Metabolomic And Antioxidant Enzyme Activity Changes In Response To Cadmium Stress Under Boron Application of Wheat (*Triticum Aestivum*)

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Abstract

Boron (B) has previously been shown to inhibit cadmium (Cd) uptake in wheat, while the underlying physiological mechanism behind this phenomenon is unclear. Here, we investigated the alteration with B application in growth, Cd uptake, MDA content, antioxidant enzyme activities and small molecule metabolites under Cd stress. The results showed that the wheat growth were significantly weaker under Cd-treated than that without Cd-treated. B application can improve the wheat growth under Cd stress. In addition, B application decreased Cd concentrations and MDA concentrations of shoot and root. These results suggest that B can inhibit the absorption of Cd and mitigation of Cd toxicity to promote plant growth, especially in the roots. Meanwhile, the key enzyme activities including SOD and POD significant increased under Cd treatments, while decreased under B treatments. Further, root differential metabolites were identified with Liquid Chromatograph Mass Spectrometer (LC-MS). A total of 198, 680 and 204 of the differential metabolites were isolated between CK+B and CK treatment, CK+Cd and CK treatment and CK+Cd+B and CK+Cd treatment respectively. The metabolites with up-accumulation in B application (CK+Cd+B) roots were mainly galactaric acid, citric acid, N6-Galacturonyl-L-lysine, D-Glucose, while the metabolites with down-accumulation were mainly threoninyl-tryptophan and C16 sphinganine. The differential metabolic pathways were mainly concentrated in linoleic acid metabolism, galactose metabolism, sphingolipid metabolism, glycolysis/gluconeogenesis, propanoate metabolism in diabetic complications between CK+Cd+B treatment and CK+B treatment. The data suggested that B alleviates Cd toxicity in winter wheat by inhibiting Cd uptake, increasing antioxidant enzyme activity and changing metabolites.

Introduction

It is well known that cadmium (Cd) is one of the heavy metals and have high toxicity for human and plant health. For human, Itai-itai is the best known Cd-induced disease, other diseases like renal dysfunction, osteoporosis, and bladder cancer (Feki-Tounsi and Hamza-Chaffai 2014). The most important cause of these diseases is due to long-term consumption of Cd-contaminated food. Studies show that food as the main source of Cd exposure contributed about 90% of the total Cd intake in the general population (Clemens et al. 2013; He et al. 2013). Therefore, it is an important measure for human food safety to prevent the absorption of Cd in crops and its migration to edible parts. In plant, Cd can enter root and be transported to the ground through the various Cd transport systems, such as transporters for uptake in roots (OsNramp5, HvNramp5, OsIRT1, OsIRT2, OsNramp1 and OsCd1), transporters for vacuolar sequestration in roots (OsHMA3), transporters involved in root-to-shoot translocation (OsHMA2, OsHMA3, OsZIP7 and OsCAL1) and transporters for distribution to the grains (OsHMA2, OsLCT1, OsZIP7, and OsCCX2) (Ma et al. 2021). Cd affects plant growth and development due to its toxic effects on different metabolic and physiological processes, such as causing leaf chlorosis, necrotic lesions, wilting, inhibited root elongation, and reduced biomass (Qin et al. 2020a; Rizwan et al. 2016). Therefore, reducing the Cd uptake or migration to edible sites is the key factor to ensure no Cd pollution in grains in polluted soil, and is also stop the migration to the food chain.

Many agronomic strategies have been shown to reduce Cd absorption in crop, such as soil removal and replacement, soil turnover and dilution, soil dressing, chemical washing, phytoremediation and inorganic amendments (Wang et al. 2011; Hseu et al. 2010; Uraguchi and Fujiwara 2012; Rizwan et al. 2016). Among these strategies, inorganic amendments was also reported as a low-cost but effective approaches. For example, there is an efficiently competes between Cd with iron (Fe), manganese (Mn), zinc (Zn), and calcium (Ca) in uptake and the application of these micronutrients commonly decreases the uptake and accumulation of Cd (Qin et al. 2020; Meda et al. 2007; Astolfi et al. 2012; Duan et al. 2018). Silicon (Si) fertilizer or nana-silicon application could reduce Cd absorption and alleviate the toxicity to crop (Ma et al. 2015; Wang et al. 2015). It was also reported that selenium (Se) fertilization could reduce Cd uptake and mitigates Cd toxicity in crop (Hu et al. 2014; Lin et al. 2012). The underlying mechanism of effects of these elements on Cd toxicity and accumulation may include 1) competitive uptake via the same transport system (Fahad et al. 2015; Qin et al. 2020a), 2) increase the antioxidative capacity and photosynthetic characteristics (Lin et al. 2012; Qin et al. 2018), 3) induce the formation of phytochelatin and cysteine-rich peptides (Jasinski et al. 2003; Semane et al. 2007).

Boron (B) is an essential micronutrient for plant growth. It is essential for the structural and functional integrity of the cell wall and membranes, photosynthesis, cell division and elongation, nitrogen and carbohydrate metabolism, etc (Shireen et al. 2018). In recent years, it has been found that B plays an important role in alleviating abiotic stress in plants. For example, B alleviates aluminum (Al) toxicity by promoting root alkalization in transition zone via polar auxin transport (Li et al. 2018) and B alleviate Cd accumulation and toxicity by improving oxidative stress and suppressing Cd uptake and transport (Chen et al. 2019). Likewise, our previous work showed that B could inhibits Cd uptake by regulating gene expression in wheat (*Triticum aestivum* L.) (Qin et al. 2020b). However, the physiological mechanism why B inhibits Cd absorption is yet to be investigated. To understand how B influences the uptake of Cd and Cd toxicity, antioxidant substances and antioxidant enzyme activity were studied and the different metabolites and metabolic pathways was analyzed.

