The Interplay between Toxic and Essential Metals for Their Uptake and Translocation Is Likely Governed by DNA Methylation and Histone Deacetylation in Maize

Sarfraz Shafiq 1,2,*, Asim Ali 3, Yasar Sajjad 3, Qudsia Zeb 2, Muhammad Shahzad 2, Abdul Rehman Khan 3○, Rashid Nazir 2 and Emilie Widemann 4○

1 Department of Anatomy and Cell Biology, University of Western Ontario, 1151 Richmond St, London, ON N6A5B8, Canada
2 Department of Environmental Sciences, COMSATS University Islamabad, Abbottabad Campus, University Road, tobe Camp, Abbottabad 22060, Pakistan; zeb_qudsia@yahoo.com (Q.Z.); mshahzad@cuiaud.edu.pk (M.S.); rashidnazir@cuiaud.edu.pk (R.N.)
3 Department of Biotechnology, COMSATS University Islamabad, Abbottabad Campus, University Road, Tobe Camp, Abbottabad 22060, Pakistan; asim21pk@hotmail.com (A.A.); yasarsajjad@cuiaud.edu.pk (Y.S.); arehman@cuiaud.edu.pk (A.R.K.)
4 Department of Biology, University of Western Ontario, 1151 Richmond St, London, ON N6A5B8, Canada; ewidema4@uwo.ca

* Correspondence: sshafiq2@uwo.ca

Received: 26 August 2020; Accepted: 18 September 2020; Published: 22 September 2020

Abstract: The persistent nature of lead (Pb) and cadmium (Cd) in the environment severely affects plant growth and yield. Conversely, plants acquire zinc (Zn) from the soil for their vital physiological and biochemical functions. However, the interplay and coordination between essential and toxic metals for their uptake and translocation and the putative underlying epigenetic mechanisms have not yet been investigated in maize. Here, we report that the presence of Zn facilitates the accumulation and transport of Pb and Cd in the aerial parts of the maize plants. Moreover, the Zn, Pb, and Cd interplay specifically interferes with the uptake and translocation of other divalent metals, such as calcium and magnesium. Zn, Pb, and Cd, individually and in combinations, differentially regulate the expression of DNA methyltransferases, thus alter the DNA methylation levels at the promoter of Zinc-regulated transporters, Iron-regulated transporter-like Protein (ZIP) genes to regulate their expression. Furthermore, the expression of histone deacetylases (HDACs) varies greatly in response to individual and combined metals, and HDACs expression showed a negative correlation with ZIP transporters. Our study highlights the implication of DNA methylation and histone acetylation in regulating the metal stress tolerance dynamics through Zn transporters and warns against the excessive use of Zn fertilizers in metal contaminated soils.

Keywords: phytotoxicity; heavy metals interplay; Zn transporters (ZIP); DNA methylation; histone deacetylases; maize

1. Introduction

Plants are exposed to diverse fluctuating environmental challenges during their development that can affect their fitness, survival and yield [1]. Among these challenges, crop plant exposure to various heavy metals in the soil is one of the leading factors that impairs morphological, physiological, biochemical, and molecular processes in plants, thus contributes to the reduction of yield [1,2]. The impacts of cadmium (Cd), lead (Pb), mercury (Hg), and chromium (Cr) heavy metals on plant
physiology, biochemical processes, and yield have been extensively studied in plants [3–5]. Apart from these heavy metals, plants acquire essential metals such as iron (Fe), zinc (Zn), manganese (Mn), copper (Cu) and nickel (Ni) from the soil for their vital physiological and biochemical functions during their development [6,7]. However, the excess of these essential metals is toxic to the plants. Zn and Fe are essential for plant metabolism and are required in a precise amount for proper development. Zn and Fe deficiency causes the reduction of plant growth, yield, and grain quality in cereals [8–10]. However, the excess of Zn and Fe in plants may cause toxicity to the biological system [8,11]. Therefore, plants have evolved a sophisticated system to balance the uptake, storage, and utilization of these metals.

Essential metals are distributed to different cells and organelles of plants depending on their needed concentration, through a variety of metal transporters [12]. Among them, the Zinc-regulated transporters, Iron-regulated transporter-like Protein (ZIP) family is reported in crops and required for the uptake of Zn [13–15]. However, the ZIP family members may also uptake Fe, Mn, and Cd [13,16,17]. Maize has nine ZIP transporters [17–19]. Maize ZIP transporters are localized in plasma membrane and endoplasmic reticulum, and ZIPs showed differential expression under Zn and Fe treatment [17]. This suggests that maize ZIPs could be functional transporters of Zn and Fe. Therefore, the exploitation of maize ZIPs would provide an excellent tool to maintain the desired amount of essential Zn and Fe in grains, which would be helpful to address the sustainable solution of malnutrition as well as to ensure the desired crop yield.

In response to fluctuating environmental and developmental cues, the gene expression is regulated by chromatin architecture, thus controls various cellular and physiological processes [20]. In this regard, DNA methylation is associated with the gene silencing involved in different biological processes, including flowering time, imprinting, flower and leaf morphogenesis, and fertility [21–23]. Maize putative DNA methyltransferases have shown differential expression in response to polyethylene glycol (PEG) and sodium chloride (NaCl) treatment [24], suggesting the involvement of DNA methylation in response to environmental stress. Pb, Cd, Zn, and Ni have been shown to induce specific DNA methylation changes in wheat, white clover, industrial hemp plants, oil seed rape, and radish [25–28]. However, the effect of heavy metal stress on DNA methylation and the underlying epigenetic mechanism in crop plants, especially in maize, is poorly understood.

DNA methylation cross-talks with histone acetylation for gene expression regulation [29]. Histone acetylation is another chromatin modification, which is associated with transcriptional activation [20]. Histone acetylation homeostasis is achieved through the action of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone deacetylation has been reported to play an important role in plant growth and development, flowering, seed development, and to deal with biotic stress and abiotic stress including salt, cold, and drought stresses [30–32]. Pb, Cd, and Zn alter the gene expression of HATs and HDACs in cotton [33,34], indicating their important role in other crops. Maize HDACs play a role in plant development [35–38]. However, the effect of heavy metals on histone acetylation in maize as well as crop plants, especially the function of maize histone deacetylases in the tolerance of metal stress, has not been studied yet. In addition, the eventual interplay and coordination among Zn, Pb, and Cd for their uptake and/or translocation and the potential involvement and epigenetic regulation of ZIP transporters in this particular interplay has not been investigated yet. We hypothesized that divalent metals (Pb, Cd, and Zn) interfere with each other’s uptake and translocation due to the un-specific nature of diveral Zn transporters. Further, we also hypothesized that heavy metals alter the epigenetic landscape of maize plants, which in return regulates the expression of Zn transporters.

By applying several metals to the maize plants, we found that Zn, Pb, and Cd combinations not only interfere with each other’s accumulation and mobility to aerial parts, but also interfere with the uptake and translocation of other divalent metals (calcium and magnesium). Moreover, our results indicate that the interplay among Zn, Pb, and Cd is regulated by ZIP transporters, which are under the control of DNA methylation and histone acetylation.
2. Results

2.1. Zn Favors the Accumulation and Mobility of Pb/Cd to the Aerial Parts of Maize Plants

As expected, we found that the exposure of plants to Pb, Cd, or Zn leads to an accumulation of the respective metals into the roots, shoots, and leaves (Table 1), showing that each metal is imported into the root and transported toward the aerial parts.

