3. Results

### *3.1. Mineral Concentrations*

#### 3.1.1. Effect of Zinc on Cadmium Concentrations

All three lines, lines 546, 667, and 428, were able to set seed without phytotoxicity in all of the treatments analyzed. Cadmium concentrations in the different tissues of control plants (c0z2) are presented in Supplementary Table S3. Consistently under c1z2 (1 μM Cd + 2 μM Zn) treatments (Figures 1 and 2), line 428 maintained the highest Cd

concentrations in all tissues except for flag leaves (Figure 2), where 667 had significantly higher Cd concentrations. Line 546 demonstrated the lowest concentrations of Cd in most tissues compared to 667 and 428. Grain Cd concentrations at full maturity were statistically different ($p < 0.05$) between all three lines, averaging 0.29, 1.09, and 2.0 mg/g DW in lines 546, 667, and 428, respectively.

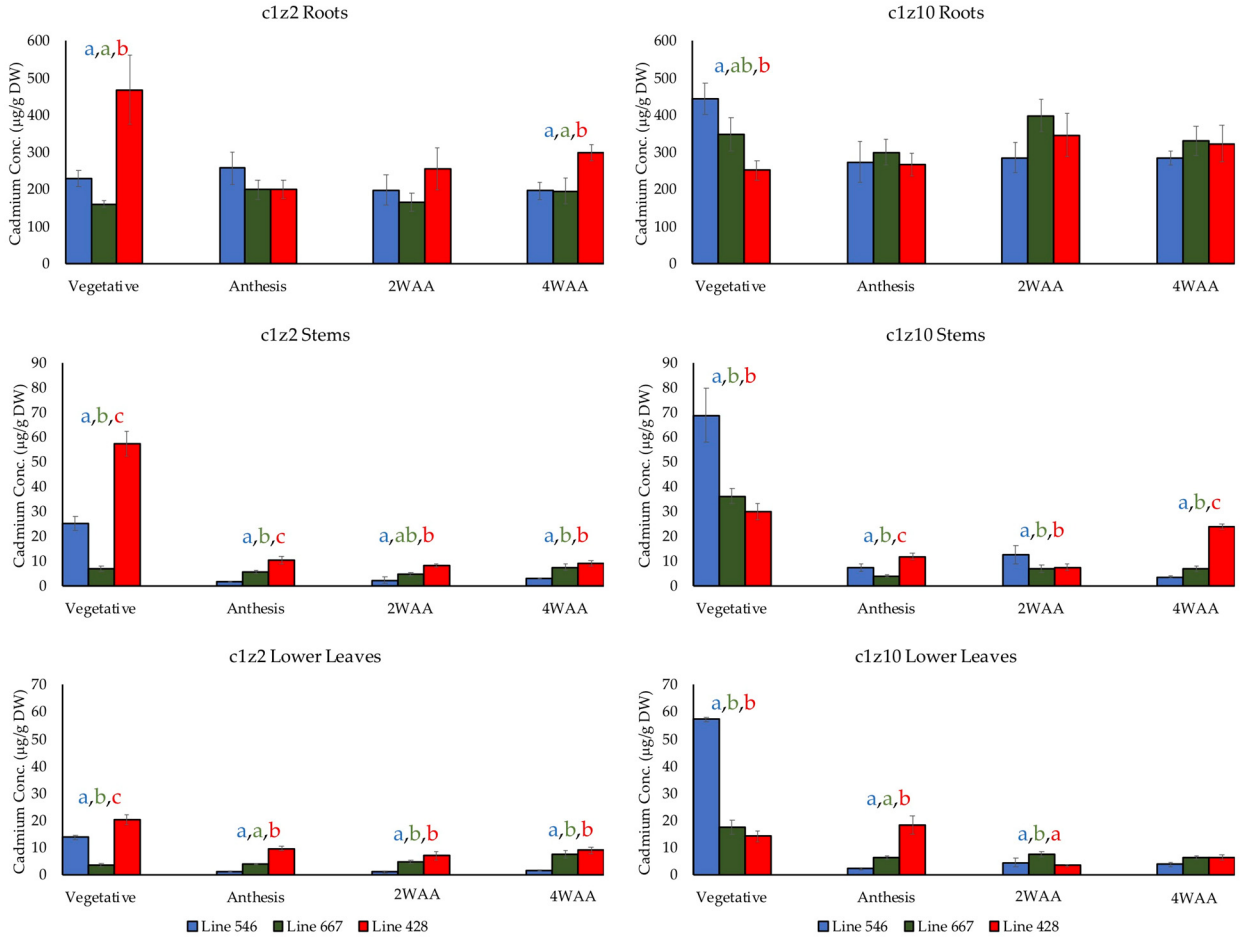


**Figure 1.** Effect of zinc fertilization on cadmium concentration in vegetative tissues: Cadmium concentrations (mg/g DW) in vegetative tissues (root, stem, and lower leaves) of rice lines 546, 667, and 428 that were harvested at 4 developmental stages (vegetative (30 days old), anthesis (first day of flowering), 2 weeks after anthesis (2WAA), and 4 weeks after anthesis (4WAA)). Plants were treated with 1 µM Cd + 2 µM Zn (c1z2) or 1 µM Cd + 10 µM Zn (c1z10). Means ± se ($n = 3$). Variance was compared using 2-way ANOVA with a multiple comparison test and Tukey correction. Different colored letters (abc) indicate significant difference between the three lines tested at a particular treatment and reproductive stage ($p < 0.05$). No letter indicates no statistical significance.

