Comparative Physiological and Transcriptomic Analyses Reveal Ascorbate and Glutathione Coregulation of Cadmium Toxicity Resistance in Wheat Genotypes

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Abstract

Background: Understanding the cadmium (Cd) resistance mechanism is crucial for combating the phytotoxicity of Cd and meeting the increasing food demand daily. A classic symptom of Cd toxicity is root growth inhibition.

Results: Using two wheat genotypes (Cd tolerant genotype T207 and Cd sensitive genotype S276) with differing root growths in response to Cd, we conducted comparative physiological and transcriptomic analyses and exogenous application tests to interpret Cd detoxification mechanisms. S276 accumulated more H_2O_2 , O_2^- , and malonaldehyde than T207. Catalase activity and levels of ascorbic acid (AsA) and glutathione (GSH) were higher, whereas superoxide dismutase and peroxidase activities were lower in T207 than in S276. Transcriptome analysis showed that the expression of *RBOHA*, *RBOHC*, and *RBOHE* significantly increased, whereas that of *RBOHB* markedly decreased by Cd treatment. The transcriptional levels of 22 genes encoding RBOH were higher, and that of 11 genes were lower in T207 than in S276. The transcription of genes involved in the AsA-GSH cycle was profoundly reshaped by Cd treatment; 124 genes were higher and 43 genes were lower in T207 than in S276. Exogenous combined application of AsA and GSH alleviated Cd toxicity by scavenging excess ROS and coordinately modulating root length and branching, especially in S276.

Conclusions: These results indicate that the AsA-GSH cycle fundamentally and vigorously influences plant defense against Cd toxicity, which provides valuable information for further clarification of the mechanisms underlying Cd detoxification.

Background

Cadmium (Cd) is a heavy metal that is harmful to animals, plants, and microorganisms. With progressing industrialization and urbanization, Cd pollution is becoming increasingly serious in farmland and irrigation water, where it is absorbed by plants. Cd is highly mobile in soil, so it is easily absorbed by plants, resulting in retarded plant growth. Cd is also highly mobile in plants and easily contaminates agricultural products through transportation [1]. It can then enter the human body via the food chain, and excessive intake or inhalation of Cd can damage the human immune, urinary, bone, nervous, reproductive, and other systems [2]. In addition, Cd has strong carcinogenic, teratogenic, and mutagenic effects [3, 4], resulting in diseases, such as “Itai-Itai disease” [5]. Hence, Cd has been listed as one of the 12 hazardous substances worldwide with environmental and food safety significance by the United Nations Environment Program since 1984.

Cd can inhibit seed germination and root growth, reduce photosynthesis, block the xylem, induce stomatal closure, and cause water and nutrient imbalances in plants [6, 7]. Besides, it can stimulate the burst of reactive oxygen species (ROS), and excessive ROS can cause the loss of cellular membrane integrity and damage the molecular structure of DNA, proteins, and lipids [1, 8]. As the main and direct site for Cd adsorption and perception, roots have evolved various strategies to cope with Cd toxicity. For

example, fixing Cd in the root cell wall firmly, reducing Cd transport to the aboveground parts, expelling Cd from the root into soils, or transporting Cd into non-metabolic organelle vacuoles [9, 10]. Another important resistance mechanism is enhancing antioxidant defense. Two ways can be used to eliminate oxidative stress, thus helping to maintain redox homeostasis in plants [11]. 1) the enzymatic antioxidant scavenging system, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione-S-transferase (GST), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR); and 2) the non-enzymatic antioxidants system, including ascorbic acid (AsA), glutathione (GSH), and nicotinamide adenine dinucleotide phosphate (NADPH) [12]. Reduced substances play key roles among these antioxidants because peroxidases can react with H_2O_2 rapidly, and their reduced forms are quickly regenerated by specific reductases [13]. AsA and GSH have strong reducibility and are localized in almost all cellular compartments, including mitochondria, chloroplasts, peroxisomes, nuclei, cytosols, endoplasmic reticulum, and vacuoles. GSH is also localized in the apoplast [14]. The roles and functions of AsA or GSH in scavenging excess ROS produced by environmental stress have been extensively studied. In a review of previous studies, Akram et al. [15] summarized the important role of AsA in reducing oxidative stress in detail and comprehensively. These studies examined the effects of AsA application on many plants under various environmental abiotic stresses, including high and low temperatures, salinity, and drought. Exogenous GSH treatment of postharvest bell pepper could alleviate chilling injury by regulating the AsA-GSH cycle [16]. For heavy metal stress, Chao et al. [17] reported that hydroponic AsA treatment reduced the production of malondialdehyde (MDA) and increased chlorophyll content, thus helping alleviate the effects of Cd poisoning in rice. In *Arabidopsis*, exogenous GSH could inhibit Cd translocation from roots to shoots, whereas endogenous GSH could not [18]. Seeds treated with AsA, proline, and GSH in turn could reduce Cd toxicity in cucumber seedlings [19]. In plants, APX and GSH do not act separately. AsA, which serves as a special electron donor for APX to reduce H_2O_2 and O_2^- content, is oxidized to dehydroascorbic acid (DHAA), and the oxidized form non-enzymatically reduced by GSH, thus forming the AsA-GSH cycle [20]. However, there are few researches, to our knowledge, studying on the interaction effect of GSH and AsA on resisting environmental stress, especially heavy metal stress in plants. Whether the ROS burst induced a decrease or increase in AsA and GSH synchronously or triggered an imbalance in the AsA-GSH cycle when plants encounter environmental stresses remains unknown. These questions need to be further studied.