Table 1. Pb, Cd, and Zn levels in roots, shoots, and leaves in response to Pb/Cd/Zn alone or in combinations.

| Treatments | Pb (mg/g) | Cd (mg/g) | Zn (mg/g) |
|------------|-----------|-----------|-----------|
|            | Leaf      | Shoot     | Root      | Total    | Leaf | Shoot | Root | Total | Leaf | Shoot | Root | Total |
| Control    | 0.10 ± c  | 0.08 ± d  | 0.08 ± d  | 0.27 ± c  | 0.01 ± c | 0.01 ± d | 0.01 ± c | 0.03 ± c | 0.10 ± c | 0.03 ± d | 0.08 ± d | 0.22 ± d |
| Pb         | 0.12 ± c  | 1.78 ± b  | 5.62 ± c  | 7.52 ± b  | 0.01 ± c | 0.01 ± d | 0.02 ± c | 0.04 ± c | 0.03 ± d | 0.04 ± d | 0.06 ± d | 0.14 ± d |
| Cd         | 0.11 ± c  | 0.22 ± d  | 0.09 ± d  | 0.42 ± c  | 0.04 ± c | 0.05 ± c | 1.92 ± b | 2.02 ± b | 0.06 ± d | 0.04 ± d | 0.07 ± d | 0.19 ± d |
| Zn         | 0.09 ± c  | 0.06 ± d  | 0.10 ± d  | 0.26 ± c  | 0.03 ± c | 0.01 ± d | 0.02 ± c | 0.06 ± d | 0.67 ± a | 0.92 ± a | 2.20 ± a | 3.80 ± a |
| Pb + Cd    | 0.09 ± c  | 0.09 ± a  | 7.23 ± b  | 7.43 ± b  | 0.05 ± c | 0.02 ± c | 1.68 ± b | 1.75 ± b | 0.03 ± d | 0.03 ± d | 0.11 ± d | 0.18 ± d |
| Pb + Zn    | 0.22 ± b  | 2.76 ± a  | 6.90 ± b  | 9.89 ± a  | 0.04 ± c | 0.03 ± ed | 0.11 ± c | 0.19 ± c | 0.24 ± c | 1.98 ± c | 2.20 ± c | 4.14 ± c |
| Cd + Zn    | 0.13 ± c  | 0.14 ± d  | 0.17 ± d  | 0.99 ± c  | 0.40 ± b  | 0.30 ± a | 3.07 ± a | 3.78 ± a | 0.20 ± bc | 0.30 ± c | 2.06 ± b | 2.57 ± b |
| Pb + Cd + Zn | 1.10 ± a | 0.77 ± c  | 8.60 ± a  | 10.49 ± a | 0.47 ± a  | 0.22 ± b | 2.88 ± a | 3.58 ± a | 0.25 ± b | 0.41 ± b | 2.11 ± b | 2.78 ± b |

Plants were grown in hydroponic culture, and metal accumulation was investigated after two weeks of treatment. The results shown are the average of three biological replicates. The values marked with different letters are statistically different (p ≤ 0.05), while the values marked with the same letters do not differ significantly. The statistical analyses were performed to examine the changes in each plant fraction i.e., shoots, roots, or leaves with applied treatments. Statistical analyses between different plant fractions are not given in the table.

Interestingly, at the same concentration, plants preferentially accumulate Pb rather than Cd and Zn compared with control. Among Pb, Cd, and Zn treatments, only Zn concentration was significantly higher in leaves and shoots compared with control. This result indicates that Zn is more mobile to aerial parts of the maize plant than Pb and Cd (Table 1). Contrary to Zn, accumulated Cd was mainly blocked in roots, while accumulated Pb was transported in shoots as well. These observations were validated by calculating the mobility index (root to shoot and root to leaf). The mobility index classifies these tested metals as Zn > Pb > Cd, where Zn is the most mobile among the three metals (Figure 1). This indicates that, at the same concentration, each metal presents different accumulation and mobility to aerial parts.

We further analyzed if the combination of metals alters their accumulation or their transport throughout the plant. Therefore, we checked the metal accumulation in the different parts of the seedling after combined treatments, e.g., Pb + Cd, Pb + Zn, Cd + Zn, and Pb + Cd + Zn. To our surprise, the combinations of Pb with Zn or with both Zn and Cd, enhanced the total Pb accumulation in plants compared with the single Pb treatment (Table 1). Moreover, the addition of Zn enhanced the Pb transport to shoots and leaves compared with Pb treatment alone (Table 1). We also found that total Cd accumulation and transport to shoots and leaves was also enhanced in the presence of Zn. The increased mobility index of Pb/Cd after the addition of Zn further supports our observations (Figure 1). Contrary to Zn, the addition of Cd mainly blocked the Pb in the roots (Table 1 and Figure 1). Together, these results indicate that Zn favors the mobility of Pb and Cd to the aerial parts of the plants.

2.2. Pb/Cd Block the Accumulation and Transport of Zn to the Aerial Parts of Maize Plants

We then investigated whether the total accumulation and mobility of Zn are altered with the addition of Cd and Pb (Table 1 and Figure 1). We found that the total accumulation as well as the transport of Zn to shoots and leaves were decreased with the addition of Pb and/or Cd compared with the individual Zn treatment. Notably, Cd only facilitates the mobility of Pb in the presence of Zn but not alone. In brief, our results unravel the dynamic interaction of Pb, Cd, and Zn for their accumulation and transport to aerial parts.
The results shown are the averages of three biological replicates. The values marked with different letters are statistically different (p ≤ 0.05), while the values marked with the same letters do not differ significantly.

2.3. Zn, Pb, and Cd Influence the Mobility of Divalent Calcium (Ca) and Magnesium (Mg) But Not the Monovalent Potassium (K)

We next investigated whether Pb, Cd, and Zn dynamics can influence the accumulation of these other divalent metals as well as their transport to aerial parts (Table 2). Total Ca and Mg accumulation was increased in the presence of Zn compared with control. However, in the presence of Cd, total Ca was decreased, while Mg was increased. The transport of Ca and Mg to aerial parts was differently affected in response to Pb, Cd, and Zn. For example, Ca and Mg were mainly blocked in the roots in the presence of Cd, whereas Mg was transported to shoots and leaves in the presence of Pb.

### Table 2. Calcium and magnesium levels in roots, shoots, and leaves in response to Pb/Cd/Zn alone or in combinations.

| Treatments | Ca (mg/g) | Mg (mg/g) |
|------------|-----------|-----------|
|            | Leaf | Shoot | Root | Total | Leaf | Shoot | Root | Total |
| Control    | 20.76 | 17.08 | 25.45 | 63.29 | 5.175 | 3.22 | 2.55 | 10.96 |
| Pb         | 21.78 | 27.59 | 10.68 | 60.06 | 3.47 | 5.53 | 3.57 | 12.57 |
| Cd         | 12.03 | 8.99  | 20.60 | 41.72 | 4.301 | 6.07 | 5.46 | 15.84 |
| Zn         | 26.29 | 26.29 | 39.34 | 91.93 | 4.98 | 4.82 | 5.67 | 15.48 |
| Pb + Cd    | 15.20 | 9.28  | 21.89 | 46.38 | 3.78 | 2.45 | 4.25 | 10.04 |
| Pb + Zn    | 36.52 | 26.90 | 25.77 | 89.21 | 5.56 | 4.03 | 4.90 | 14.51 |
| Cd + Zn    | 21.05 | 29.07 | 19.24 | 69.37 | 4.54 | 5.40 | 5.47 | 15.42 |
| Pb + Cd + Zn | 26.62 | 27.86 | 16.32 | 70.81 | 5.56 | 5.46 | 5.98 | 17.01 |

Plants were grown in hydroponic culture, and metal accumulation was investigated after two weeks of treatment. The results shown are the averages of three biological replicates. The values marked with different letters are statistically different (p ≤ 0.05), while the values marked with the same letters do not differ significantly. The statistical analyses were performed to examine the changes in each plant fraction i.e., shoots, roots, or leaves with applied treatments. Statistical analyses between different plant fractions are not given in the tables.
We further analyzed if the combination of Pb, Cd, and Zn alters the accumulation of Ca and Mg as well as their transport in aerial parts of the plants. We found that the levels of Ca and Mg in shoots and leaves were different between the Pb/Cd/Zn individual treatments and their combinations (Pb + Cd, Pb + Zn, Cd + Zn, and Pb + Cd + Zn). For example, in the presence of Pb + Zn, Ca and Mg accumulation in leaves was further increased compared with Zn treatment alone. These results show that Pb, Cd, and Zn dynamics influence the Ca and Mg mobility to aerial parts of the plants. However, we found that K levels did not change in response to Pb, Cd, and Zn treatments (Table S1), suggesting that Pb, Cd, and Zn dynamics are specific to divalent metals.

2.4. Antioxidant Activity Is Altered in Response to Zn, Pb, and Cd Applied Alone and in Combinations

Metal stress has been reported to alter the activity of Peroxidase (POD), Superoxide dismutase (SOD), and Catalase (CAT) antioxidant enzymes [39,40]. We then investigated whether the combinations of Zn, Pb, and Cd interfere with the POD, SOD, and CAT levels (Figure 2). The results show that POD and SOD activities were enhanced in response to Cd and Pb alone, while CAT activity was reduced compared with control. However, POD, SOD, and CAT activities did not change in response to Zn compared with control. POD and SOD levels were higher in response to the treatments with Pb + Cd and Zn + Pb + Cd compared with control. However, CAT level was decreased in response to Zn + Pb + Cd compared with control. Moreover, the addition of Zn with Pb or Cd resulted in a slight decrease of POD and SOD activities compared with individual Pb or Cd, while the levels of CAT were similar to individual Pb or Cd. Together, upon the combination of metals, the antioxidants activities were somehow similar to the individual metals.