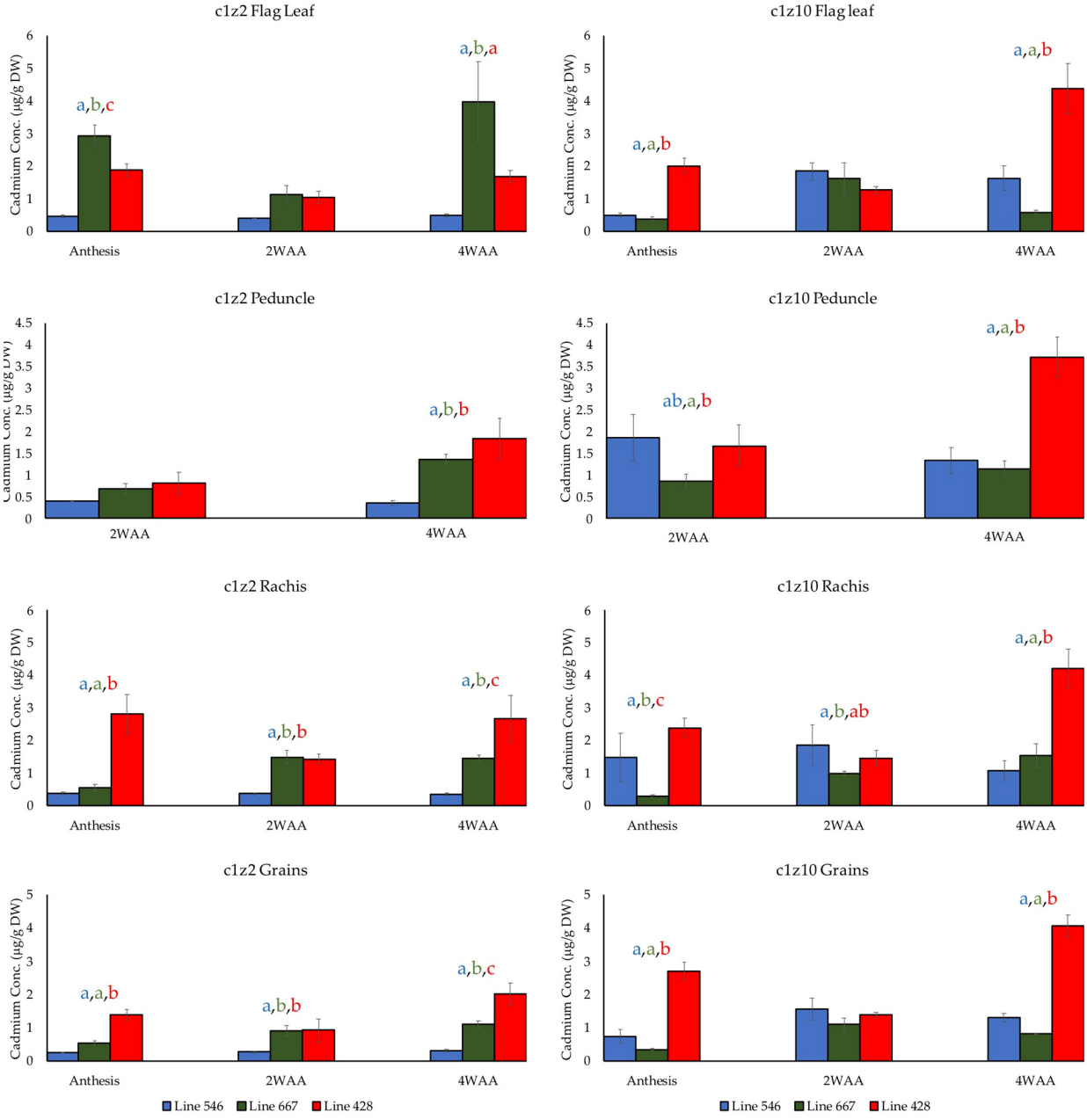


**Figure 2.** Effect of zinc fertilization on cadmium concentration in reproductive tissues: Cadmium concentrations (mg/g DW) in reproductive tissues (flag leaf, peduncle, rachis, and grains) of rice lines 546, 667, and 428 that were harvested at 4 developmental stages (vegetative (30 days old), anthesis (first day of flowering), 2 weeks after anthesis (2WAA), and 4 weeks after anthesis (4WAA)). Plants were treated with 1 µM Cd + 2 µM Zn (c1z2) or 1 µM Cd + 10 µM Zn (c1z10). Means ± se ($n$ = 3). Variance was compared using 2-way ANOVA with a multiple comparison test and Tukey correction. Different colored letters (abc) indicate significant difference between the three lines tested at a particular treatment and reproductive stage ($p < 0.05$). No letter indicates no statistical significance.