Currently, studies on Cd accumulation and tolerance mechanisms have mainly focused on the model plants, such as *Arabidopsis thaliana* and rice. Extending the scope to other food crops, such as wheat, is desired. Wheat (*Triticum aestivum* L.) is the predominant cereal crop and the main staple food for more than 50% of the world's population [21]. In some areas, wheat and its products are vital contributors to dietary Cd intake by people, especially in countries lacking water, where farmers use wastewater to irrigate wheat, and in areas with high soil Cd concentrations [22, 23]. Cd is more toxic to wheat than other heavy metals, such as chromium [24]. Cd hinders wheat shoot growth, causes chlorosis and necrosis of leaves, decreases leaf number and leaf area, and decreases yield. As the major perception and adsorption site of Cd, the root growth was also severely affected [25–27]. Here, we selected two wheat genotypes differing in root growth under Cd toxicity, investigated their ROS generation and enzymatic and non-

enzymatic antioxidant systems, analyzed the transcriptional response of genes involved in the ROS-producing and AsA-GSH cycle to Cd toxicity, studied the interaction effect of AsA and GSH on Cd toxicity, and selected the optimal concentration combination to alleviate Cd toxicity. These findings will provide new insight for improving wheat growth in the Cd-contaminated areas.

Results

Growth of the wheat under Cd treatment

As the Cd concentration in the solution increased from 0 to 100 μM , the shoot height and biomass and root length and biomass decreased gradually compared with the control group (Fig. 1A–F). Regardless of the Cd concentrations, under Cd treatment for 12 d, the leaf number per plant was three and had two fewer leaves than plants without Cd treatment (Fig. 1G). Since 5 μM Cd can cause significant adverse effects on wheat, we selected 5 μM Cd as the Cd treatment in the following experiments.

Cd treatment decreased the shoot biomass to 59.4% and 34.2% and decreased the shoot height to 78.8% and 62.7% for T207 and S276, respectively, compared to the control (Fig. 2B, C). Similarly, Cd treatment decreased the root biomass of T207 and S276 to 73.8% and 49.7%, respectively, compared to the control (Fig. 2D). Cd treatment also decreased the maximum root length of T207 and S276 to 80.7% and 53.0%, respectively (Fig. 2E). The shoot height and biomass and root length and biomass were all significantly higher in T207 than S276, under Cd treatment (Fig. 2B–E). These results indicated that the root growth was very sensitive to Cd toxicity, and the tolerance index of T207 was higher than that of S276.

Response of lipid peroxidation and ROS to Cd stress

After Cd treatment, the concentration of MDA, an indicator of lipid peroxidation, was measured in the roots of the two genotypes (Fig. 3A). MDA levels significantly increased following Cd treatment. Under Cd toxicity, the MDA level of S276 was 35% higher than that of T207. O_2^- and H_2O_2 production in the root markedly increased in the S276 after 3 d of Cd treatment, especially the H_2O_2 concentration (Fig. 3B and 3C). The H_2O_2 and O_2^- concentrations in S276 were 47% and 15% higher, respectively, than those of T207 root under Cd treatments, indicating that distinct oxidative stress may be an important contributor to the phenotypic differences between T207 and S276 under Cd stress.

Antioxidative enzyme activities and antioxidants levels under Cd stress

We examined the activities of several key antioxidant enzymes and concentrations of antioxidants in T207 and S276. The activities of SOD, POD, and CAT were significantly increased by Cd stress, whereas

the opposite trend was observed for APX (Fig. 4). Under Cd stress, CAT activity was significantly higher in T207 than in S276 (Fig. 4B); however, SOD and POD activities were significantly higher in S276 than in T207 (Fig. 4A, C). The APX activities showed no significant differences between S276 and T207 (Fig. 4D). The non-enzymatic antioxidants AsA concentrations were 305% and 303% higher than those of the control after Cd treatment in T207 and S276, respectively (Fig. 5A); another non-enzymatic antioxidant GSH concentrations were 1380% and 764% higher than those in T207 and S276, respectively (Fig. 5B). These two antioxidants exhibited significantly higher concentrations in T207 than in S276 under Cd stress (Fig. 5).