![Figure 2](image_url) 

**Figure 2.** The activities of Peroxidase (POD), Superoxide dismutase (SOD), and Catalase (CAT) do not differ much between Pb/Cd/Zn applied alone and in combinations. The results shown are the average of three biological replicates. Bars represent mean ± SD. The values marked with different letters are statistically different \((p < 0.05)\), while the values marked with the same letters do not differ significantly.

2.5. Pb, Cd, and Zn Alone and in Combinations Differentially Regulate the Expression of ZIP Transporters

The increased concentration of Pb/Cd in shoots and leaves with the addition of Zn led us to hypothesize that ZIP transporters may play a role in the transport of Pb/Cd to aerial parts of plants. We therefore investigated the expression of ZIP transporters in response to Zn, Pb, and Cd applied alone and in combinations. The results show that ZIP transporters present a differential expression in response to different metals, applied alone and in combinations (Figure 3). In response to Zn, *Iron-Regulated Transporter 1 (IRT1)* expression was increased, whereas the expression of other ZIP transporters was decreased compared with control. In response to Pb treatment, the expression of *IRT1*,
ZIP1, and ZIP6 was increased compared with control, whereas the expression of ZIP2, ZIP3, and ZIP4 was decreased compared with control. In response to Cd, the expression of IRT1, ZIP2, and ZIP8 was increased compared with control, whereas the expression of other ZIP transporters was decreased compared with control. The expression of some genes was either increased or decreased in response to Pb, Cd, and Zn, but their levels were significantly different in many cases, e.g., IRT1 expression was highest in response to Pb; ZIP2 and ZIP8 expressions were highest in response to Cd; and ZIP1 and other ZIP gene expression was lower in response to Cd than that of Zn.

**Figure 3.** The expression of ZIP transporters in response to Pb/Cd/Zn applied alone and in combinations. (A) The expression of ZIP transporters was normalized with ZmUBQ5. The data presented are the averages of three biological replicates. Bars represent mean ± SD. The values marked with different letters are statistically different (p ≤ 0.05), while the values marked with the same letters do not differ significantly. (B) Multiple linear regression (MLR) equations for uptake of each heavy metal and expression of ZIP transporters. (C) Cumulative percentage contribution (CPC) for the expression of each ZIP transporter in response to the uptake of heavy metals.
We next investigated whether the expression of ZIPs is altered when Pb, Cd, and Zn were combined. To our surprise, we found that the dynamics of ZIPs expression were changed in response to Pb + Cd, Pb + Zn, Cd + Zn, and Pb + Cd + Zn. In the case of Zn + Pb, the expression of IRT1 and ZIP1 was increased compared with control, but still lower than that of Pb alone. Furthermore, the expression of ZIP6 did not change compared with control, while the expression of ZIP2, ZIP3, ZIP4, ZIP5, and ZIP7 was similar to that of Zn alone. These results indicate that Zn encountered the increased expression of IRT1, ZIP1, and ZIP6 compared with Pb alone. In the case of Cd + Zn, similar to Cd, the expression of IRT1, ZIP2, and ZIP8 was increased compared with control, whereas the expression of other ZIPs was decreased. However, increased expression of ZIP2 and ZIP8 was lower than that of Cd alone, suggesting that Zn antagonizes the increased expression of ZIP2 and ZIP8 from Cd. In addition, the expression of other ZIP genes was at the similar level than that of Cd and/or Zn alone. In the case of Pb + Cd, the expression of ZIP2 and ZIP8 was increased compared with control, but their expression was lower than that of Cd treatment, suggesting that Pb antagonized the expression of ZIP2 and ZIP8 compared with Cd. However, the expression of other ZIP transporters was similar to that of Cd in Pb + Cd treatment. Furthermore, we combined all three metals and found that, similar to Cd and Zn + Cd, the expression of IRT1, ZIP2, and ZIP8 was increased compared with control but still was lower than that of Cd, whereas the expression of other ZIPs was decreased to levels similar to Pb, Cd, and/or Zn levels. Together, these results show that, in addition to Zn, the expression of ZIP transporters is also regulated by Pb and Cd. Furthermore, metal combinations differently regulate the expression of IRT1, ZIP1, ZIP2, ZIP6, and ZIP8 compared with individual metals.

The Multiple Linear Regressions (MLR) method was applied to find the linear relationship between explanatory variables (heavy metals uptake) and response variables (ZIP expression). The R² value for the MLR equations between Pb, Cd, and Zn uptake models and ZIPs expression were 0.82, 0.80, and 0.95, respectively. This indicates a good fit of the model for all cases (Figure 3B). The highest percentage contribution in Pb uptake was with ZIP5 and ZIP6 (23%); for Cd with ZIP4 (36%); and for Zn with ZIP6 (26%) (Figure 3C). This further suggests the specific regulation of ZIP transporters in response to Pb and Cd.

2.6. Pb, Cd, and Zn Alone and in Combinations Differentially Regulate the Expression of Histone Deacetylases (HDACs)

Because heavy metals were reported to cause DNA hypo-acetylation in human [19], we hypothesized that HDACs could also play a role in plant adaptation under these metal stresses. Therefore, we checked the expression of all HDACs in maize in response to Pb, Cd, or Zn applied alone and in combination (Figure 4). We found that all HDACs respond differently to each metal. In response to Zn, the expression of HDAC102, HD2b, HD2c, and HDAC106 was decreased compared with control, whereas the expression of HD2a was increased. In response to Pb, the expression of all HDACs was downregulated compared with control. On the contrary, in response to Cd, the expression of all the HDACs was increased compared with control and the increase in expression of HD2a was even more than that of Zn alone. These results indicate the different regulation of HDACs in response to Pb, Cd, and Zn, as well as suggest different acetylation levels. For example, Pb and Cd could result in histone hyperacetylation and histone hypoacetylation, respectively.
HD2a was even more than that of Zn alone. These results indicate the different regulation of HDACs in response to Pb, Cd, and Zn, as well as suggest different acetylation levels. For example, Pb and Cd could result in histone hyperacetylation and histone hypoacetylation, respectively.

We next investigated the expression of HDACs in response to the combination of Pb, Cd, and Zn (Figure 4). In response to Cd + Zn, the expression of HDA102, HDA110, HD2a, HD2b, HD2c, and HDA106 was increased compared with control, while their expression was still lower than that of Cd alone. In response to Cd + Pb, the expression of HD1b, HDA102, HDA110, HD2a, HD2c, and HDA106 was increased compared with control but still lower than that of Cd alone. This indicates that Zn and Pb antagonize the Cd-mediated increase in the expression of all HDACs. In response to Pb + Zn, the expression of HDA102, HD2b, HD2c, and HDA106 decreased compared with control, to similar levels as for Pb or Zn alone. However, we did not detect the expression of the histone deacetylases RPD3, HDA1, and HDA108 in maize roots treated with Pb, Cd, and Zn, either alone or in combination.

We next applied the MLR equation on heavy metal uptake and HDACs expression. The $R^2$ value for the MLR equations between Pb, Cd, and Zn uptake models and HDACs expression were 0.55, 0.94, and 0.78, respectively. This indicates a good fit of the model for all cases (Figure 4B). The highest percentage contribution in Pb uptake was with HDA102 (39%); for Cd with HDA102 (27%); and for Zn with HDA106 (47%) (Figure 4C). This further suggests the specific regulation of HDACs in response to Pb, Cd, and Zn.

Figure 4. The expression of histone deacetylases in response to Pb/Cd/Zn applied alone and in combinations. (A) The expression of histone deacetylases was normalized with ZmUBQ5. The data presented are the averages of three biological replicates. Bars represent mean ± SD. The values marked with different letters are statistically different ($p \leq 0.05$), while the values marked with the same letters do not differ significantly. (B) Multiple linear regression (MLR) equations for uptake of each heavy metal and expression of HDACs. (C) Cumulative percentage contribution (CPC) for expression of each HDAC transporter in response to the uptake of heavy metals.
We next applied the MLR equation on heavy metal uptake and HDACs expression. The $R^2$ value for the MLR equations between Pb, Cd, and Zn uptake models and HDACs expression were 0.55, 0.94, and 0.78, respectively. This indicates a good fit of the model for all cases (Figure 4B). The highest percentage contribution in Pb uptake was with HDA102 (39%); for Cd with HDA102 (27%); and for Zn with HDA106 (47%) (Figure 4C). This further suggests the specific regulation of HDACs in response to Pb, Cd, and Zn.