When grown with increased Zn levels (c1z10; 1 µM Cd + 10 µM Zn) (Figures 1 and 2), there were no differences between the lines in root Cd concentration except the vegetative stage. The high line (428) had significantly higher Cd concentrations in stems, flag leaves, peduncles, rachis, and grains. There were no differences between the medium and the low Cd lines in most of the tissues. Cadmium concentrations in grains were 4.0, 1.3, and 0.8 µg/g DW in lines 428, 546, and 667, respectively. There were some differences between c1z2 and c1z10 in the vegetative tissues, but differences were not consistent

(Table S4). It is interesting to note that the flag leaf Cd concentration was significantly reduced with increased Zn in the medium line (667) when compared to lower Zn treatments (Figure 2; Supplementary Table S5). Zinc fertilization significantly increased grain Cd concentration by 348% in the low line (546) and by 102% in the high line (428) but resulted in a slightly lower Cd concentration in the medium line (667), although the difference was not statistically significant.

### 3.1.2. Effect of Cadmium and Zinc Fertilization on Zinc Concentrations

Zinc concentrations were comparable between all lines under control (c0z2; 0 mM Cd + 2 mM Zn) treatments (Figures 3 and 4). In the presence of cadmium (c1z2; 1 mM Cd + 2 mM Zn), the high line (428) had significantly higher grain Zn concentrations when compared to the other two lines. However, there were no differences in Zn concentrations between the control and the Cd-treated lines (Supplementary Tables S6 and S7).

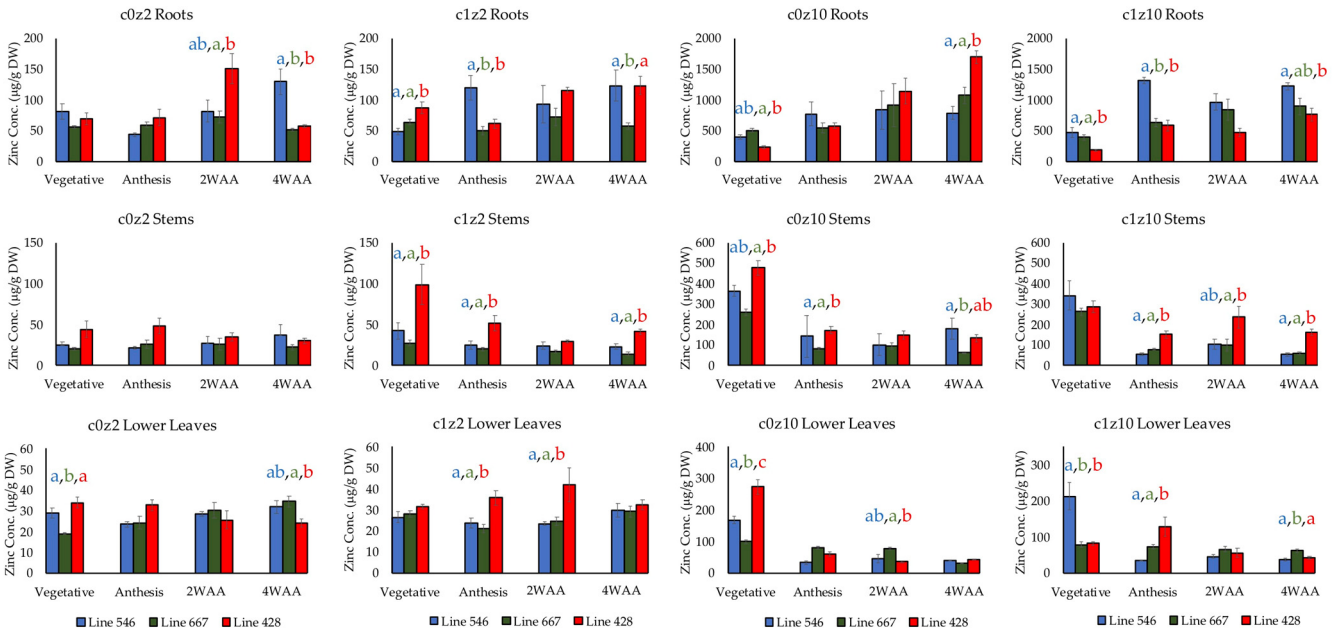


**Figure 3.** Effect of cadmium and zinc fertilization on zinc concentration in vegetative tissues: Zinc concentrations (mg/g DW) in vegetative tissues (root, stem, and lower leaves) of rice lines 546, 667, and 428 that were harvested at 4 developmental stages (vegetative (30 days old), anthesis (first day of flowering), 2 weeks after anthesis (2WAA), and 4 weeks after anthesis (4WAA)). Plants were treated with 0 µM Cd + 2 µM Zn (c0z2), 1 µM Cd + 2 µM Zn (c1z2), 0 µM Cd + 10 µM Zn (c0z10), or 1 µM Cd + 10 µM Zn (c1z10). Means ± se ($n$ = 3). Variance was compared using 2-way ANOVA with a multiple comparison test and Tukey correction. Different colored letters (abc) indicate significant difference between the three lines tested at a particular treatment and reproductive stage ($p < 0.05$). No letter indicates no statistical significance.