Materials And Methods

Plant material and experimental conditions

Hydroponic experiment was carried out on Henan Agricultural University, Zhengzhou, China. Wheat seeds (cv Zhengmai 379, *Triticum aestivum* L.) were surface-sterilized in 0.5% Na-hypochlorite for 15 min and seeds were then rinsed carefully with deionized water. The seeds were germinated on a plastic net floating on deionized water and seven-day-old rice seedlings with similar size were transfer to the incubators to growth in a controlled chamber with photoperiod of 16 h/8 h (day/night) at 25°C/20°C, RH 75%, and light intensity of $200 \pm 25 \mu\text{mol m}^{-2} \text{s}^{-1}$. The full nutrient solution (FNS) contained: 4.0 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 6 mM KNO_3 , 1 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 46.2 μM H_3BO_3 , 9.1 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.3 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.8 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 100 μM FeNaEDTA, 0.2 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$. The solution pH was adjusted to 6.5 using 1 M HCl or NaOH. Half-strength nutrient solution was applied for the first 7 d and changed to full strength solution for another 4 d. Each treatment was replicated four times. Five treatments was set as follow: (1) CK (without B); (2) CK+B (B was added after 7 days of culture); (3) CK+Cd (Cd was added after 7 days of culture); (4) CK+Cd+B (Cd and B were added after 7 days of culture); (5) preB+Cd (B seven-day pretreatment before Cd exposure). Cd was applied as CdCl_2 solution with a final Cd concentration of 5 μM . After 23 d Cd treatments, root and leaf samples rinsed in distilled water were harvested and used fresh or kept frozen in liquid N_2 for various biochemical assays.

Cd concentration analysis

Plants were divided into root and shoot fractions and oven-dried at 70°C to constant weight. These fractions were then digested with mixed acid (HNO₃:HClO₄ (4:1, v/v)) in a Microwave Digestion System. Cd concentration was determined by graphite furnace atomic absorption spectrometry (GFAAS). The translocation factor (TF) was calculated as follows: $TF = \text{shoot } C_{Cd} / \text{root } C_{Cd}$.

Malondialdehyde (MDA) determination

The level of lipid peroxidation is estimated as the amount of MDA determined by the thiobarbituric acid (TBA) reaction as described by Qin et al. (2018). Briefly, 500 mg of fresh root or shoot samples was homogenized with 2 mL 5% v/v trichloroacetic acid (TCA) and centrifuged at 10,000×g for 10 min. Then, 1 mL 0.5% v/v TBA in 20% v/v TCA was added to 1 mL supernatant and the homogenate was boiled for 30 min at 95 °C. The mixture was followed by an immediate cooling on ice to stop the reaction and centrifuged at 10,000×g for 5 min. The absorbance was determined at 450 nm, 532 nm and 600 nm and MDA concentration was estimated by the formula: $MDA (\mu\text{mol/g FW}) = 6.45(OD_{532}-OD_{600})-0.56 OD_{450}$.

Antioxidant enzyme activities

Antioxidant enzyme activities (SOD, CAT and POD) in roots and leaves were determined spectrophotometrically according to previous literatures and make some modifications (Molins et al. 2013; Wu et al. 2017). Fresh tissues (0.5 g) were ground in liquid nitrogen to a fine powder using a mortar and pestle. The powder was transferred to a pre-cooled (4°C) mortar and pestle with 5 mL of 50 mM phosphate buffer (pH 7.8), containing 0.12 mM EDTA and 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 12,000×g at 4°C for 20 min. The supernatant was used for assays of the antioxidant enzyme activities. The assay of SOD was carried out briefly as follows: the assay mixture (total 3 mL) for SOD contained 0.05 M phosphate buffer, containing 12 μM EDTA and 13 mM L-methionine, 75 μM nitroblue tetrazolium (NBT), 2 μM riboflavin in 5 mM KOH and 0.05 mL enzyme extract. Reaction was initiated by placing the glass test tubes in between two fluorescent tubes. By switching the light on and off, the reaction was started and terminated, respectively. The increase in absorbance due to formazan formation was read at 560 nm. The activity was expressed as $\Delta A_{560} (\text{U mg}^{-1} \text{ protein min}^{-1})$. CAT activity was assayed. The reaction mixture for CAT comprised of 1.5 mL of 25 mM phosphate buffer (pH 7.0), 0.3 mL of 0.1 M H₂O₂ and 0.2 mL of enzyme extract. The change of absorbance value within 210s was measured at 240 nm with the blank setting of deionized water. The CAT activity was expressed as $\text{U mg}^{-1} \text{ protein min}^{-1}$. Guaiacol peroxidase activity was determined spectrophotometrically by measuring the oxidation of guaiacol to tetraguaiacol at 470 nm. The reaction mixture contained 1 mL of 50 mM phosphate buffer (pH 5.5), 1 mL of 0.3% H₂O₂, 0.95 mL 0.2% (v/v) guaiacol, and 0.05 mL enzyme extract. The change rate of absorbance value was recorded at 470nm within 210s, and the number of reads was recorded every 30s. A peroxidase activity unit (U) was determined by ΔA_{470} changing by 0.01 per minute and the activity was expressed as $\text{U mg}^{-1} \text{ protein min}^{-1}$.