2.7. Pb, Cd, and Zn Alone and in Combinations Differentially Regulate the Expression of DNA Methyltransferases

Pb, Cd, and Zn phytotoxicity has been shown to alter the DNA methylation levels at metal transporters to confer the metal tolerance in wheat [28]. We hypothesized that Pb, Cd, and Zn alone and in combinations could alter the DNA methylation levels at the promoter of ZIP transporters through the regulation of DNA methyltransferases in maize. Therefore, we checked the expression of maize DNA methyltransferases [24]. The results show that in response to Zn the expression of *Methyltransferase 1* (*MET1*), *MET2a*, and *MET2b* was decreased compared with control, whereas the expression of *MET3a* and *MET3c* was increased (Figure 5). In response to Pb, the expression of *MET1*, *MET2b* and *MET4* was downregulated compared with control, whereas the expression of *MET2a* and *MET3b* was increased. Contrary to Pb and Zn, the expression of all the DNA methyltransferases was increased in response to Cd. Although the expression of DNA methyltransferases was increased with Pb/Cd/Zn, their levels were still significantly different among Pb, Cd, and Zn. For example, *MET2a* and *MET3b* expression levels were the highest in response to Cd compared with Pb. In response to Cd, the expression of *MET3a* and *MET3c* was more than that of Zn, which indicates the different regulation of DNA methyltransferases depending on the metal stress.

We also evaluated the expression of DNA methyltransferases in combination of metals (Figure 5). In response to Cd + Zn, the expression of all the methyltransferases was increased compared with control. However, the expression of *MET1*, *MET2b*, and *MET4* was lower than that of Cd, and the expression of *MET2a*, *MET3a* and *MET3b* was even higher than that of Cd. In response to Pb + Cd, the expression of all the DNA methyltransferases was higher than that of control, except for *MET2a*. Furthermore, the expression of *MET1*, *MET3a*, and *MET3b* was higher than that of Cd and the expression of *MET2b* and *MET3c* was lower than that of Cd. However, the expression of *MET4* was similar to Cd. In response to Pb + Zn, *MET3c* showed a strong increase in the expression compared with control and Pb/Zn. In response to Pb + Cd + Zn, the expression of all DNA methyltransferases was increased compared with control but the expression was either similar to Zn + Cd or Cd alone. Interestingly, the expression of *MET3a* was higher than that of Cd or Zn + Cd. Together, the expression levels of DNA methyltransferases are different among Pb, Cd, and Zn individual treatments and their combinations.

We next applied the MLR equation on heavy metal uptake and DNA methyltransferases expression. The $R^2$ value for the MLR equations between Pb, Cd, and Zn uptake models and *METs* expression were 0.57, 0.97, and 0.84, respectively. This indicates a good fit of the model for all cases (Figure 5B). The highest percentage contribution in Pb uptake was with *Met2b* (32%); for Cd with *Met4* (29%); and for Zn with *Met1* (29%) (Figure 5C). This further suggests the specific regulation of DNA methyltransferases in response to Pb, Cd, and Zn.
Figure 5. The expression of DNA methyltransferases in response to Pb/Cd/Zn applied alone and in combinations. (A) The expression of DNA methyltransferases was normalized with ZmUBQ5. The data presented are the averages of three biological replicates. Bars represent mean ± SD. The values marked with different letters are statistically different (p ≤ 0.05), while the values marked with the same letters do not differ significantly. (B) Multiple linear regression (MLR) equations for uptake of each heavy metal and expression of DNA methyltransferases. (C) Cumulative percentage contribution (CPC) for expression of each DNA methyltransferase in response to the uptake of heavy metals.

2.8. Zn, Pb, and Cd Combinations Alter the DNA Methylation Levels at the Promoter of ZIP Transporters

Because Pb, Cd, and Zn alone and in combination differently regulate the expression of DNA methyltransferases (Figure 5), we therefore investigated the DNA methylation levels at the promoter of ZIP transporters. We employed Chop-PCR to detect the non-methylated DNA levels at the promoter of some selected candidates, i.e., IRT1, ZIP1, ZIP2, ZIP6, and ZIP8 (Figure 6 and Figure S1). We used these ZIP transporters because their gene expression was more dynamic in response to the combination of Pb, Cd, and Zn (Figure 3). Consistent with the upregulation of all the DNA methyltransferases in
response to Cd, DNA methylation levels were increased at IRT1, ZIP1, ZIP2, and ZIP6 compared with the control (Figure 6). However, DNA methylation at ZIP8 was decreased compared to the control in response to Cd. In response to Pb, DNA methylation was increased at ZIP2 and ZIP8 compared to control. In response to Zn, DNA methylation levels were also increased at IRT1, ZIP1, ZIP2, and ZIP6 compared to control, but decreased at ZIP8. These results indicate that each metal leads to distinct DNA methylation levels at specific loci.

![Figure 6. DNA methylation levels at the promoter of selected ZIP transporters in response to Pb/Cd/Zn applied alone and in combinations. DNA was digested with McrBc and equal amounts of digested or undigested DNA were used as template for PCR. McrBc digests the methylated DNA; therefore, lighter band intensity reflects higher DNA methylation level.](image)

In general, the expression of all the DNA methyltransferases was increased in response to the combination of metals compared with Cd alone, e.g., Pb + Cd, Cd + Zn, and Pb + Cd + Zn (Figure 5). Consistent with this observation, in response to Pb + Cd, the methylation levels were further increased at IRT1, ZIP1, ZIP2, and ZIP6 compared to Cd, but decreased at ZIP8 (Figure 6). In response to Cd + Zn and Pb + Cd + Zn, DNA methylation levels were also increased at IRT1, ZIP1, and ZIP6 compared with control as well as Zn and Pb. These results indicate that Pb, Cd, and Zn interaction further alters the DNA methylation levels at specific loci compared with individual metals.

Pearson’s correlation coefficient ($r$) is a measure of the strength of the association between two variables. We therefore calculated the Pearson correlation between the expression of ZIPS and the expression of HDACs/DNA methyltransferases (Figure 7). The expression of IRT1, ZIP1, ZIP3, ZIP5, ZIP6, and ZIP7 showed negative correlation with the expression of HDACs and DNA methyltransferases. These results suggest that HDACs and DNA methyltransferases together negatively regulate the expression of some ZIPS. Furthermore, the expression of ZIP2 and ZIP8 showed a positive correlation with the expression of HDACs and DNA methyltransferases, suggesting that the regulation of ZIP2 and ZIP8 could be independent of HDACs and DNA methyltransferases.

**Int. J. Mol. Sci. 2020, 21, x FOR PEER REVIEW**

...methyltransferases in response to Cd, DNA methylation levels were increased at IRT1, ZIP1, ZIP2, and ZIP6 compared with the control (Figure 6). However, DNA methylation at ZIP8 was decreased compared to the control in response to Cd. In response to Pb, DNA methylation was increased at ZIP2 and ZIP8 compared to control. In response to Zn, DNA methylation levels were also increased at IRT1, ZIP1, ZIP2, and ZIP6 compared to control, but decreased at ZIP8. These results indicate that each metal leads to distinct DNA methylation levels at specific loci.

**Figure 6.** DNA methylation levels at the promoter of selected ZIP transporters in response to Pb/Cd/Zn applied alone and in combinations. DNA was digested with McrBc and equal amounts of digested or undigested DNA were used as template for PCR. McrBc digests the methylated DNA; therefore, lighter band intensity reflects higher DNA methylation level.

In general, the expression of all the DNA methyltransferases was increased in response to the combination of metals compared with Cd alone, e.g., Pb + Cd, Cd + Zn, and Pb + Cd + Zn (Figure 5). Consistent with this observation, in response to Pb + Cd, the methylation levels were further increased at IRT1, ZIP1, ZIP2, and ZIP6 compared to Cd, but decreased at ZIP8 (Figure 6). In response to Cd + Zn and Pb + Cd + Zn, DNA methylation levels were also increased at IRT1, ZIP1, and ZIP6 compared with control as well as Zn and Pb. These results indicate that Pb, Cd, and Zn interaction further alters the DNA methylation levels at specific loci compared with individual metals.