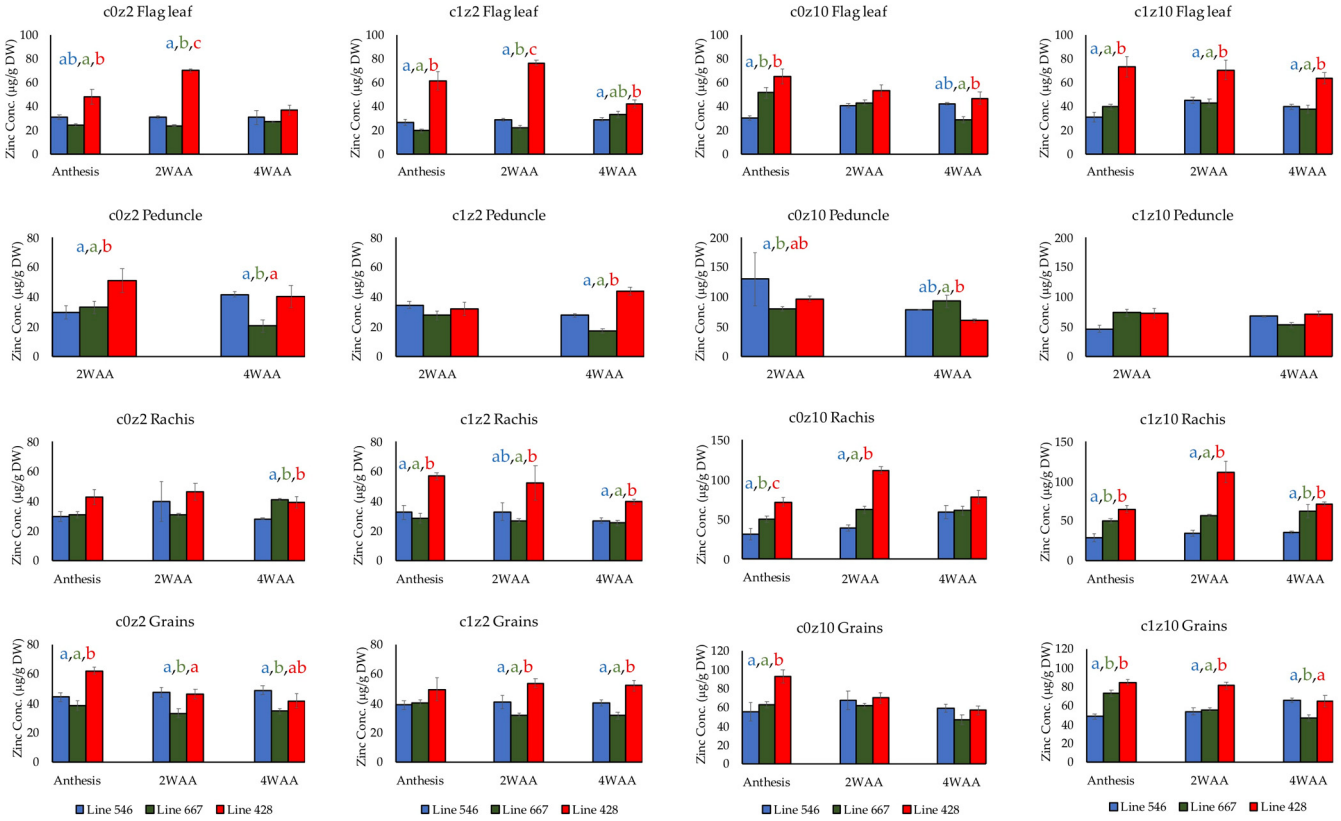


**Figure 4.** Effect of cadmium and zinc fertilization on zinc concentration in reproductive tissues: Zinc concentrations (mg/g DW) in reproductive tissues (flag leaf, peduncle, rachis, and grains) of rice lines 546, 667, and 428 that were harvested at 4 developmental stages (vegetative (30 days old), anthesis (first day of flowering), 2 weeks after anthesis (2WAA), and 4 weeks after anthesis (4WAA). Plants were treated with 0 µM Cd + 2 µM Zn (c0z2), 1 µM Cd + 2 µM Zn (c1z2), 0 µM Cd + 10 µM Zn (c0z10), or 1 µM Cd + 10 µM Zn (c1z10). Means ± se ($n$ = 3). Variance was compared using 2-way ANOVA with a multiple comparison test and Tukey correction. Different colored letters (abc) indicate significant difference between the three lines tested at a particular treatment and reproductive stage ($p < 0.05$). No letters indicate no statistical significance.