Metabolite extraction and metabolite profiling analysis

The identification of differential metabolites was performed by Shanghai Lu Ming Biological Technology Co. Ltd. 80 mg accurately weighed sample was transferred to a 1.5 mL Eppendorf tube. Two small steel balls were added to the tube. 20 μL of 2-chloro-L-phenylalanine (0.3 mg/mL) dissolved in methanol as internal standard and 1 mL mixture of methanol and water (7/3, v/v) were added to each sample, samples were place at -20°C for 2 min. Then grinded at 60 HZ for 2 min, and ultrasonicated at ambient temperature for 30 min after vortexed, then placed at -20°C for 20 min. Samples were centrifuged at 13,000 rpm, 4°C for 10 min. 0.3 mL of supernatant in a brown and glass vial was dried in a freeze concentration centrifugal dryer. 0.4 mL mixture of mixture of methanol and water (1/4, v/v) were added to each sample, samples vortexed for 30s, then placed at 4°C for 2 min. Samples were centrifuged at 13,000 rpm, 4°C for 5 min. The supernatants (150 μL) from each tube were collected using crystal syringes, filtered through 0.22 μm microfilters and transferred to LC vials. The vials were stored at -80°C until LC-MS analysis. QC samples were prepared by mixing aliquots of the all samples to be a pooled sample.

An ACQUITY UHPLC system (Waters Corporation, Milford, USA) coupled with an AB SCIEX Triple TOF 5600 System (AB SCIEX, Framingham, MA) was used to analyze the metabolic profiling in both ESI positive and ESI negative ion modes. An ACQUITY UPLC BEH C18 column (1.7 μm, 2.1×100 mm) were employed in both positive and negative modes. The binary gradient elution system consisted of (A) water (containing 0.1% formic acid, v/v) and (B) acetonitrile (containing 0.1% formic acid, v/v) and separation was achieved using the following gradient: 0 min, 5% B; 2 min, 20% B; 4 min 25% B; 9 min 60% B; 14 min 100% B; 18 min 100% B; 18.1 min 5% B and 19.5 min 5% B. The flow rate was 0.4 mL/min and column temperature was 45°C. All the samples were kept at 4°C during the analysis. The injection was 10 μL. Data acquisition was performed in full scan mode (m/z ranges from 70 to 1000) combined with IDA mode. The QCs were injected at regular intervals (every 10 samples) throughout the analytical run to provide a set of data from which repeatability can be assessed.

The acquired LC-MS raw data were analyzed by the proggenesis QI software (Waters Corporation, Milford, USA). The internal standard was used for data QC (reproducibility). Metabolites were identified by proggenesis QI (Waters Corporation, Milford, USA) Data Processing Software, based on public databases such as <http://www.hmdb.ca/>; <http://www.lipidmaps.org/> and self-built databases. The positive and negative data were combined to get a combine data which was imported into R ropls package. Principle component analysis (PCA) and (orthogonal) partial least-squares-discriminant analysis (O)PLS-DA were carried out to visualize the metabolic alterations among eperimental groups, after mean centering and Pareto variance (Par) scaling, respectively. The Hotelling's T2 region, shown as an ellipse in score plots of the models, defines the 95% confidence interval of the modeled variation. Variable importance in the projection (VIP) ranks the overall contribution of each variable to the OPLS-DA model, and those variables with VIP > 1 are considered relevant for group discrimination. The differential metabolites were selected on the basis of the combination of a statistically significant threshold of variable influence on projection (VIP) values obtained from the OPLS-DA model and p values from a two-tailed Student's t test on the normalized peak peak areas, where metabolites with VIP values less than 0.05 were considered as differential metabolites.

Statistical analysis

Analysis of variance (ANOVA) was computed for statistically significant differences using the statistical package SPSS, version 20.0 (SPSS, Chicago, IL, USA). All data are the means $\pm$ SE of four independent replicates; the mean differences were compared utilizing the LSD's multiple range test ($P < 0.05$).

Results

Effect of different treatments on wheat growth and Cd accumulation

From the perspective of growth, the wheat growth under Cd-treated, including CK + Cd, CK + Cd + B and PreB + Cd, were significantly weaker than that without Cd-treated (CK and CK + B) (Fig. 1a). Boron (B) application can improve the wheat growth under Cd stress (Fig. 1b). Similar trend was observed in PreB + Cd treatment compared with CK + Cd + B treatment (Fig. 1c). Compared with CK + Cd, the fresh weight (FW) increased by 15.22% and 56.52% in root under CK + Cd + B and PreB + Cd treatment, where decreased by 10.59% and 45.88% in root respectively (Table 1). Cd treatment lead to a dramatic reduction of root and shoot water content, while little effect on water content with B treatment. Compared with CK + Cd, shoot Cd concentrations decreased by 8.78% and 3.99% under CK + Cd + B and PreB + Cd treatment, where root Cd concentrations decreased by 26.34 and 26.30%, respectively. However, the transport coefficients of Cd transport from roots to shoots increased 10.57% and 35.07% under CK + Cd + B and PreB + Cd treatment compared with CK + Cd treatment. These results suggest that B can inhibit the absorption of Cd and promote plant growth, and B pretreatment is more effective in alleviating Cd-inhibited wheat.