Pearson’s correlation coefficient ($r$) is a measure of the strength of the association between two variables. We therefore calculated the Pearson correlation between the expression of ZIPS and the expression of HDACs/DNA methyltransferases (Figure 7). The expression of IRT1, ZIP1, ZIP3, ZIP5, ZIP6, and ZIP7 showed negative correlation with the expression of HDACs and DNA methyltransferases. These results suggest that HDACs and DNA methyltransferases together negatively regulate the expression of some ZIPS. Furthermore, the expression of ZIP2 and ZIP8 showed a positive correlation with the expression of HDACs and DNA methyltransferases, suggesting that the regulation of ZIP2 and ZIP8 could be independent of HDACs and DNA methyltransferases.
3. Discussion

In response to fluctuating environment, chromatin landscape is achieved through post-transcriptional histone modifications to regulate the gene expression. Here, we studied the dynamic metal interactions among Zn, Pb, and Cd in maize and found that the presence of Zn facilitates the accumulation and transport of Pb and Cd in the aerial parts of the maize plants. Furthermore, we showed that Pb, Cd, and Zn alone and in combinations alter the expression of DNA methyltransferases, which consequently change the DNA methylation levels at the promoter of some ZIP transporters to regulate their expression.

Arabidopsis MET1 (mammalian DNA Methyltransferase 1 (Dnmt1)) and plant specific Chromomethylases (CMTs) are reported to maintain the CG and CHG methylation, respectively [41,42]. Moreover, Arabidopsis Domains Rearranged Methylases (DRMs) (mammalian Dnmt3) are reported to establish all the methylation contexts, especially CHH methylation [43]. ZmMET1 is the homolog of MET1 and Dnmt1; ZmMET2a and ZmMET2b are the homologs of plant specific Arabidopsis CMTs; and ZmMET3a, ZmMET3b, and ZmMET3c are homologs of Arabidopsis DRMs.
and ZmMET3a, ZmMET3b, and ZmMET3c are homologs of Arabidopsis DRM and mammalian Dnmt3 de novo DNA methyltransferases. Our results show that Pb, Cd, and Zn alone and in combination regulate the expression of maize DNA methyltransferases. Furthermore, MLR analysis between the expression of DNA methyltransferases and uptake of Pb/Cd/Zn showed that the highest percentage of expected contribution in Pb uptake was with Met2b and Met1 (32% and 22%, respectively); for Cd with Met4 and Met3a (29% and 26%, respectively); and for Zn with Met1 and Met3c (29% and 24%, respectively). This indicates the specific regulation of each DNA methyltransferase in response to Pb, Cd, and Zn. It also indicates that one DNA methyltransferase could be regulated by more than one metal. In response to metal stress, changes in DNA methylation have been reported in wheat, Vicia faba, rape seedlings, white clover, industrial hemp hyper-accumulators plants, and Arabidopsis [25,26,28,44–46]. Interestingly, DNA methylation changes in response to Cd stress depend on the plants, e.g., DNA hypomethylation in rape seedling, white clover and industrial hemp [25,26], while DNA hypermethylation in Vicia faba [46]. Our data show that the expression of all DNA methyltransferases is increased in response to Cd treatment, suggesting DNA hypermethylation in response to Cd in maize. Indeed, DNA methylation levels at IRT1, ZIP1, ZIP2, and ZIP6 confirm the DNA hypermethylation at these loci in response to Cd. Furthermore, DNA methylation levels were also slightly increased in response to Zn or Pb at specific ZIP transporters. These results indicate the specific DNA methylation changes in response to Pb, Cd, and Zn. Notably, we also observed further increase in DNA methylation levels at ZIP transporters in response to the combination of metals, e.g., DNA methylation levels at IRT1, ZIP1, ZIP2, and ZIP6 in response to Pb + Cd. This indicates that the combination of metals could produce different DNA methylation levels compared with individual metals. However, further studies are required to investigate the genome wide DNA methylation status as well as targets of DNA methyltransferases in response to metals applied individually or in combination.

Similar to DNA methylation, the expression of all HDACs also responds differently to the individual metals as well as the combination of metals. Furthermore, MLR analysis also suggests the specific regulation of HDACs in response to Pb, Cd, and Zn uptake. These observations suggest that Pb, Cd, and Zn could cause different histone acetylation profiles. Interestingly, our Pearson correlation analysis showed a negative correlation between the expression of HDACs/DNA methyltransferases and the expression of IRT1, ZIP1, ZIP3, ZIP5, ZIP6, and ZIP7. This suggests that altered DNA methylation and histone acetylation levels, alone or together, could regulate the ZIPs gene expression. However, the expression of ZIP2 and ZIP8 showed a positive correlation with the expression of HDACs and DNA methyltransferases, suggesting that the regulation of these ZIP transporters could be independent of HDACs and DNA methyltransferases. However, further studies are required to validate the crosstalk between histone acetylation and DNA methylation to regulate the ZIPs gene expression.

Our results also show an interesting plant preference of metal accumulation and mobility to different parts of the plants. To our surprise, the addition of Zn facilitates the accumulation of Pb and Cd to the aerial parts of the plants. Furthermore, Pb, Cd, and Zn dynamics seem specific to divalent metals, as K levels did not change after treatments. There are two possible explanations for this phenomenon. Firstly, Pb and Cd could be potentially co-transported with Zn. Secondly, Pb and Cd could allosterically regulate the ability of some ZIP transporters to transport Pb and Cd. Notably, we also observed that Pb and Cd also regulate the expression of ZIP transporters, e.g., IRT1, ZIP1, and ZIP6 expression increased in response to Pb, while the expression of ZIP2, ZIP3, and ZIP4 decreased compared with control. Furthermore, MLR analysis also suggests that Pb, Cd, and Zn specifically regulate the expression of certain ZIP transporters. Interestingly, the ZIP family has been reported to uptake Fe, Mn, and Cd [13,16,17,47] in other plants, questioning the specificity of ZIP metal transporters. Moreover, Arabidopsis IRT1, a ZIP transporter, has also been reported to transport multiple metals, including Fe, Zn, Mn, and Cd [48,49]. These observations suggest that maize ZIP transporters may also transport Pb/Cd. Therefore; we propose that heavy metals alter the DNA methylation and histone acetylation levels through the activity of DNA methyltransferases and histone
deacetylases (Figure 8). The resulting chromatin landscape may control the expression of certain ZIP transporters that may carry Pb and Cd along with Zn into the cell. Furthermore, the accumulation of Pb and/or Cd will disturb the cellular homeostasis of other essential divalent metals. Consequently, maize plant tries to antagonize the metal toxicity through the activity of SOD, POD, and CAT in response to Pb, Cd, and Zn. However, upon the combination of metals, the antioxidants activities were somehow similar to the individual metals. This suggests that maximum activity of CAT, SOD, and POD was already achieved in response to individual metals. Therefore, the combination of metals would not further elevate their response. Eventually, the excess of these toxic metals might then be loaded to xylem, and transported to aerial parts through the activity of multiple transporters, for example Heavy Metal ATPases (HMAs), Yellow Stripe-Likes (YSLs), etc. [50–52]. In this scenario, achieving the specificity of ZIP metal transporters that specifically uptake Zn and block Pb and Cd is desired and need further investigations. Zn is being applied as a fertilizer. In the light of our results, if the agriculture land is already polluted with divalent metals, the application of Zn could make toxic metals more mobile to aerial parts of plants. Furthermore, the presence of Pb/Cd in the soil can block the Zn accumulation and transport to aerial parts of the plants. However, how plant imports preferentially one metal over the other metals, and how the metal combinations change metal accumulation and mobility dynamic need further investigations.

![Proposed model for the uptake and translocation interplay of Pb, Cd, and Zn through ZIP transporters.](image)

**Figure 8.** Proposed model for the uptake and translocation interplay of Pb, Cd, and Zn through ZIP transporters. Heavy metals may alter the DNA methylation and histone acetylation levels through the activity of DNA methyltransferases and histone deacetylases, respectively. The resulting chromatin landscape may control the expression of certain ZIP transporters that may carry Pb and Cd along with Zn into the cell. Consequently, other metal transporters may facilitate the loading of toxic metals to xylem, and subsequent transport to shoots that result in the increased concentration of toxic metals in aerial parts of the plants. Moreover, the enrichment of toxic metals into the cell disturbs the uptake and translocation of other essential divalent metals, e.g., Ca/Mg. The gradient represents the metal concentration, and cylindrical structures represent the metal transporters that can load the metals to the xylem.