Zinc fertilization increased Zn concentration in specific tissues but differed between lines. When extra Zn was supplied in the absence of Cd (c0z10; 0 mM Cd + 10 mM Zn), Zn concentrations were significantly higher when compared to the controls (c0z2; 0 mM Cd + 2 mM Zn) in most tissues (Figures 3 and 4; Supplementary Tables S6 and S7). Although the Zn concentrations were higher in the Zn fertilization treatments, there were no differences between the lines for most tissues.

When Zn fertilization treatments with and without Cd (c0z10 vs. c1z10) were compared, although the root Zn concentrations were significantly lower in the high line (428) in the presence of Cd, there were no differences in most aboveground tissue Zn concentrations between c0z10 and c1z10 treatments (Supplementary Tables S6 and S7).

When grown in the presence of Cd (c1z2 and c1z10), Zn concentration patterns differed between the two treatments (Figures 3 and 4). Zn fertilization (c1z10) led to significant increases in Zn in some tissues in all three lines, and significantly increased in the grains of both the low and high lines. Zinc concentrations were significantly higher in most of the vegetative tissues in the Zn fertilization treatment (c1z10) when compared to c1z2, but that difference was not consistent in the reproductive tissues (Supplementary Tables S6 and S7).

### 3.1.3. Other Grain Mineral Concentrations

The grain mineral concentrations of nine of the essential minerals were also measured in the three lines in all treatment conditions (Supplementary Table S8). The effects of

Zn fertilization were unique to the line and mineral tested. As a general trend, line 428 accumulated the highest concentrations of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), and manganese (Mn) under the varying treatments. For all lines, Zn fertilization alone caused no changes in the concentrations of Ca, Cu, Fe, Mg, Mn, Ni, or P, but Cd or Cd and Zn treatments did. Cadmium treatments (c1z2) increased the grain concentrations of Ca, Fe, and Mg in high-line (428) grains compared to controls (c0z2), but there was no difference in these minerals between the control (c0z2) and zinc fertilization (c0z10 and c1z10) treatments. Interestingly, Mg concentrations increased in the presence of Cd in the high line, while Mg concentration was lower in low and medium lines. Pearson's correlation test revealed significant positive correlations between Cd and Ca and Cd and Ni, while there was significant negative correlation between Cd and P in the medium (667) line (Supplementary Table S9). When the plants were supplied with extra Zn (c1z10), there was strong positive correlation between Cd and Mn in the high Cd line.

### *3.2. Gene Expression*

Gene expression relative to ubiquitin was analyzed in the roots of plants harvested in the vegetative stage (Figure 5) or roots and flag leaves of plants harvested at the anthesis stage (Figure 6). In the vegetative tissues, the gene expression of all transport genes examined were highest in line 667 when cadmium and/or zinc were supplied. At anthesis, however (Figure 6), OsNRAMP5 and OsHMA3 expression were higher in the low line (546) when extra Zn was supplied. In flag leaves, OsHMA3 expression was higher in the medium line (667) under c1z2 treatments (Figure 6).

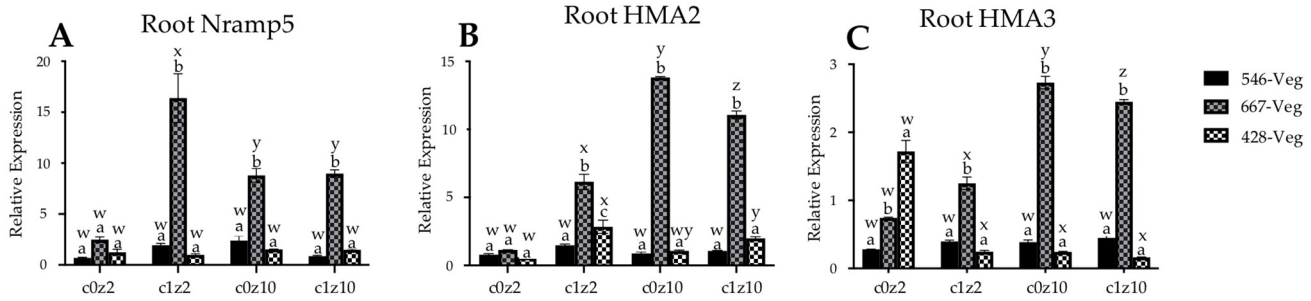


**Figure 5.** Gene expression of transport proteins OsNramp5 (**A**), OsHMA2 (**B**), and OsHMA3 (**C**), in roots lines 546, 667, and 428 harvested at the vegetative reproductive stages: Quantitative real-time PCR was used to quantify mean relative gene expression ($n$ = 3) using the delta-delta Ct method with standard error. Rice ubiquitin was used as the housekeeping gene. Two-way ANOVA multiple comparisons test with Tukey correction was used to analyze significant differences in gene expression. Statistical significance ($p < 0.05$) between the three lines at a particular treatment are annotated by (a, b, c) and differences between the four treatments within a rice line are annotated by (w, x, y, z) above bars.