Table 1
Biomass, water content and Cd concentration in tissues of wheat

	CK	CK + B	CK + Cd	CK + Cd + B	PreB + Cd
Shoot FW (g plant ⁻¹)	1.57 $\pm$ 0.11ab	1.82 $\pm$ 0.04a	0.85 $\pm$ 0.01c	0.94 $\pm$ 0.16c	1.24 $\pm$ 0.14bc
Root FW (g plant ⁻¹)	0.81 $\pm$ 0.09ab	1.03 $\pm$ 0.03a	0.46 $\pm$ 0.05c	0.53 $\pm$ 0.11bc	0.72 $\pm$ 0.06abc
Shoot water content (%)	92.55 $\pm$ 0.54ab	93.34 $\pm$ 0.10a	91.18 $\pm$ 0.53abc	89.94 $\pm$ 0.73c	91.44 $\pm$ 0.51abc
Root water content (%)	88.54 $\pm$ 0.27a	88.70 $\pm$ 0.23a	85.99 $\pm$ 0.55b	85.17 $\pm$ 0.19b	86.06 $\pm$ 0.34b
Shoot Cd concentration (mg kg ⁻¹)	nd	nd	225.8 $\pm$ 12.8a	206.0 $\pm$ 11.7a	216.8 $\pm$ 12.6a
Root Cd concentration (mg kg ⁻¹)	nd	nd	1216.5 $\pm$ 73.1a	896.1 $\pm$ 71.2b	896.6 $\pm$ 35.9b
Transport coefficient	-	-	20.16 $\pm$ 1.20b	22.29 $\pm$ 0.3ab	27.23 $\pm$ 2.08a
Data are means $\pm$ standard error of four replications and different letters denote significant differences between different treatments according to LSD's multiple comparison test at $P < 0.05$. 'nd' indicates 'not detected'. Transport coefficient indicates as the ratio of shoot Cd concentration/root Cd concentration.					

Effect of different treatments on MDA concentration and antioxidant enzymes activities

Lipid peroxidation measured in terms of MDA increased in all the Cd stressed plants comparing with control. Compared with CK treatment, MDA concentration significant increased with 120.8%, 62.00% and 122.68% in root, and 188.86%, 122.25% and 221.72% in shoot at CK + Cd, CK + Cd + B and PreB + Cd treatments respectively (Fig. 2). The MDA concentration decreased 26.62% ($P < 0.05$) in root and 23.06% in shoot respectively under CK + Cd + B treatment compared with CK + Cd treatment. However, the MDA concentrations have no significantly difference between PreB + Cd treatment and CK + Cd treatment.

In the present study, the key enzymes including SOD, POD and CAT exhibited differential responses to Cd treatments in shoots and roots of wheat (Fig. 3). It was found that SOD activity significant increased by 84.11%, 54.19% and 48.44% at CK + Cd, CK + Cd + B and PreB + Cd compared with CK in root respectively (Fig. 3a), while no significant differences in shoot (Fig. 3b). Compared with CK + Cd treatment, SOD activity significant decreased by 16.25% and 19.37% at CK + Cd + B and PreB + Cd, respectively. Similarly, POD activity under Cd-treatment showed an increased tendency, especially increased by 359.52%, 300.49% and 366.21% at CK + Cd, CK + Cd + B and PreB + Cd compared with CK in shoot (Fig. 3d). However, there was no significant difference in POD activity between CK + Cd + B and PreB + Cd compared with CK + Cd. Different from SOD and POD, CAT activity of the Cd-treated plants was decreased compared with the CK in both shoot and root (Fig. 3e and f). Especially at the root, lower activity of CAT was observed where 29.58%, 51.58% and 79.81% decreased respectively at CK + Cd, CK + Cd + B and PreB + Cd compared with CK. In addition, the CAT activity decreased 31.24% and 71.33% in root at CK + Cd + B and PreB + Cd compared with CK + Cd.

Effect of different treatments on root extract metabolite

To better explore the differential metabolites in B alleviates the Cd toxicity metabolites, the differential metabolites of wheat under CK, CK + Cd and CK + Cd + B were analyzed by LC-MS. Our data show that the parameters of the OPLS-DA for the classification were expressed by the R2Y (cum) of 0.866 and Q2 (cum) of 0.368 for CK + B and CK treatment (Fig. 4a), 0.983 and 0.941 for CK + Cd and CK treatment (Fig. 4b), 0.81 and 0.0107 for CK + Cd + B and CK + B treatment (Fig. 4c), respectively. In addition, methods of 7-fold cross validation and response permutation testing (RPT) were used to investigate the quality of the model, indicating that there was no overfitting in the measurement model. These parameters representing the exhibited a better stability and predictability, and effectively reflected the metabolic differences between treatment.