4. Materials and Methods

4.1. Hydroponics

Maize seeds (*Zea Mays* L. cv. NK-8441 Syngenta) were placed on a filter paper humidified with 0.5 mM solution of CaSO₄ and placed in incubator at 28 °C for 48 h to germinate. After germination,
two-day-old young seedlings of similar size were transferred to hydroponics medium containing 0.2 mM KH$_{2}$PO$_{4}$, 1 mM K$_2$SO$_4$, 2 mM Ca(NO$_3$)$_2$, 2 mM CaCl$_2$, 0.5 mM MgSO$_4$. 7H$_2$O, 5 µM H$_3$BO$_3$, 2 µM MnSO$_4$, 0.5 µM ZnSO$_4$, 0.3 µM CuSO$_4$, and 0.01 µM (NH$_4$)$_2$Mo$_7$O$_{24}$. Hydroponics medium was changed twice a week. The heavy metal treatment was applied at three leaf stage by adding Cd (100 µM), Pb (100 µM), and Zn (100 µM) alone or in combination using completely randomized design (CRD). Cd, Pb, and Zn were added as CdCl$_2$, Pb(NO$_3$)$_2$, and ZnSO$_4$, respectively, and their concentrations were selected based on previously published reports [53–55]. The seedlings were grown in a greenhouse under an average day/night temperature of 28/18 °C, a photoperiod of 14 h light (≥350 µmol m$^{-2}$ s$^{-1}$ PAR)/10 h dark, and a relative humidity of 65% ± 5%.

4.2. Atomic Absorption Analysis

Two weeks after treatment with Pb, Cd, or Zn, freshly harvested maize plants were washed with double distilled water (distilled water passed through Millipore filters) to remove any soil particles and air-borne pollutants. The plants were divided into roots, shoots, and the whole 4th leaf for atomic absorption analysis. After taking their fresh weight, the samples were placed in an oven at 60 °C for 72 h until a constant dry weight was attained using weight balance (Panther-USA, model-BM-32). Plant materials were ground (<0.2 mm) and about 150 mg of dried plant material were used for the analysis of Pb, Cd, and Zn. The material was burned into ashes at 550 °C for 5 h in the furnace and digested with 2 mL of 4 M HNO$_3$ for 3 h. Subsequently, double distilled water (∼8 mL) was used to make a final volume of 10 mL. The digested plant material from different parts of the plants was filtered (i.e., using Whatman filter paper No. 21) and analyzed for Pb, Cd, and Zn concentrations through the atomic absorption spectrophotometer (Perkin Elmer A Analyst 700), as suggested by A.O.C.A (association of official analytical chemist) protocol [56]. The instrument was calibrated with calibration blank and three series of working standard solutions of studied metals. Atomic absorption spectroscopy standards of Merck for each analysed metal were used in this study.

4.3. Extraction and Quantification of Antioxidant Enzymes

Green leaf samples were harvested from plants and quickly immersed in liquid nitrogen. The frozen samples (0.5 g) were homogenized in freshly prepared potassium phosphate buffer (100 mM potassium phosphate pH 7 and 0.1 mM EDTA). After centrifugation at 4 °C and 13,000×g for 5 min, the supernatant was collected in eppendorf tubes and used for the analysis of antioxidant enzymes. Catalase, peroxidase and superoxide dismutase activities were determined as described previously [57]. The CAT activity was determined by monitoring the decomposition of H$_2$O$_2$ at 240 nm through spectrophotometer (U2020 IRMECO, Germany), while the activity of POD was measured by using guaiacol as substrate. The reaction mixture contained 0.1 M phosphate buffer of pH 7, 1% guaiacol, 0.4 M H$_2$O$_2$, and the enzyme extract. Change in absorbance per unit time was measured at 470 nm. SOD activity was measured by photoreduction of nitroblue tetrazolium (NBT). Reaction mixture comprised 50 mM phosphate buffer of pH 7.8, 0.1 mM EDTA, 20 mM L-methionine, 750 µM NBT, 20 µM riboflavin, and the enzyme extract. The amount of protein was also determined according to the protocol of Lowry [58].

4.4. Gene Expression Analysis

After 48 h of treatment with Pb, Cd, and Zn applied alone and in combinations, total RNA from roots was extracted by using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. DNase I treatment was performed with DNase (Takara, Shiga, Japan), and reverse transcription was performed with Superscript III (Invitrogen, Carlsbad, CA, USA) using the gene-specific primers as described previously [59]. RT-qPCR was performed with the gene-specific primers using SYBR green Master Mix (Roche, Basel, Switzerland) as described in [59]. Maize Ubiquitin 5 (ZmUBQ5) was used as an internal reference gene to normalize the data. Primer sequences for qRT-PCR are listed in Table S2.
4.5. ChoP-PCR

Chop-PCR was performed as previously described [60]. Briefly, genomic DNA was extracted with CTAB from roots after 48 h of treatment with Pb, Cd, and Zn applied alone and in combinations. Then, the DNA was digested with a methylation-sensitive restriction enzyme (McrBc). Equal amounts of digested and undigested DNA were used as templates for 32 cycles of PCR amplification, followed by agarose gel electrophoresis and ethidium bromide staining. Chop-PCR primers are listed in Table S2.

4.6. Statistical Analysis

Each treatment was applied in three biological replicates and there were three technical replicates in each biological replicate regarding the biochemical analysis. Data of means from each biological replicates were subjected to the General Linear Model (GLM) to know the effect of treatments on each studied parameter. Furthermore, after doing analysis of variance (ANOVA), post hoc multiple comparison (least significant difference test) was performed to compare and rank the treatments means value by using alphabets. Statistical analyses were performed at significance level of \( p \leq 0.05 \). In the next step, correlation between studied parameters was found by using Pearson Correlation. Multiple linear regressions (MLR) analysis was constructed between dependent variables (heavy metal uptake) and predictors (ZIP expression, DNA methyltransferases and HDACs expression). The MLR equation was used as described below [61]

\[
Y = b_0 + b_1x_1 + b_2x_2 + \ldots + b_{x_{th}}x_{x_{th}}
\]  

(1)

\( Y \) is the predicted value of the dependent variable (heavy metal uptake), \( b_0 \) is the value of multiple regression constant, \( b_1-x_{th} \) is the value of regression constant of studied parameters, and \( x_1-x_{th} \) represents the studied variables of ZIP expression, DNA methyltransferases, and HDACs expression.

The \( R^2 \) value represents the coefficient of multiple linear regression and it denotes worthwhile competence between predicted and quantified values. A higher \( R^2 \), more than 0.75, is considered a good fit model of MLR for metal uptake and studied parameters. Cumulative percentage contributions (CPC) represents the contribution of studied parameters against heavy metal uptake and calculated by using the following equation;

\[
B = \frac{b_i}{\sum b_i} \times 100
\]  

(2)

\( B \) is the cumulative percentage contribution of studied parameter, \( b_i \) is the value of MLR coefficients of each studied parameter, and \( \sum b_i \) is the value of sum of MLR coefficients of all studied parameters.

Mobility Index was calculated by using the following formula;

\[
\text{Mobility Index} (%) = \frac{\text{Concentration of metal in the receiving level}}{\text{Concentration of metal in the source level}} \times 100
\]  

(3)

All the statistical analyses were performed using IBM SPSS statistical software.

5. Conclusions

This study show that Zn, Pb, and Cd interfere with each other’s accumulation and transport to aerial parts of the maize plants. Further, the interplay among Zn, Pb, and Cd specifically alters the uptake and translocation of divalent calcium and magnesium but not the monovalent potassium. Finally, we showed that DNA methyltransferases and histone deacetylases together regulate the expression of ZIP transporters, which are most likely implicated in the transport of Pb, Cd, and Zn. This study demonstrates that epigenetic regulators may play an important role against the tolerance of heavy metals in maize. However, further studies, such as on genome wide DNA methylation changes and histone acetylation changes in response to metal stress alone and in combination, will advance our understandings regarding their role in the tolerance to metal stress.
Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/18/6959/s1.

Author Contributions: Conceptualization, S.S.; Data curation, S.S. and A.A.; Formal analysis, S.S., A.A., Y.S., Q.Z., M.S., A.R.K., R.N. and E.W.; Funding acquisition, S.S.; Methodology, S.S. and M.S.; Supervision, S.S.; Writing—original draft, S.S.; and Writing—review and editing, S.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the grant from Higher Education Commission of Pakistan (PD-IPFP/HRD/HEC/2013/1129).