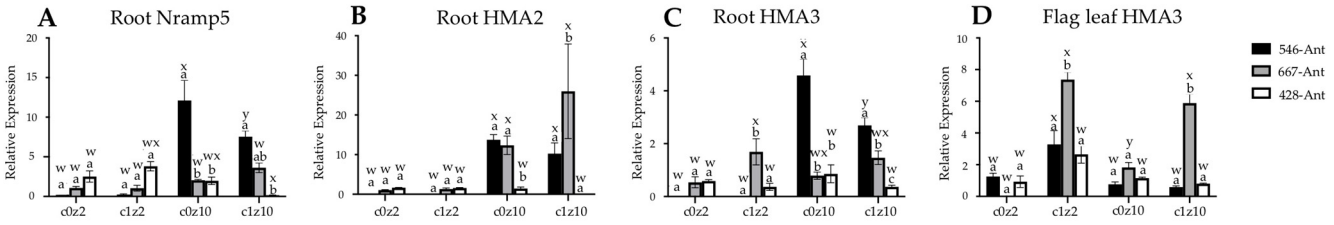


**Figure 6.** Gene expression of transport proteins OsNramp5 (**A**), OsHMA2 (**B**), OsHMA3 (**C**), in roots and OsHMA3 (**D**) in flag leaves of lines 546, 667, and 428 harvested at the anthesis reproductive stages: Quantitative real-time PCR was used to quantify mean relative gene expression ($n$ = 3) using the delta-delta Ct method with standard error. Rice ubiquitin was used as the housekeeping gene. Two-way ANOVA multiple comparisons test with Tukey correction was used to analyze significant differences in gene expression. Statistical significance ($p < 0.05$) between the three lines at a particular treatment are annotated by (a, b, c) and differences between the four treatments within a rice line are annotated by (w, x, y) above bars.

The genes involved in the synthesis of non-protein thiols glutathione and phytochelatin were also analyzed in tissues harvested at the vegetative and anthesis stages. At anthesis, root GSH ligase expression was significantly higher in the high (428) line when compared to the other two lines (Figure 7A). PCS1 expression was increased in the roots of line 667 as a response to all treatments but only increased in line 546 roots as a response to Zn (Figure 7B). In flag leaves, GSH ligase, GSHS and PCS1 were consistently highest in the medium line (667) (Figure 7C–E) under c1z2 treatments.