The Volcano plot were used to visualize the P value and the fold change value to screen the differential metabolites under positive ion mode and negative ion mode. As shown in the Fig. 4, the red origin represents the significantly up-regulated differential metabolites in the experimental group, the blue origin represents the significantly down-regulated differential metabolites, and the gray point represents the non-significantly different metabolites. Using VIP screening (variable importance in the projection) > 1 at a level of $P < 0.05$, a total of 198 of the differential metabolites were isolated between CK + B and CK

treatment, in which a total of 91 of the differential metabolites were up-regulated (red dots, Fig. 4d) and a total of 107 of the differential metabolites were down-regulated (blue dots, Fig. 4d) in the CK + B treatment compared with CK treatment, respectively. In the same way, a total of 680 of the differential metabolites were isolated between CK + Cd and CK treatment, in which a total of 341 of the differential metabolites were up-regulated (red dots, Fig. 4e) and a total of 339 of the differential metabolites were down-regulated (blue dots, Fig. 4e) in the CK + Cd treatment compared with CK treatment, respectively. A total of 204 of the differential metabolites were isolated between CK + Cd + B and CK + Cd treatment, in which a total of 132 of the differential metabolites were up-regulated (red dots, Fig. 4f) and a total of 72 of the differential metabolites were down-regulated (blue dots, Fig. 4f) in the CK + Cd + B treatment compared with CK + Cd treatment, respectively. Furthermore, using screening conditions with more than 1 of VIP and fold change at a level of *P* less than 0.05, we isolated out a total of top 50 of the differential metabolites as show in Table 2. Analyses show that the B application (CK + Cd + B) not only obviously up-regulated accumulation of galactaric acid, citric acid, N6-Galacturonyl-L-lysine, D-Glucose, 3,4,5-trihydroxy-6- $\{[4\text{-methoxy-6-(3-oxoprop-1-en-1-yl)-2H-1,3-benzodioxol-5-yl]oxy\}$ oxane-2-carboxylic acid, N-Acetyldjenkolic acid, and 2-Methylthiophene, but also down-regulated the accumulation of threoninyl-tryptophan and C16 sphinganine in the roots compared with CK + Cd (VIP > 5).

Table 2
Identification of metabolites (Top 50) under different treatments

Metabolites	Ck + B vs CK		Metabolites	Ck + Cd vs CK		Metabolites
	VIP	log ₂ (FC)		VIP	log ₂ (FC)	
Glucocerebrosides	9.05	0.64	p-Coumaroylputrescine	32.95	1.98	Galactaric acid
Cer(d18:0/15:0)	8.10	0.89	Rutalinium	25.77	2.65	Citric acid
PI(20:1(11Z)/22:2(13Z,16Z))	7.90	0.35	4-Coumaroyl-2-hydroxyputrescine	21.72	3.97	N6-Galacturonyl-L-lysine
N-stearoyl alanine	7.25	0.23	Threoninyl-Tryptophan	10.28	6.26	D-Glucose
Drotaverine	5.98	3.41	Ribalinium	9.70	3.30	3,4,5-trihydroxy-6-[[4-methoxy-6-(3-oxoprop-1-er 1-yl)-2H-1,3-benzodioxol-5-yl]oxy]oxane-2-carboxylic acid
GlcCer(d18:2(4E,8Z)/16:0(2OH[R]))	5.83	0.52	N1-trans-Feruloylagmatine	7.80	5.24	N-Acetylidenkolic acid
Cer(d16:1/17:0)	5.42	0.87	Beta-D-Glucopyranuronic acid	7.64	1.25	2-Methylthiophene
Glyuranolide	4.98	0.31	1-(m-Methoxycinnamoyl)pyrrolidine	7.16	1.68	Diferuloylputrescine
DG(18:4(6Z,9Z,12Z,15Z)/24:1(15Z)/0:0)	4.37	1.43	N,N'-Bis(gamma-glutamyl)cystine	6.03	5.41	PI(20:1(11Z)/22:2(13Z,16Z))
1α-hydroxy-26,27-dimethylvitamin D3 / 1α-hydroxy-26,27-dimethylcholecalciferol	4.00	0.82	Cefuroxime	5.96	0.44	9S-hydroxy-10S,11S-epoxy 12Z,15Z-octadecadienoic acid
TG(14:0/18:1(11Z)/o-18:0)	3.56	0.36	6-[3-(6,7-dimethoxy-2H-1,3-benzodioxol-5-yl)oxirane-2-carbonyloxy]-3,4,5-trihydroxyoxane-2-carboxylic acid	5.72	5.50	4-O-Caffeoyl-3-O-feruloylquinic acid
N-Oleoyl-D-erythro-Sphingosine (C18:1 Ceramide)	3.46	1.08	Acacetin 7-(4"-Acetylrrhamnosyl)-(1->6)-glucosyl-(1->3)-(6"-acetylglucoside)	5.67	2.20	D-Myoinositol 4-phosphate
PC(20:0/P-18:0)	3.35	0.22	Coumarin	5.19	1.88	12,13-Epoxy-9,15-octadecadienoic acid
PE(14:1(9Z)/15:0)	3.15	0.51	Cefpodoxime	4.78	5.42	2-Hydroxyadipic acid
PE-GlcGlcDG(P-14:0/16:1(9Z))	2.78	0.30	Safynol	4.64	2.02	4R-aminopentanoic acid
Vinaginsenoside R14	2.64	2.09	Phenylacetyl glycine dimethylamide	4.43	2.24	Desglucocoroloside
Cer(d18:1/17:0)	2.57	1.27	Methyl 2-(10-heptadecenyl)-6-hydroxybenzoate	3.98	1.40	3,4-dimethyl-5-propyl-2-furanoctanoic acid;
Cer(d18:0/14:0)	2.50	0.79	Embinin	3.68	1.98	3-methyl-5-pentyl-2-furanoctanoic acid
DG(20:0/0:0/18:2n6)	2.48	1.25	N-stearoyl valine	21.62	-0.37	13R,23S-Dimethylpentatriacontane
13R-HOME(11E)	2.47	0.37	9,10,13-TriHOME	16.02	-1.58	3-methoxy Prostaglandin F1α
PS(18:4(6Z,9Z,12Z,15Z)/20:5(5Z,8Z,11Z,14Z,17Z))	2.44	7.85	Azelaic acid	11.12	-1.12	beta-D-Xylopyranosyl-(1->5)-alpha-L-arabinofuranosyl-(1->5)-L-arabinose
2-hydroxyhexadecanoic acid	2.43	0.20	12,13,15-trihydroxy-9E-octadecenoic acid	10.81	-1.71	Prostaglandin PGE2 1-glycerol ester
Densipolic acid	2.36	2.27	Porson	10.58	-0.67	Aspartyl-Cysteine
Notoginsenoside H	2.32	2.55	Citric acid	10.11	-1.87	(-)-Enunicelline
N-(2R-Hydroxyhexadecanoyl)-2S-amino-9-methyl-4E,8E-octadecadiene-1,3R-diol	2.31	0.96	PI(0-16:0/15:1(9Z))	9.05	-0.23	12-amino-octadecanoic acid