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Singh, S.; Parihar, P.; Singh, R.; Singh, D.; Prasad, S.M. Heavy Metal Tolerance in Plants: Role of Transcriptomics, Proteomics, Metabolomics, and Ionomics. Front. Plant Sci. 2016, 6, 1143. [CrossRef] [PubMed]
2. Oveˇcka, M.; Takáˇc, T. Managing heavy metal toxicity stress in plants: Biological and biotechnological tools. Biotechnol. Adv. 2014, 32, 73–86. [CrossRef] [PubMed]
3. Wang, H.; Zhao, S.; Liu, R.; Zhou, W.; Jin, J. Changes of photosynthetic activities of maize (Zea mays L.) seedlings in response to cadmium stress. Photosynthetica 2009, 47, 277–283. [CrossRef]
4. Cheng, W.-D.; Zhang, G.; Yao, H.-G.; Wu, W.; Xu, M. Genotypic and environmental variation in cadmium, chromium, arsenic, nickel, and lead concentrations in rice grains. J. Zhejiang Univ. Sci. B 2006, 7, 565–571. [CrossRef]
5. Fahr, M.; Laplaze, L.; Bendaud, N.; Hocher, V.; El Mzibri, M.; Bogusz, D.; Smouni, A. Effect of lead on root growth. Front. Plant Sci. 2013, 4, 175. [CrossRef]
6. Kobayashi, T.; Nishizawa, N.K. Iron Uptake, Translocation, and Regulation in Higher Plants. Annu. Rev. Plant Biol. 2012, 63, 131–152. [CrossRef]
7. Vatansever, R.; Ozyigit, I.I.; Filiz, E. Essential and Beneficial Trace Elements in Plants, and Their Transport in Roots: A Review. Appl. Biochem. Biotechnol. 2016, 181, 464–482. [CrossRef]
8. Broadley, M.R.; White, P.J.; Hammond, J.P.; Zelko, I.; Lux, A. Zinc in plants. New Phytol. 2007, 173, 677–702. [CrossRef]
9. Morrissey, J.; Guerinot, M.L. Iron Uptake and Transport in Plants: The Good, the Bad, and the Ionomome. Chem. Rev. 2009, 109, 4553–4567. [CrossRef]
10. Sadeghzadeh, B.; Rengel, Z. Zinc in Soils and Crop Nutrition. In The Molecular and Physiological Basis of Nutrient Use Efficiency in Crops; Wiley: Chichester, UK; Hoboken, NJ, USA, 2011; pp. 335–375.
11. Küpper, H.; Andresen, E. Mechanisms of metal toxicity in plants. Metallomics 2016, 8, 269–285. [CrossRef]
12. Song, W.-Y.; Park, J.; Eisenach, C.; Maeshima, M.; Lee, Y.; Martinova, E. ABC Transporters and Heavy Metals. In Plant ABC Transporters. Signaling and Communication in Plants; Springer: Cham, Switzerland, 2014; pp. 1–17.
13. Milner, M.J.; Seamon, J.; Craft, E.; Kochian, L. Transport properties of members of the ZIP family in plants and their role in Zn and Mn homeostasis. J. Exp. Bot. 2012, 64, 369–381. [CrossRef] [PubMed]
14. Tiong, J.; McDonald, G.; Genç, Y.; Shirley, N.J.; Langridge, P.; Huang, C.Y. Increased expression of sixZIPfamily genes by zinc (Zn) deficiency is associated with enhanced uptake and root-to-shoot translocation of Zn in barley (Hordeum vulgare). New Phytol. 2015, 207, 1097–1109. [CrossRef] [PubMed]
15. Evens, N.P.; Buchner, P.; Williams, L.E.; Hawkesford, M.J. The role of ZIP transporters and group F bZIP transcription factors in the Zn-deficiency response of wheat (Triticum aestivum). Plant J. 2017, 92, 291–304. [CrossRef] [PubMed]
16. Guerinot, M.L. The ZIP family of metal transporters. Biochim. Biophys. Acta (BBA) Biomembr. 2000, 1465, 190–198. [CrossRef]
17. Li, S.; Zhou, X.; Huang, Y.; Zhu, L.; Zhang, S.; Zhao, Y.; Guo, J.; Chen, J.; Chen, R. Identification and characterization of the zinc-regulated transporters, iron-regulated transporter-like protein (ZIP) gene family in maize. BMC Plant Biol. 2013, 13, 114. [CrossRef]
18. Mäser, P.; Thomine, S.; Schroeder, J.I.; Ward, J.M.; Hirschi, K.D.; Sze, H.; Talke, I.N.; Amtmann, A.; Maathuis, F.J.; Sanders, D.; et al. Phylogenetic Relationships within Cation Transporter Families of Arabidopsis. Plant Physiol. 2001, 126, 1646–1667. [CrossRef]
19. Arita, A.; Costa, M. Epigenetics in metal carcinogenesis: Nickel, arsenic, chromium and cadmium. Metallomics 2009, 1, 222–228. [CrossRef]
20. Berr, A.; Shafiq, S.; Shen, W.-H. Histone modifications in transcriptional activation during plant development. Biochim. Biophys. Acta (BBA) Bioenergy 2011, 1809, 567–576. [CrossRef]
21. Finnegan, E.J.; Peacock, W.J.; Dennis, E.S. Reduced DNA methylation in Arabidopsis thaliana results in abnormal plant development. Proc. Natl. Acad. Sci. USA 1996, 93, 8449–8454. [CrossRef]
22. Gehring, M. Genomic Imprinting: Insights From Plants. Annu. Rev. Genet. 2013, 47, 187–208. [CrossRef]
23. Kakutani, T.; Jeddeloh, J.A.; Flowers, S.K.; Munakata, K.; Richards, E.J. Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. Proc. Natl. Acad. Sci. USA 1996, 93, 12406–12411. [CrossRef]
24. Qian, Y.; Xi, Y.; Cheng, B.; Zhu, S. Genome-wide identification and expression profiling of DNA methyltransferase gene family in maize. Plant Cell Rep. 2014, 33, 1661–1672. [CrossRef] [PubMed]
25. Aina, R.; Sgorbati, S.; Santagostino, A.; Labra, M.; Ghiani, A.; Citterio, S. Specific hypomethylation of DNA is induced by heavy metals in white clover and industrial hemp. Physiol. Plant. 2004, 121, 472–480. [CrossRef]
26. Filek, M.; Keskinen, R.; Hartikainen, H.; Szarejko, I.; Janiak, A.; Miszalski, Z.; Golda, A. The protective role of selenium in rape seedlings subjected to cadmium stress. J. Plant Physiol. 2008, 165, 833–844. [CrossRef]
27. Yang, J.-L.; Liu, L.; Gong, Y.; Huang, D.-Q.; Wang, F.; He, L.-L. Analysis of genomic DNA methylation level in radish under cadmium stress by methylation-sensitive amplified polymorphism technique. Zhi Wu Sheng Li Yu Fen Zi Sheng Wu Xue Xue Bao = J. Plant Physiol. Mol. Biol. 2007, 33, 219–226.
28. Shafiq, S.; Zeb, Q.; Ali, A.; Sajjad, Y.; Nazir, R.; Widemann, E.; Liu, L. Lead, Cadmium and Zinc Phytotoxicity Alter DNA Methylation Levels to Confer Heavy Metal Tolerance in Wheat. Int. J. Mol. Sci. 2019, 20, 4676. [CrossRef]
29. Aoyama, T.; Okamoto, T.; Kohno, Y.; Fukiage, K.; Otsuka, S.; Furu, M.; Itó, K.; Jin, Y.; Nagayama, S.; Nakayama, T.; et al. Cell-specific epigenetic regulation of ChM-I gene expression: Crosstalk between DNA methylation and histone acetylation. Biochem. Biophys. Res. Commun. 2008, 365, 124–130. [CrossRef]
30. Tian, L.; Chen, Z.J. Blocking histone deacetylation in Arabidopsis induces pleiotropic effects on plant gene regulation and development. Proc. Natl. Acad. Sci. USA 2001, 98, 200–205. [CrossRef]
31. Luo, M.; Wang, Y.-Y.; Liu, X.; Yang, S.; Li, Y.; Wu, K. HD2C interacts with HDA6 and is involved in ABA and salt stress response in Arabidopsis. J. Exp. Bot. 2012, 63, 3297–3306. [CrossRef]
32. Cigliano, R.A.; Cremona, G.; Paparo, R.; Termolino, P.; Perrella, G.; Gutziat, R.; Consiglio, F.; Conicella, C. Histone Deacetylase AtHDA7 Is Required for Female Gametophyte and Embryo Development in Arabidopsis. Plant Physiol. 2013, 163, 431–440. [CrossRef]
33. Imran, M.; Shafiq, S.; Naeem, M.K.; Widemann, E.; Munir, M.Z.; Jensen, K.B.; Wang, R.R.-C. Histone Deacetylase (HDAC) Gene Family in Allotetraploid Cotton and Its Diploid Progenitors: In Silico Identification, Molecular Characterization, and Gene Expression Analysis under Multiple Abiotic Stresses, DNA Damage and Phytohormone Treatments. Int. J. Mol. Sci. 2020, 21, 321. [CrossRef] [PubMed]
34. Imran, M.; Shafiq, S.; Farooq, M.A.; Naeem, M.K.; Widemann, E.; Bakhsh, A.; Jensen, K.B.; Wang, R.R.-C. Comparative Genome-wide Analysis and Expression Profiling of Histone Acetyltransferase (HAT) Gene Family in Response to Hormonal Applications, Metal and Abiotic Stresses in Cotton. Int. J. Mol. Sci. 2019, 20, 5311. [CrossRef] [PubMed]
35. Varotto, S.; Locatelli, S.; Canova, S.; Pipal, A.; Motto, M.; Rossi, V. Expression Profile and Cellular Localization of Maize Rpd3-Type Histone Deacetylases during Plant Development1. Plant Physiol. 2003, 133, 606–617. [CrossRef] [PubMed]
36. Rossi, V.; Locatelli, S.; Varotto, S.; Donn, G.; Pirona, R.; Henderson, D.A.; Hartings, H.; Motto, M. Maize Histone Deacetylase hda101 Is Involved in Plant Development, Gene Transcription, and Sequence-Specific Modulation of Histone Modification of Genes and Repeats. Plant Cell 2007, 19, 1145. [CrossRef]
37. Yang, H.; Liu, X.; Xin, M.; Du, J.; Hu, Z.; Peng, H.; Rossi, V.; Sun, Q.; Ni, Z.; Yao, Y. Genome-Wide Mapping of Targets of Maize Histone Deacetylase HDA101 Reveals Its Function and Regulatory Mechanism during Seed Development. Plant Cell 2016, 28, 629–645. [CrossRef]
38. Hou, H.; Zheng, X.; Zhang, H.; Yue, M.; Hu, Y.; Zhou, H.; Wang, Q.; Xie, C.; Wang, P.; Li, L. Histone Deacetylase Is Required for GA-Induced Programmed Cell Death in Maize Aleurone Layers. Plant Physiol. 2017, 175, 1484–1496. [CrossRef]
39. Ekmekçi, Y.; Tanyolaç, D.; Ayhan, B. Effects of cadmium on antioxidant enzyme and photosynthetic activities in leaves of two maize cultivars. *J. Plant Physiol.* 2008, 165, 600–611. [CrossRef]