Digital gene expression profiling of ROS-related genes

The significant increase in non-enzymatic antioxidants AsA and GSH concentrations by Cd treatment indicated that the AsA-GSH cycle may contribute significantly to wheat Cd resistance. The AsA-GSH cycle is an important part of ROS regulation network that include ROS-producing and ROS-detoxifying proteins in the cell. The major ROS-producing genes were the respiratory burst oxidase homologs (*RBOHs*). The major ROS-detoxifying genes include genes encoding iron (Fe)-, manganese (Mn)-, and copper-zinc (CuZn)-SODs, CATs, APXs, glutathione peroxidases (GPXs), and peroxiredoxins (PrxR). We summarized the expression of genes involved in the AsA-GSH cycle by RNA-sequencing. A total of 336 genes were involved in the AsA-GSH cycle in wheat. In general, a higher percentage of genes had more abundant transcripts in T207 than in S276 under Cd treatment (Fig. 6A). The expression of *RBOHA*, *RBOHC*, and *RBOHE* was significantly increased by Cd treatment, whereas that of *RBOHB* was significantly decreased (Fig. 6B). Except for the *RBOHB* subfamily, almost all other genes had higher transcript abundance in T207 than in S276. A total of 22 *RBOH* genes were significantly more abundant in S276 than in T207, and another 11 showed the opposite trend (Fig. 6A). Among the 18 *SOD* genes, the three *SOD-4As* in wheat were the most abundant and were significantly induced by Cd treatment (Additional file 1).

Under Cd treatment, eight genes exhibited significantly higher expression while one showed significantly lower expression in T207 than in S276. Among the 10 *CAT* genes, the expression of *CAT2* and *CATA* was the most induced by Cd treatment. Under Cd treatment, three *CAT* gene expressions were significantly higher and that of two were significantly lower in T207 than in S276. Among the 26 *APX* genes in wheat, the expressions of 13 were significantly higher and that of 2 were significantly lower in T207 than in S276 under Cd treatment. Among the 16 *MDAR* genes in wheat, eight exhibited significantly higher expression and one showed significantly lower expression in T207 than in S276 under Cd treatment. For the 13 *GPX* genes in wheat, eight were significantly highly expressed in T207 than in S276 under Cd treatment. Among the nine *GR* genes in wheat, one was significantly highly expressed and two were significantly lowly expressed in T207 than in S276 under Cd treatment. The other genes involved in the AsA-GSH cycle included *PrxR*, *Trx*, and *GLR*. Most of these genes showed higher expressions in T207 than in S276 (Fig. 6A, C; Additional file 1).

Root growth, lipid peroxidation, and ROS level in response to exogenous AsA and GSH

According to the results above, Cd stress exhibited a positive effect on the synthesis and accumulation of the antioxidants AsA and GSH. Thus, we investigated the role of these two substances in reducing the Cd toxicity-mediated oxidative damage. GSH application alone (T1-T3) significantly increased root biomass, total root length, root tips number, total root surface, and total root volume of S276 (Fig. 7A–C, E, F). AsA application alone (T4-T6) had relatively weaker effects on RSA, and it even decreased the total root length and increased the root mean diameter significantly at a high concentration (T6: 0.8 mM AsA) (Fig. 7B, D), which usually meant more severe Cd toxicity. The integrated use of AsA and GSH significantly changed RSA. Application of 50 μ M GSH+0.1 mM AsA (T8) to Cd-stressed wheat significantly increased the root biomass, total root length, root tip numbers, total root surface, and total root volume (Fig. 8A–C, E, F). Meanwhile, the mean root diameter did not change (Fig. 7D). Therefore, T8 (50 μ M GSH + 0.1 mM AsA) was the best combination among the nine combinations. T9 (100 μ M GSH + 0.1 mM AsA) treatment had similar effects to T8. The effect of improving wheat growth is mainly reflected in the genotype S276, which exhibits a greater vulnerability to Cd toxicity. Fig. 8 shows the phenotype of Cd-free, 5 μ M Cd, 5 μ M Cd + T8 (50 μ M GSH + 0.1 mM AsA), and 5 μ M Cd + T9 (100 μ M GSH + 0.1 mM AsA)-treated wheat under Cd stress. Shoot and root growth was improved by the two treatments.

Further observation after DAB, NBT, and Evans blue staining were performed to identify the H_2O_2 and O_2^- levels and cell viability. For all the above staining methods, the roots were stained stronger following Cd treatment in both genotypes, especially in S276 (Fig. 9), which was consistent with the ROS and MDA levels (Fig. 3). The exogenous application of 50 μ M GSH + 0.1 mM AsA and 100 μ M GSH + 0.1 mM AsA could significantly decrease the degree of DAB, NBT, and Evans blue staining, especially in S276 (Fig. 9).