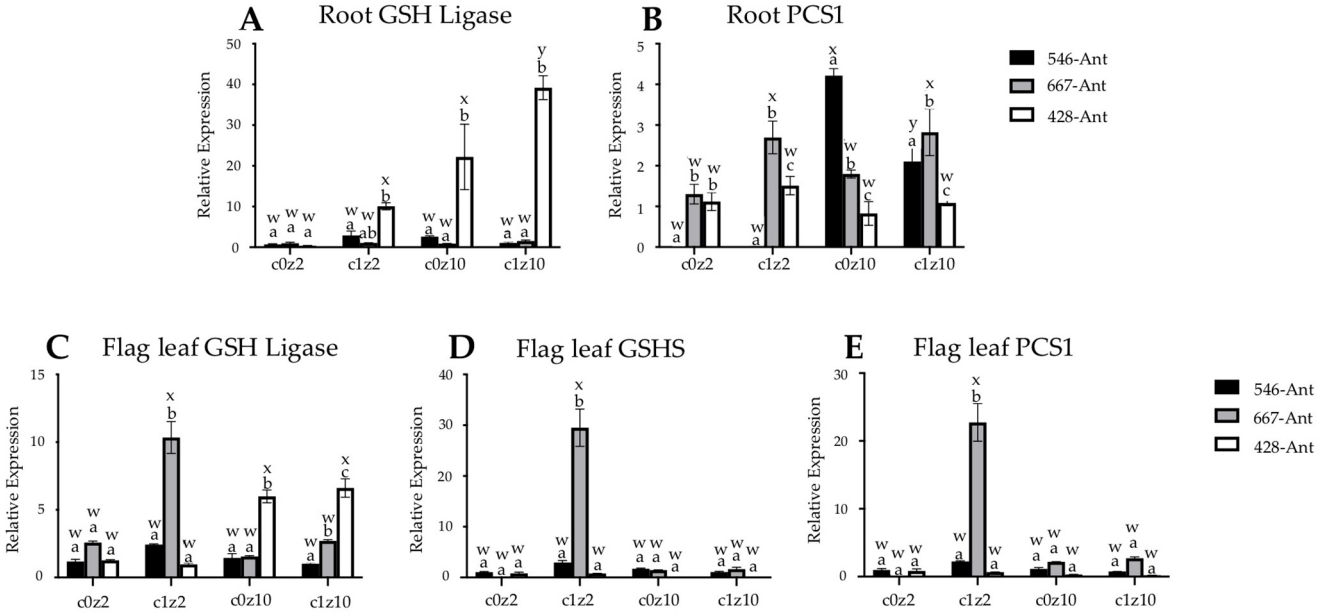


**Figure 7.** Relative expression of non-protein thiols γ-Glutamyl cysteine ligase (GSH ligase) (**A**,**C**), GSHS (**D**), and Phytochelatin synthase (PCS1) (**B**,**E**) from roots (**A**,**B**) and flag leaves (**C**–**E**) of lines 546, 667, and 428 harvested at anthesis stage: Quantitative real-time PCR was used to quantify mean relative gene expression ($n$ = 3) using the delta-delta Ct method with standard error. Rice ubiquitin was used as the housekeeping gene. Two-way ANOVA multiple comparisons test with Tukey correction was used to analyze significant differences in gene expression. Statistical significance ($p < 0.05$) between the three lines at a particular treatment are annotated by (a, b, c) and differences between the four treatments within a rice line are annotated by (w, x, y) above bars.

## 4. Discussion

Interactions between minerals can significantly alter their distribution and accumulation in plant tissues [67–70]. Given the importance of rice as a major food source, it is crucial to produce low Cd cultivars to meet the demand of customers. There is an urgent need to elucidate the mechanisms responsible for differentiating between toxic cadmium and beneficial zinc in uptake, sequestration, and bioavailability. The goal of this work was to assess the influence of Zn levels on Cd and mineral transport and partitioning in three varieties of rice that differed in grain Cd accumulation. To accomplish this goal, we used Cd and Zn treatments that were similar to the environmental concentrations available to plants [71]. All three rice lines were able to successfully reach full reproductive maturity without showing any signs of phytotoxicity.

Our study showed that there was no difference in root Cd concentrations in response to Zn fertilization in all the lines. Some previous studies have shown that Zn fertilization can reduce Cd translocation and accumulation in different tissues and grains [72–75]. However, our study showed that when increasing the external zinc concentration (c1z10), grain Cd concentrations increased in the high (428) and the low (546) lines. This suggests that either there is a different route of translocation for Cd and Zn or that these concentrations of Zn are not effective in restricting Cd uptake and translocation. This is consistent with some previous studies that showed that Zn does not always competitively inhibit Cd accumulation and it is concentration-dependent [31,76–78]. Interestingly, in the medium line, Cd concentration in the flag leaves was significantly lower in response to Zn fertilization and in turn did not cause an increase in grain Cd, which seems to indicate that Zn competitively inhibits Cd transport in this line. Most of the cadmium was accumulated in the stems and lower leaves, consistent with previous studies in cereal crops due to increased transpiration-driven flow in these tissues [48,67,79]. Our study showed only some remobilization of cadmium from the vegetative tissues, which indicates that Cd could be stored in non-labile subcellular compartments such as the vacuoles and thus is not loaded into the phloem [48,67,80].

In terms of the Zn concentration, our results showed that Cd did not influence Zn concentration in any of the lines. It was also interesting to note that in Zn fertilization treatments without Cd (c0z10), there was an increase in Zn concentration in many of the tissues but there was no increase in the grain Zn concentration, especially in the low and medium lines. A previous study has also shown that when Zn was supplied to the leaves of rice during flowering and grain fill, 90% of this Zn was translocated to the vegetative organs and not to the grains [81].

The route that a mineral takes to reach grains is influenced by several factors, including mineral availability, interactions with other minerals, and gene expression, which may be specific to genotype [82]. Transport proteins are critical for the uptake, translocation, and sequestration of minerals in plant tissues [83–85]. The regulation of transport proteins at the molecular level can be used to identify the critical mechanisms involved in mineral transport to edible tissues of plants [86–88]. The expression of transport proteins can be regulated by mineral availability [89–91]. In order to assess Cd and Zn interactions in mineral transport, we analyzed how the gene expression of key transport proteins was affected by Cd, Zn, or combination treatments. While several transport proteins have been identified, we focused on key transport proteins that have been implicated in Cd or Zn transport in rice. In the high-, medium-, and low-grain Cd-accumulating lines, the expressions of OsNRAMP5, OsHMA2, and OsHMA3 were all influenced by treatment (Figure 5). Additionally, expression patterns were unique at reproductive stages. OsNRAMP5 is a root uptake transport protein involved in the transport of cadmium, manganese, and iron [65,92]. In this study, although OsNRAMP5 expression was the highest in the medium line or low line depending on the treatment and developmental stage, there was no difference in root Cd concentrations between the lines. At any given time, minerals are being translocated out of the roots to shoots. Thus, similar Cd and Zn concentrations in roots could be due to the differential expression of other genes as well.