40. Al-Mureish, K.; Othman, N.A.R.M.; Al-Hakimi, A.M.A. Salicylic Acid-Mediated Alleviation of Cadmium Toxicity in Maize Leaves. *J. Plant Sci. (Sci. Publ. Group)* 2014, 2, 276–281. [CrossRef]

41. Kankel, M.W.; Ramsey, D.E.; Stokes, T.L.; Flowers, S.K.; Haag, J.R.; Jeddelyoh, J.A.; Riddle, N.C.; Verbisky, M.L.; Richards, E.J. Arabidopsis MET1 cytosine methyltransferase mutants. *Genetics* 2003, 163, 1109–1122.

42. Lindroth, A.M.; Cao, X.; Jackson, J.P.; Zilberman, D.; McCallum, C.M.; Henikoff, S.; Jacobsen, S.E. Requirement of CHROMOMETHYLASe3 for Maintenance of CpXpG Methylation. *Science* 2001, 292, 2077–2080. [CrossRef]

43. Cao, X.; Jacobsen, S.E. Role of the Arabidopsis DRM Methyltransferases in De Novo DNA Methylation and Gene Silencing. *Curr. Biol.* 2002, 12, 1138–1144. [CrossRef]

44. Taspinar, M.S.; Agar, G.; Alpsoy, L.; Yildirim, N.; Bozari, S.; Sevsay, S. The protective role of zinc and calcium in Vicia faba seedlings subjected to cadmium stress. *Toxicol. Ind. Health* 2010, 27, 73–80. [CrossRef] [PubMed]

45. Li, Z.; Chen, X.; Li, S.; Wang, Z. Effect of nickel chloride on Arabidopsis genomic DNA and methylation of 18S rDNA. *Electron. J. Biotechnol.* 2015, 18, 51–57. [CrossRef]

46. Ogutcu, H.; Arslan, E.; Agar, G.; Gulluce, M.; Turan, M.; Sahin, F. Protective Role of Calcium on DNA Methylation Caused Cadmium Stress in Vicia faba Seedlings. In Proceedings of the International Conference on Agricultural, Ecological and Medical Sciences, Bali, Indonesia, 6–7 February 2014.

47. Li, X.; Yang, Y.; Jia, L.; Chen, H.; Wei, X. Zinc-induced oxidative damage, antioxidant enzyme response and proline metabolism in roots and leaves of wheat plants. *Ecotoxicol. Environ. Saf.* 2013, 89, 150–157. [CrossRef]

48. Vert, G.; Grotz, N.; Déldaléchéamp, F.; Gaymard, F.; Guerinot, M.L.; Briat, J.-F.; Curie, C. IRT1, an Arabidopsis Transporter Essential for Iron Uptake from the Soil and for Plant Growth. *Plant Cell* 2002, 14, 1223–1233. [CrossRef] [PubMed]

49. Korsunova, Y.O.; Eide, D.; Clark, W.G.; Guerinot, M.L.; Pakrasi, H.B. The IRT1 protein from Arabidopsis thaliana is a metal transporter with a broad substrate range. *Plant Mol. Biol.* 1999, 40, 37–44. [CrossRef]

50. Rascio, N.; Navari-Izzo, F. Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? *Plant Sci.* 2011, 180, 169–181. [CrossRef]

51. Palmer, C.; Guerinot, M.L. A Question of Balance: Facing the challenges of Cu, Fe and Zn Homeostasis. *Nat. Chem. Biol.* 2009, 5, 333–340. [CrossRef] [PubMed]

52. Hossain, M.A.; Piyatida, P.; Da Silva, J.A.T.; Fujita, M. Molecular Mechanism of Heavy Metal Toxicity and Tolerance in Plants: Central Role of Glutathione in Detoxification of Reactive Oxygen Species and Methylglyoxal and in Heavy Metal Chelation. *J. Bot.* 2012, 2012, 1–37. [CrossRef]

53. Dresler, S.; Hanaka, A.; Bednarek, W.; Maksymiec, W. Accumulation of low-molecular-weight organic acids in roots and leaf segments of Zea mays plants treated with cadmium and copper. *Acta Physiol. Plant* 2014, 36, 1565–1575. [CrossRef]

54. Ridošková, A.; Sobrova, P.; Krystofová, O.; Sochor, J.; Zitka, O.; Babula, P.; Adam, V.; Docekalová, H.; Kizek, R. Cadmium(II) and Zinc(II) Ions Effects on Maize Plants revealed by Spectroscopy and Electrochemistry. *Int. J. Electrochem. Sci.* 2011, 6, 6011–6031.

55. Malikowski, E.; Kita, A.; Galas, W.; Karcz, W.; Kuperberg, J.M. Lead distribution in corn seedlings (Zea mays L.) and its effect on growth and the concentrations of potassium and calcium. *Plant Growth Regul.* 2002, 37, 69–76. [CrossRef]

56. Helrich, K. *Official Methods of Analysis of the Association of Official Analytical Chemistry*, 15th ed.; The Association: Arlington, VA, USA, 1990.

57. Csiszar, J.; Lantos, E.; Tari, I.; Madosa, E.; Wodala, B.; Vashegyi, Á.; Horváth, F.; Pecsvaradi, A.; Szabó, M.; Bartha, B.; et al. Antioxidant enzyme activities in Allium species and their cultivars under water stress. *Plant Soil Environ.* 2008, 53, 517–523. [CrossRef]

58. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951, 193, 265–275.

59. Berr, A.; Shafiq, S.; Pinon, V.; Dong, A.; Shen, W.-H. The trxG family histone methyltransferase SET DOMAIN GROUP 26 promotes flowering via a distinctive genetic pathway. *Plant J.* 2014, 81, 316–328. [CrossRef]
60. Zhang, H.; Tang, K.; Wang, B.; Duan, C.-G.; Lang, Z.; Zhu, J.-K. Protocol: A beginner's guide to the analysis of RNA-directed DNA methylation in plants. *Plant Methods* **2014**, *10*, 18. [CrossRef]

61. Khan, A.H.A.; Nawaz, I.; Qu, Z.; Butt, T.A.; Yousaf, S.; Iqbal, M. Reduced growth response of ornamental plant Nicotiana alata L. upon selected heavy metals uptake, with co-application of ethylenediaminetetraacetic acid. *Chemosphere* **2019**, *241*, 125006. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).