Discussion

Numerous studies have reported that Cd-contaminated lands adversely affect all aspects of plant. Root growth inhibition is one of the primary symptoms of Cd toxicity and can be used to establish phenotypic and genotypic differences in Cd sensitivity or tolerance [28]. In this study, two wheat genotypes with differential root growth responses to Cd tolerance were selected. Subsequently, a series of physiological and transcriptome experiments were conducted to explain the mechanisms underlying their contrasting Cd tolerance.

Physiological and biochemical studies indicated that Cd induced considerable ROS accumulation in the roots. Less ROS was produced in the Cd-tolerant genotype T207 than in the Cd-sensitive genotype S276 (Figs. 3 and 9). Besides, the MDA content and intensity of Evans blue staining, indicators of plasma membrane damage and cell death, were consistently higher in S276 than in T207 (Figs. 3 and 9), which may explain the Cd inhibition of root elongation in S276. RBOHs are the primary source of plasma membrane-associated ROS [29]. *Arabidopsis* contains 10 *RBOH* homologous genes (*AtRBOH A-J*) [30].

Different *RBOH* copy numbers exist in other plant species, including six in barley [31] and twenty in *Nicotiana tabacum* [32]. Differentially expressed in different plant tissues, *RBOH*-mediated ROS burst significantly affect plant physiological processes such as pollen tube growth and root hair cell expansion; and plant defense against biotic and abiotic stresses, including heavy metals [33, 34]. Using diphenylene iodonium (DPI), an oxidase inhibitor, the authors found that NADPH oxidase (*RBOH*) could be the main source of Cd-induced H_2O_2 [35]. However, the authors did not characterize which *RBOH* gene was the main contributor. In *Arabidopsis*, Cd induced the expression of *RBOHC* and *RBOHD* in leaves and *RBOHF* in roots exposed to low Cd concentrations [36]. Using *Arabidopsis* mutants *rbohC*, *rbohD*, and *rbohF*, Gupta et al. [33] found that compared with the wild type, the roots of these mutants grew poorly, while their leaves showed better growth after 5-d Cd treatment. These studies suggest that different *RBOH* genes differentially regulate ROS metabolism under Cd toxicity. Among the 45 *RBOH* genes in wheat, in general, *RBOHA*, *RBOHC*, and *RBOHE* were significantly induced by Cd treatment, and levels of 22 *RBOH* genes were higher in S276 than T207, which was consistent with the higher Cd-induced ROS content in S276 than T207 (Fig. 3B, C). Interestingly, *RBOHB* was significantly decreased by Cd treatment, and its level was higher in T207 than in S276 (Fig. 6; Additional file 1). *RBOHB* is a root-specific protein in *Arabidopsis* [37] that functions in lateral root development in *Phaseolus vulgaris* [38]. Here, we found that the root tip numbers were significantly decreased by Cd treatment, especially in S276 (Fig. 7C), which may be related to the decreased expression of *RBOHB*. This phenomenon of the contrasting function of *RBOHB* compared with *RBOHD* and *RBOHF* has also been reported in *Arabidopsis* resistance against nematodes [37]. These results indicate that there is an intricate and fine transcriptional regulation mechanism that restricts ROS production under Cd stress in wheat.

To minimize the ROS-induced oxidative damage, plants have evolved a sophisticated defense antioxidative system consisting of enzymatic and non-enzymatic components. Among antioxidative enzymes, SOD and POD are the first line of defense against ROS. SOD catalyzes the dismutation of O_2^- to H_2O_2 and molecular oxygen [11], while CAT and APX subsequently convert H_2O_2 to H_2O [39]. A previous study considered Cd as a metal without redox-active properties, which therefore did not participate directly in Fenton-type reactions—the source of ROS [40]. Therefore, Cd is assumed to cause the indirect accumulation of ROS by inhibiting antioxidant enzymes. However, some studies have also shown that variations in antioxidative enzyme activities under oxidative stress can be contradictory: increase, decrease, or no change [41–43]; this is attributable to different plant species, plant tissues, or durations and concentrations of metal exposure. In this study, except for APX, activities of SOD, POD, and CAT were all significantly induced by Cd (Fig. 4). This may be attributable to the increased ROS production. Between the two genotypes, SOD and POD activities were significantly higher, while CAT activity was significantly lower in the Cd-sensitive genotype S276 than in the Cd-tolerant genotype T207. The basal levels of these enzymes showed similar trends (Fig. 4). These results were similar to the results for different wheat genotypes in response to Al toxicity [44]. This suggests common mechanisms underlying wheat resistance to Cd and Al stress. Among the *APX* genes in *Arabidopsis*, cytosolic *APX1* is the central component of the ROS gene network [45]. In this study, *APX1* in wheat was significantly upregulated by Cd treatment. However, APX activity was significantly decreased, which is consistent with