Note: VIP indicates the weight value of the variable from the OPLS-DA model. Generally, the metabolites of VIP > 1 are considered as differential metabolites. the greater the contribution of the variable to the grouping. FC: fold change. log₂(FC): the ratio of the average expression of metabolites in the two groups of : means up-regulation, negative value means down-regulation.

Metabolites	Ck + B vs CK		Metabolites	Ck + Cd vs CK		Metabolites
	VIP	log ₂ (FC)		VIP	log ₂ (FC)	
9-oxo-9,11-seco-campest-5-en-3beta,11-diol	2.26	0.75	Galactaric acid	8.78	-2.00	2-deoxy-20-hydroxyecdysone 22-phosphate
p-Coumaroylputrescine	23.74	-0.58	9,10-DHOME	7.65	-1.38	N2-Acetyl-L-aminoadipate
Galactaric acid	12.60	-0.83	{[3-(2-hydroxyphenyl)prop-2-en-1-yl]oxy)sulfonic acid	7.36	-3.58	Monic acid
N-palmitoyl alanine	7.37	-0.19	N-palmitoyl alanine	6.99	-0.55	20-hydroxyecdysone 22-phosphate
1-(m-Methoxycinnamoyl)pyrrolidine	6.70	-0.71	Margaroylglycine	5.66	-0.65	(±)12(13)-EpOME
1alpha,25-dihydroxy-23-azavitamin D3 / 1alpha,25-dihydroxy-23-azacholecalciferol	6.60	-2.75	9S-hydroxy-10S,11S-epoxy-12Z,15Z-octadecadienoic acid	5.48	-1.30	N-Gluconyl ethanolamine
D-Glucose	5.94	-0.48	9,10,18-TriHOME(12)	5.42	-1.36	Camellenodiol
Acetylcholine	5.68	-1.34	12-hydroxy-8E,10E-heptadecadienoic acid	5.10	-0.94	1-(sn-Glycero-3-phospho)-1D-myo-inositol
3-amino-2-naphthoic acid	5.63	-0.47	12-oxo-PDA	5.05	-1.57	O-Demethylfonsecin
N1-(alpha-D-ribose)-5,6-dimethyl-benzimidazole	4.57	-0.49	D-Glucose	4.89	-1.16	Avenoleic acid
N-Acetyldjenkolic acid	4.45	-0.63	Guanine	4.80	-1.20	17-hydroxy-linolenic acid
Spermic acid 2	4.15	-0.31	α-9(10)-EpODE	4.66	-1.74	8,13-DiHODE
Eicosanoyl-EA	3.80	-0.28	(±)13-HpODE	4.62	-1.39	Adenine
Coumarin	3.75	-0.51	3-Deoxyguanosine	4.57	-1.62	Threoninyl-Tryptophan
4R-aminopentanoic acid	3.73	-0.87	16-L1-PhytoP	4.39	-1.28	C16 Sphinganine
Tricosanoylglycine	3.57	-0.36	cis-Hydroxy Perhexiline	4.36	-0.66	17-hydroxy stearic acid
Linoleic acid	2.97	-0.23	16-F1-PhytoP	4.35	-1.03	TG(14:0/18:1(11Z)/o-18:0
9S-HpOTrE	2.95	-0.58	9,12,13-TriHOME	4.24	-1.86	L-Phenylalanine
Spermine	2.95	-0.56	Isoleucylproline	4.08	-0.60	Embinin
(Z)-4-Hepten-1-ol	2.85	-0.44	N-Hexadecyl-L-hydroxyproline	4.05	-0.48	1-Phenyl-1,3-octadecanedione
12-amino-octadecanoic acid	2.77	-0.26	2-Methylthiophene	4.03	-1.30	Histidinyl-Isoleucine
27-Nor-5b-cholestane-3a,7a,12a,24,25-pentol	2.71	-0.34	9(S)-HpODE	3.98	-1.36	1,N2-propanodeoxyguanosine
delta10-13-PhytoF	2.67	-0.36	OKODA-PA	3.95	-4.94	Mizoribine
6-Deoxyfagomine	2.53	-1.82	Lymecycline	3.78	-1.82	Spermic acid 2
Traumatic Acid	2.28	-0.46	OHODA-PA	3.71	-4.64	20:0 Campesteryl ester
Note: VIP indicates the weight value of the variable from the OPLS-DA model. Generally, the metabolites of VIP > 1 are considered as differential metabolites. the greater the contribution of the variable to the grouping. FC: fold change. log ₂ (FC): the ratio of the average expression of metabolites in the two groups of : means up-regulation, negative value means down-regulation.						