One such gene, OsHMA2, is a xylem loading transport protein that has been shown to be essential for Zn homeostasis and root to shoot translocation of both Zn and Cd in rice [32]. Mutations in OsHMA2 were responsible for restricting the translocation of both minerals in rice [93]. The low expression of OsHMA2 in the low Cd-accumulating line, line 546, could explain the reduced Cd in aerial tissues. In rice roots, OsHMA3, is localized to the tonoplast and responsible for vacuolar sequestration of Cd and excess Zn, restricting their transport to aboveground tissues [15,16,18,94–96]. In our study, the expression of OsHMA3 was also found to be significantly higher in the flag leaves of the medium accumulating line, 667 (Figure 5). Interestingly, this can probably explain the difference between the high (428) and medium (667) Cd lines, which differed in grain Cd concentration in both c1z2 and c1z10 treatments. Transport proteins including OsZIP3, OsZIP4, Cation Exchange Proteins (CAX), Low Cation Transporters (LCT), Metal Tolerance Proteins (MTP), and ATP Binding Cassette transporters (ABCC) have also been implicated in the transport of Cd and/or Zn, but have been omitted from this study. We did, however, analyze the genes involved in the production of non-protein peptides, glutathione and phytochelatin, which assist in the detoxification of Cd [97]. The three genes tested, GSH ligase, GSHS, and PCS1 were explored in flag leaves and roots at the vegetative and anthesis stages (Figure 6). The expression patterns of all genes were affected by treatment in one or more lines. The differential expression between the three lines suggests that Cd–Zn interactions can influence transport by modifying production of thiolate compounds [98–100]. It is interesting to note that the expression of the three genes in the medium-line (667) c1z2 treatment was significantly higher in the flag leaves when compared to the other two lines, which correlates with the high Cd concentration in the flag leaves. This could be the reason for restricted translocation of cadmium from the flag leaf to the reproductive tissues since the concentrations of Cd in the peduncles and rachis were similar to the other two lines. Experiments analyzing the role of glutathione and phytochelatin in cadmium retention suggest that the non-protein peptides play a key role in limiting the translocation of the toxin [61,98].

Concentrations of essential minerals Ca, Cu, Fe, Mg, Mn, Ni, and P were also significantly affected by the Cd and Zn treatments, although effects were specific to the line tested. The high line (428) had the highest concentrations of Ca, Cu, Fe, Mg, and Mn in c1z2 treatments. When Zn fertilization was supplied, Ca, Fe, and P concentrations were lower, but Mn and Ni concentrations in grains increased. We also found strong positive and negative correlations between Cd and essential minerals, but, once again, the specific mineral interactions differed between the lines. Positive or negative correlations between the minerals may be the result of shared transport systems [20,24,36,101]. Our results confirm the importance of assessing the interactions between other essential minerals in attempts to biofortify for specific traits.

Limitations of this work include the inability to measure mineral content or measure precise tissue accumulations or translocation patterns since we used part of the harvested tissue for gene expression studies. We also analyzed the gene expression of only selected transport proteins and stress genes that have been implicated in both cadmium and zinc transport in rice. More robust techniques such as RNA Seq may be able to identify even more genetic differences controlling differential Cd and Zn accumulation.

## 5. Conclusions

In conclusion, there is a dire need to produce cereals with improved nutritional quality and, to do so, a detailed understanding of mineral interactions on transport is needed. We have shown here that increased Zn supply does not decrease Cd concentration but in fact increases grain Cd concentration and that an increased Zn supply does not increase grain Zn concentrations in these three rice lines. Cadmium and zinc tissue concentrations differed between the lines at different stages of reproductive maturity. These accumulation patterns are likely due to differential gene expression controlling mineral translocation within the plant. The difference in the expression patterns of OsNRAMP5, OsHMA2,

OsHMA3, GSH ligase, GSHS, and PCS in our study could be used to explain the difference in Cd translocation and accumulation between the lines. In particular, our study indicates the need to identify genes specifically involved in the export of minerals from flag leaves to grains, which may help us understand the route of mineral translocation into the grains, further assisting in improving strategies to minimize Cd while increasing nutrient density in rice.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12092182/s1. Table S1: A and B: Experimental design: List of lines, treatments, reproductive stages, and tissues analyzed in experiment; Table S2: List of primers used; Table S3: Cadmium concentrations in (A) vegetative and (B) reproductive tissues of 3 rice lines grown in the absence of cadmium; Table S4: Effect of zinc fertilization on cadmium concentration in vegetative tissues; Table S5: Effect of zinc fertilization on cadmium concentration in reproductive tissues; Table S6: Effect of cadmium and zinc fertilization on zinc concentration in vegetative tissues; Table S7: Effect of cadmium and zinc fertilization on zinc concentration in reproductive tissues; Table S8: Mineral concentrations in grains of 3 rice cultivars grown c0z2, c1z2, c0z10, and c1z10; Table S9: Correlations between grain Cd and essential minerals.

**Author Contributions:** R.P.S. conceived and designed the experiments; M.T. performed the experiments and analyzed the data; M.A.G. performed the ICP-OES analysis and contributed to the manuscript; M.T. and R.P.S. wrote the paper. This work is part of the doctoral dissertation work by M.T.: Understanding the influence of zinc on grain cadmium accumulation and bioaccessibility in rice. 2021. Doctoral dissertation, City University of New York. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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