the response of wheat to Cu toxicity [42], suggesting post-transcriptional regulation of APX activity in wheat. The AsA-GSH cycle — an important pathway to control H_2O_2 levels in cells — is present in all cell compartments. AsA and GSH react directly with H_2O_2 to eliminate its toxicity, and the regeneration of AsA requires GSH as a reductant [39]. In this study, AsA and GSH concentrations increased greatly on exposure to Cd (Fig. 5). Similar results have been reported in *Arabidopsis* [33]. The AsA and GSH concentrations were significantly higher in T207 than in S276 under Cd treatment (Fig. 5). Cd stress also disturbed the expression of genes involved in the AsA-GSH cycle. A greater number of upregulated genes were involved in the AsA-GSH cycle in T207 than that in S276 (Figs. 4–6). These observations indicated that the AsA-GSH cycle in T207 was more efficient than that in S276 under Cd stress.

Both exogenous AsA and GSH can increase the cell division activity and influences cell differentiation in the root apical meristem of *Arabidopsis* root apical meristem [46, 47]. However, their applications do not have exactly the same effects on the RSA: AsA treatment promotes root length [46], while GSH promotes both the number and length of lateral roots [48]. Recent studies have demonstrated that the exogenous application of AsA or GSH can alleviate heavy metal stress. For example, Jung et al. found that exogenous AsA [49] or GSH [50] could alleviate arsenic toxicity in rice seedlings. Alamri et al. found that AsA and GSH independently alleviated arsenate toxicity in Brinjal [51]. The role of AsA and GSH in alleviating Cd toxicity in wheat plants remains unclear. Cd seriously hinders plant root growth, including reducing root length, increasing its diameter [52], and inhibiting branching [53]. In this study, under Cd treatment, exogenous GSH mainly increase total root numbers, thus increasing the total root surface, total root volume and root biomass. Exogenous ASA mainly increase root mean diameters, and decrease root length and diameter, showing no significantly effect on root surface, total root volume and root biomass (Fig. 7). A previous study showed that spraying ASA alone could alleviate Cd-induced oxidative stress in rapeseed plants, while only the aboveground biomass was significantly affected [54]. This is similar to our results that the effect of AsA on the root biomass was slight (Fig. 7A), and high ASA even caused shorter and thicker roots (Fig. 7B, D). Although AsA alone did not improve the architecture of Cd-treated roots, the combined application of AsA and GSH significantly relieved the adverse growth of root induced by Cd, better than GSH addition alone (Fig. 7). The combined application of AsA and GSH to Cd-stressed wheat seedlings increased root biomass, total root length, and total root tip numbers; and decreased the levels of MDA, O_2^- , and H_2O_2 compared to those under Cd treatment (Figs. 7 and 9). This study demonstrates that Cd-induced root growth retardation in wheat was correlated with the AsA and GSH contents. Plants might enhance the activities of the AsA-GSH cycle to overcome Cd toxicity (Fig. 4). AsA and GSH contribute significantly to ROS scavenging and redox regulation under heavy metal stress [17, 55, 56] have been extensively studied. Whereas their combined effects were studied for the first time, and a 0.1 mM AsA + 50 μM GSH was found to be the best for Cd toxicity alleviation. Integrating the above theoretical analyses and experimental results, the combination application of exogenous AsA and GSH to Cd stressed wheat, not only enhanced the metabolic intensity of the AsA-GSH cycle to scavenge excess ROS, but also fine-tuned the root growth synergistically, eventually alleviating Cd toxicity greatly.

Conclusions

The physiological and transcriptional experiments conducted on two wheat genotypes, T207 and S276, revealed that the excessive production of ROS by Cd primarily elicited root growth inhibition. The Cd tolerant genotype T207 showed a better root architecture system; lower O_2^- , H_2O_2 , and MDA concentrations; higher CAT activity; and more abundant AsA and GSH contents than the Cd-sensitive genotype S276 following Cd treatment. Root transcriptome profiles between T207 and S276 demonstrated that Cd-induced the transcriptional rearrangement of root genes involved in ROS burst and the AsA-GSH cycle. Overall, genes encoding *RBOH* were more highly expressed in S276 than in T207 and a more active ROS-scavenging AsA-GSH cycle was present in T207 than in S276. Our results also demonstrate that the exogenous application of AsA and GSH alleviates Cd toxicity in wheat plants by scavenging excess ROS and regulating root growth synergistically, especially in the Cd-sensitive genotype. The combination of 0.1 mM AsA with 50 μ M GSH exhibits the best effect. The results reported here provide valuable insights into the critical role of AsA-GSH cycle in Cd detoxification in wheat plants, and such information would be useful in developing safer food with high yield and a sustainable agricultural production system in Cd-contaminated lands. Further genetic manipulation of the candidate genes is required to better understand the Cd-detoxifying mechanisms.