The pathway enrichment analysis of differential metabolites

The pathway enrichment analysis of differential metabolites was carried out according to the Kyoto Encyclopedia of Genes and Genomes (KEGG, <https://www.kegg.jp/>) database. Pathway enrichment analysis show that the differential metabolites (top 50) were involved glycerophospholipid metabolism, linoleic acid metabolism, one carbon pool by folate, glycosylphosphatidylinositol (GPI)-anchor biosynthesis, autophagy-other, pentose phosphate pathway, alpha-Linolenic acid metabolism, galactose metabolism, aminoacyl-tRNA biosynthesis, AGE-RAGE signaling pathway in diabetic complications between CK + B treatment and CK treatment (Fig. 5a); linoleic acid metabolism, pyrimidine metabolism, glycerophospholipid metabolism, purine metabolism, one carbon pool by folate, beta-Alanine metabolism, alpha-Linolenic acid metabolism, cyanoamino acid metabolism, aminoacyl-tRNA biosynthesis, sphingolipid metabolism for CK + Cd treatment and CK treatment (Fig. 5b); linoleic acid metabolism, galactose metabolism, sphingolipid metabolism, glycolysis/gluconeogenesis, propanoate metabolism, fructose and mannose metabolism, AGE-RAGE signaling pathway in diabetic complications, pyrimidine metabolism, phenylalanine metabolism, arachidonic acid metabolism for CK + Cd + B treatment and CK + B treatment (Fig. 5c).

Discussion

Cadmium (Cd) is a toxic heavy metal that has been known causing many physiological disorders such as a decrease in photosynthesis, sugars and soluble proteins and antioxidant enzyme activities, and also increased the production of reactive oxygen species (ROS) which caused oxidative stress in plants (Abbasi et al. 2015; Chen et al. 2018). This would result in a retardation of plant growth, reduction in biomass and grain yield. Boron (B) is an essential micronutrient for the structural and functional integrity of the cell wall and membranes, photosynthesis, cell division and elongation, nitrogen and carbohydrate metabolism, etc. (Shireen et al. 2018). Meanwhile, studies have also found that B plays a crucial role in alleviated Cd accumulation and toxicity (Chen et al. 2019; Qin et al. 2020b). Here, we determined the responses of wheat against Cd stress under B supply or pre-supply, with respect to their biomass, ROS scavenging mechanism and differential metabolite mechanism.

The present study revealed that Cd application and B deficiency inhibited the growth of wheat, while B supply or pre-supply could improve the wheat growth under Cd stress. In addition, the root and shoot Cd concentration were decreased under B supply or pre-supply compared with CK + Cd treatment, especially at the root. While the transport coefficients of Cd transport from roots to shoots increased under CK + Cd + B and PreB + Cd treatment compared with CK + Cd treatment. Similar result were also found in our previous study (Qin et al. 2020b). These results suggest that B can inhibit the absorption of Cd and promote its migration from root to aboveground, where B pretreatment is more effective in inhibiting Cd absorption and promote Cd migration.

Oxidative stress is inevitably related to Cd stress in plants and the antioxidant defense machinery protects plants against oxidative stress damages (Gill and Tuteja 2010). For example, malondialdehyde (MDA) represents the degree of lipid peroxidation and reflects the degree of cell damage. Antioxidant enzymes can remove reactive oxygen species (ROS) from plants and reduce the abiotic stress caused by Cd. In our study, MDA concentration significant increase in root and shoot at CK + Cd treatment compared with CK treatment respectively, while the MDA concentration decreased under CK + Cd + B treatment compared with CK + Cd treatment. This results indicates that B could reduce the accumulation of MDA to alleviate Cd toxicity in wheat. In order to better understand the role of B in antioxidant enzymes, the antioxidant enzymes activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were detected. SOD is a key enzyme that constitutes the first line of defense against ROS, where converts superoxide radicals into less toxic agents, producing H_2O_2 (Gill et al. 2010). CAT and POD are important enzymes that scavenge excess of H_2O_2 by catalyzing it into water and divalent oxygen for ROS detoxification (Garg and Manchanda 2009). Here, it was found that SOD and POD activity significant increased at Cd treatment. However, lower SOD and POD activity were found under CK + Cd + B and PreB + Cd compared with CK treatment. In addition, lower activity of CAT was also observed at B application compared with Cd treatment in root. The results suggested that SOD and POD activity have been greatly activated under Cd stress condition and almost all the antioxidant enzymes moderately reduced after B application.

Some small molecular compounds play an important role in plant transport heavy metals and resistance to heavy metal stress. For instance, Zn hyperaccumulation was associated with enhanced xylem transport and phloem remobilization of Zn by bound to organic acid and citric acid in *Sedum alfredii* (Lu et al. 2013). For Cd, studies show that glutathione (GSH), metallothioneins (MTs), and phytochelatins (PCs) are the most important ligands involved in Cd detoxification in plants (Gallego et al. 2012; Nakamura et al. 2020). In addition, phytosiderophores, organic acids, amino acids, peptides and proteins have also been discovered as important ligands in inducing Cd precipitation by chelation (Bali et al. 2020). Within plant cells, Cd can bind to these small molecule compounds to protect the cytosol from free Cd ions and sequester Cd in the vacuole to increase the tolerance of plants to Cd (Schat et al. 2002; Zimeri et al. 2005; Ernst et al. 2008). In the present study, a total of 198, 680 and 204 of the differential metabolites were isolated between CK + B and CK treatment, CK + Cd and CK treatment and CK + Cd + B and CK + Cd treatment respectively. B application (CK + Cd + B) not only obviously up-regulated accumulation of galactaric acid, citric acid, N6-Galacturonyl-L-lysine, D-Glucose, 3,4,5-trihydroxy-6-([4-methoxy-6-(3-oxoprop-1-en-1-yl)-2H-1,3-benzodioxol-5-yl]oxy)oxane-2-carboxylic acid, N-Acetyldjenkolic acid, and 2-Methylthiophene, but also down-regulated the accumulation of threoninyl-tryptophan and C16 sphinganine in the roots compared with CK + Cd (VIP > 5). Furthermore, differential metabolic pathways were mainly concentrated in linoleic acid metabolism, galactose metabolism, sphingolipid metabolism, glycolysis/gluconeogenesis, propanoate metabolism, fructose and mannose metabolism, AGE-RAGE signaling pathway in diabetic complications between CK + Cd + B treatment and CK + B treatment. A previous study has also shown that B nutrition could change the organic carbon structure of cell walls and enhanced the contents of amino acid, cellulose, phenols and lignin in the cell wall (Dong et al. 2016; Wu et al. 2018). In addition, B nutrition could induce changes in pectin composition and architecture of components in root cell walls, which have been shown to play an important role in inhibiting Cd absorption (Wu et al. 2016; Wu et al. 2017). Therefore, we hypothesize that these differential metabolites, including carbohydrate and amino acid compounds, play a key role in mitigation of Cd toxicity with B application. Nevertheless, other metabolites and metabolic pathways may also be involved in B mitigation of Cd toxicity in wheat, which still needs further verification.

Conclusions

In this study, we demonstrated that B application reduces Cd absorption, MDA content and mitigates Cd toxicity in wheat. In addition, the key enzymes activity including SOD and POD significant increased with Cd application, while decreased under B application. The changes observed in metabolites of galactaric acid, citric acid, N6-Galacturonyl-L-lysine, D-Glucose, 3,4,5-trihydroxy-6-([4-methoxy-6-(3-oxoprop-1-en-1-yl)-2H-1,3-benzodioxol-5-yl]oxy)oxane-2-carboxylic acid, N-Acetyldjenkolic acid, 2-Methylthiophene, threoninyl-tryptophan and C16 sphinganine (VIP > 5) involved in linoleic acid metabolism, galactose metabolism, sphingolipid metabolism, glycolysis/gluconeogenesis, propanoate metabolism, fructose and mannose metabolism, AGE-RAGE signaling pathway in diabetic complications of roots in response to B application under Cd stress. The overall results indicated that B can change antioxidant enzyme activity and small molecule metabolites to affect plant stress to Cd.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to Publish

Not applicable.

Authors Contributions

Shiyu Qin and Peng Zhao conceived and designed the research. Shiyu Qin, Zhaojun Nie, Chang Li and Yafang Xu perform all the experiments, including supervised the study, implemented the method and performed data analysis, interpreted the data and wrote the manuscript. Wei Gao and Hongen Liu supervised the study and checked the results. All authors read and approved the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Figures

Figure 1

The growing conditions for (a) all treatment, (b) CK+Cd and CK+Cd+B and (c) PreB+Cd and CK+Cd+B.

Figure 2

The MDA concentration of root (a) and shoot (b) of wheat under different treatment. Data are means $\pm$ SE. Bar with different letters are significantly different at $P < 0.05$ in multiple comparison by LSD's test.

Figure 3

Superoxide dismutase (SOD) (a and b), peroxidases (POX) (c and d) and catalase (CAT) (e and f) activity in root and shoot respectively under different treatments. Data are means $\pm$ SE. Bar with different letters are significantly different at $P < 0.05$ in multiple comparison by LSD's test.

Figure 4

Metabolomics analyses of the different treatments. Score scatter plot of OPLS-DA model for group CK+B vs CK (a), CK+Cd vs CK (b) and CK+Cd+B vs CK+Cd (c); Volcano plot for group CK+B vs CK (d), CK+Cd vs CK (e) and CK+Cd+B vs CK+Cd (f).

Figure 5

Metabolic pathway enrichment diagram of differential metabolites between CK+B and CK (a), CK+Cd and CK (b), and CK+Cd+B and CK+B (c). The p-value of the metabolic pathway is the significance of the enrichment of the metabolic pathway. The red line indicates a P value of 0.01, and the blue line indicates a P value of 0.05. It is significant for the signal pathway when the top of the bar is higher than the blue line.

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