Methods

Plant material

Wheat seeds were provided by Wheat Research Institute, Henan Academy of Agricultural Sciences.

Plant growth

Hydroponic culture was used for seed germination and seedling growth. Plump wheat seeds were surface-sterilized using 0.5% (w/v) NaClO for 10 min and then rinsed completely with pure water. After being allowed to germinate in a gauze for 5 d, uniform seedlings were transplanted into black plastic containers with Hoagland and Arnon [57] solution. The full-strength solution contained 5.0 mM KNO_3 , 5.0 mM $Ca(NO_3)_2$, 2.0 mM $MgSO_4 \cdot 7H_2O$, 1.0 mM KH_2PO_4 , 50 μ M EDTA-Fe, 46 μ M H_3BO_3 , 9.0 μ M $MnCl_2 \cdot 4H_2O$, 0.80 μ M $ZnSO_4 \cdot 7H_2O$, 0.37 μ M $Na_2MoO_4 \cdot 2H_2O$, and 0.30 μ M $CuSO_4 \cdot 5H_2O$. The nutrient solution was replaced every 3 d. The wheat seedlings were first grown in one-quarter-strength, one-half-strength, and eventually full-strength solutions. The plants were grown in a climate chamber using a temperature setting of 24/22 °C (day/night), a photoperiod of 14/10 h (day/night), and light intensity of 300–320 μ mol $m^{-2} s^{-1}$.

Experimental design

1. Cd concentration gradient treatments

5-d-old wheat genotype seedlings were treated with CdCl_2 at six concentrations: 0 μM , 5 μM , 10 μM , 20 μM , 50 μM , and 100 μM for 12 d. Shoot height and biomass, root length and biomass, and leaf number were recorded.

2. Selection of tolerant and sensitive cultivars

A total of 126 wheat genotypes were cultured in a nutrient solution containing 5 μM Cd^{2+} . Root biomass and length were measured after 12 d.

3. Physiological and transcriptional responses to Cd^{2+} in both cultivars

Wheat seedlings of 12-d were divided into two experimental groups based on Cd-free (normal culture) and Cd (5 μM Cd^{2+}) treatments. After 3 d, the samples were harvested to determine ROS concentrations, antioxidative enzyme activities, antioxidant concentrations, and transcriptome sequencing.

4. Addition of exogenous antioxidants

Wheat seedlings of 12-d-old were separated into 17 experimental groups based on the following treatments: Cd-free (normal culture), Cd (5 μM Cd^{2+}), T1 (20 μM GSH + 5 μM Cd^{2+}), T2 (50 μM GSH + 5 μM Cd^{2+}), T3 (100 μM GSH + 5 μM Cd^{2+}), T4 (0.1 mM AsA + 5 μM Cd^{2+}), T5 (0.4 mM AsA + 5 μM Cd^{2+}), T6 (0.8 mM AsA + 5 μM Cd^{2+}), T7 (20 μM GSH + 0.1 mM AsA + 5 μM Cd^{2+}), T8 (50 μM GSH + 0.1 mM AsA + 5 μM Cd^{2+}), T9 (100 μM GSH + 0.1 mM AsA + 5 μM Cd^{2+}), T10 (20 μM GSH + 0.4 mM AsA + 5 μM Cd^{2+}), T11 (50 μM GSH + 0.4 mM AsA + 5 μM Cd^{2+}), T12 (100 μM GSH + 0.4 mM AsA + 5 μM Cd^{2+}), T13 (20 μM GSH + 0.8 mM AsA + 5 μM Cd^{2+}), T14 (50 μM GSH + 0.8 mM AsA + 5 μM Cd^{2+}), and T15 (100 μM GSH + 0.8 mM AsA + 5 μM Cd^{2+}). After 7 d, the root system architecture (RSA) parameters were analyzed and root microscopy was performed.

Determination of ROS concentrations

The O_2^- level was measured by the hydroxylamine oxidation method according to the method of Ma et al. [58]. H_2O_2 reacts with titanium oxysulfate to produce pertitanic acid, which is yellow, and at 410 nm is proportional to the H_2O_2 concentration.

Determination of lipid peroxidation

The level of lipid peroxidation is usually represented by the MDA content [59]. MDA was determined by condensation with thiobarbituric acid (TBA) to form a red product [59].

Antioxidative enzyme activities and antioxidants assays

Root samples were homogenized with a 50-mM phosphate buffer (PBS, pH = 7.0) containing 1 μ M EDTA. The homogenates were centrifuged at $14000 \times g$ for 20 min at 4 °C. Subsequently, the supernatant was used for the enzyme assays. O_2^- reduces nitroblue tetrazolium chloride (NBT) to produce formazan. SOD can clean up O_2^- , thus formazan content could represent SOD activity [60]. One unit (U) of SOD activity represents 50% inhibition of formazan production. POD catalyzes H_2O_2 and forms guaiacol dehydrogenation. Through monitoring the formation of guaiacol dehydrogenation in 470 nm, POD activity was determined. The CAT activity was determined by monitoring the decomposition of H_2O_2 at 240 nm. APX activity was determined by observing the decrease of AsA in absorbance at 290 nm over 2 min. AsA was determined by reacting the supernatant with Fast Blue B salt and assayed spectrophotometrically at 420 nm. GSH was determined by reacting with 5,5'-Dithiobis-2-nitrobenzoic acid and assayed spectrophotometrically at 412 nm. These substances were determined using an assay kit (Coming Medical Technology Co., Ltd, Suzhou, China).

Microscopic analysis

H_2O_2 was revealed in root samples by immersion in 0.5% (w/v) 3,3-N-diaminobenzidine tetrahydrochloride (DAB), and then incubation at room temperature until the root turned brown [35]. O_2^- was detected by reacting with NBT to produce a blue formazan precipitate in the root [61]. The loss of cell viability was observed after staining with 0.25% (w/v) Evans blue solution for 5 min and washing three times with 100 μ M $CaCl_2$ solution (pH = 5.6) [62]. A fluorescent microscope (LSM800, Carl Zeiss, Oberkochen, Germany) was used for observation.

High-throughput RNA-seq

Wheat roots were harvested from CK and Cd-treated plants for transcriptome analysis, with three biological replicates each. Using an Illumina HiSeq X Ten platform (Illumina Inc., San Diego, CA, USA), a total of 12 RNA samples were analyzed, which generated 6.0-Gb of sequencing data with 150-bp paired-end (PE) reads per sample. Transcript abundances (FPKM values) were calculated from RNA-seq data using the method described by Zhou et al. [63].

Determination of root architecture

WinRHIZO (Pro 2013a, Regent Instrument Inc.), a root analysis software, was used to measure the RSA parameters, including total root length, average root diameter, number of root tips, total root surface area, and total root volume.

Statistical analysis

Using GraphPad Prism 5.01, the differences among Cd concentration gradient treatments were evaluated with Tukey test. The differences between control and treatment (Cd-free and Cd treatment) or between genotypes (T207 and S276) were evaluated with the Student's t-test. Differences were considered statistically significant at $p < 0.05$.

Abbreviations

Cd: cadmium; AsA: ascorbic acid; GSH: glutathione; ROS: reactive oxygen species; SOD: superoxide dismutase; POD: peroxidase; CAT: catalase; APX: ascorbate peroxidase; GST: glutathione-S-transferase; MDHAR: monodehydroascorbate reductase; GR: glutathione reductase; NADPH: nicotinamide adenine dinucleotide phosphate; MDA: malondialdehyde; DHAA: dehydroascorbic acid; RBOH: respiratory burst oxidase homolog; GPX: glutathione peroxidase; PrxR: peroxiredoxins; DPI: diphenylene iodonium; RSA: root system architecture; TBA: thiobarbituric acid; PBS: phosphate buffer; NBT: nitroblue tetrazolium chloride; DAB: 3,3-N-diaminobenzidine tetrahydrochloride.

Declarations

-Ethics approval and consent to participate

Not applicable.

-Consent for publication

Not applicable.

-Availability of data and material

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

-Competing interests

The authors declare that the 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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-Authors' contributions

Tao Zhang, Jingui Xiao, Yongsheng Zhao, Yifan Zhang, and Yaqi Jie conducted the experiments, analyzed the data, and drafted the manuscript. Dandan Shen edited the manuscript. Caipeng Yue provided conceptual advice. Jinyong Huang supervised the research work. Yingpeng Hua designed the study and supervised the research work. Ting Zhou conceived the idea, provided critical review, and edited the manuscript.

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Not applicable.

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Figures

Figure 1

Characterization of wheat growing under different Cd concentrations. A-B, Images of wheat shoots (A) and roots (B) growing under different Cd concentrations for 12 d. From left to right, represent the phenotypes of plants grow in 0 μM (control), 5 μM, 10 μM, 20 μM, 50 μM, and 100 μM Cd²⁺. C, Shoot height of wheat growing under different Cd concentrations. D, Shoot biomass of wheat growing under different Cd concentrations. E, Maximum root length of wheat growing under different Cd concentrations. F, Root biomass of wheat growing under different Cd concentrations. G, Leaf number of wheat growing under different Cd concentrations. Results are means ± SE of six biological replicates.

Figure 2

Characterization of wheat genotypes T207 and S276 growing under normal or Cd treatment. A, Images of T207 and S276 growing under normal or Cd treatment for 12 d. B, Shoot biomass of genotypes T207 and S276. C, Shoot height of genotypes T207 and S276. D, Root biomass of genotypes T207 and S276. E, Maximum root length of genotypes T207 and S276. Results are means $\pm$ SE of six biological replicates. The black column represents T207 and the white column represents S276. The symbols # and * indicate statistically significant differences between treatments (normal and Cd conditions) (#, $P < 0.05$, ##, $P < 0.01$ and ###, $P < 0.001$) and between genotypes (T207 and S276) (*, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$), respectively. FW, fresh weight.

Figure 3

MDA, O₂⁻, and H₂O₂ concentrations of wheat genotypes T207 and S276 roots growing under normal or Cd treatment. A, MDA concentration of genotypes T207 and S276. B, O₂⁻ concentration of genotypes T207 and S276. C, H₂O₂ concentrations of genotypes T207 and S276. Results are means $\pm$ SE of four biological replicates. The black column represents T207 and the white column represents S276. The symbols # and * indicate statistically significant differences between treatments (normal and Cd

conditions) (#, $P < 0.05$, ##, $P < 0.01$ and ###, $P < 0.001$) and between genotypes (T207 and S276) (*, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$), respectively. FW, fresh weight.

Figure 4

SOD, POD, CAT, and APX activities of wheat genotypes T207 and S276 roots growing under normal or Cd treatment. A, SOD activities of genotypes T207 and S276. B, POD activities of genotypes T207 and S276. C, CAT activities of genotypes T207 and S276. D, APX activities of genotypes T207 and S276. Results are means $\pm$ SE of four biological replicates. The black column represents T207 and the white column represents S276. The symbols # and * indicate statistically significant differences between treatments (normal and Cd conditions) (#, $P < 0.05$, ##, $P < 0.01$ and ###, $P < 0.001$) and between genotypes (T207 and S276) (*, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$), respectively. FW, fresh weight.

Figure 5

AsA and GSH concentrations of wheat genotypes T207 and S276 roots growing under normal or Cd treatment. A, AsA concentration of T207 and S276. B, GSH concentration of genotypes T207 and S276. Results are means $\pm$ SE of four biological replicates. The black column represents T207 and the white column represents S276. The symbols # and * indicate statistically significant differences between treatments (normal and Cd conditions) (#, $P < 0.05$, ##, $P < 0.01$ and ###, $P < 0.001$) and between genotypes (T207 and S276) (*, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$), respectively. FW, fresh weight.

Figure 6

The expression of genes involved in AsA-GSH cycle. A, Genes involved in AsA-GSH cycle in wheat root. The red box indicates the numbers of genes that showed significantly higher expression in T207 than in S276 under Cd treatment. The blue box indicates the numbers of genes that showed significantly higher expression in S276 than in T207 under Cd treatment. B, The heatmap of RBOH expression in wheat root. C, The heatmap of genes involved in AsA-GSH cycle expression in wheat root.

Figure 7

Root architecture analysis of wheat genotypes T207 and S276. Root biomass (A), total root length (B), total root tips number (C), root mean diameter (D), total root surface area (E), total root volume (F) of genotypes T207 and S276. Results are means $\pm$ SE of four biological replicates. The black column represents T207 and the white column represents S276. The symbols # indicates statistically significant differences between genotypes (T207 and S276) under Cd treatment and * indicate statistically significant differences between treatments (Cd-free and T1-15 to Cd treatment) (#, $P < 0.05$, ##, $P < 0.01$ and ###, $P < 0.001$), respectively. FW, fresh weight.

Figure 8

The phenotypes of wheat genotypes T207 and S276 in response to exogenous AsA and GSH. A, Whole plant phenotypes. B, Root phenotypes. Cd: 5 μ M Cd²⁺, T8: 50 μ M GSH + 0.1 mM AsA + 5 μ M Cd²⁺, T9: 100 μ M GSH + 0.1 mM AsA + 5 μ M Cd²⁺.

Figure 9

Histochemical detection of H₂O₂, O₂⁻, and cell death of wheat genotypes T207 and S276 in response to exogenous AsA and GSH. A, DAB staining of roots. B, NBT staining of roots. C, Evan's blue staining of roots. Cd: 5 μ M Cd²⁺, T8: 50 μ M GSH + 0.1 mM AsA + 5 μ M Cd²⁺, T9: 100 μ M GSH + 0.1 mM AsA + 5 μ M Cd²⁺.

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