



Full Length Article

How Exogenous Cadmium Affects Micronutrients Accumulation and the Related Gene Expression Regulation in *Brassica juncea*

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Abstract

Cd accumulation in plants not only affects the plant growth, but also further endangers human healthy by plant-derived food process. *Brassica juncea* L. has been appreciated as the potential Cd hyperaccumulator plant as it is suitable for bioremediation purposes. The previous studies revealed that *B. juncea* biomass and metabolism were severely affected by Cd stress. However, the relationship between Cd accumulation and micronutrient accumulation in *B. juncea* remains unknown. Cd shares chemical similarity to Zn, Fe, and Mn and can thus replace them in the ionic related groups of many proteins in plant cells. In the present study, the micronutrients Mn, Zn, Fe, and Cu contents and Cd contents in *B. juncea* were detected under gradient Cd treatment condition. The result showed that Zn, Mn and Cu accumulation in roots were significantly affected by Cd stress. The main metal and heavy metal transporter family candidates were then identified according to the phylogenetic analysis of related homologues in *Arabidopsis thaliana*, *B. napus* and *B. juncea*. We further investigated the expression level of *BjNramp*, *BjHMA*, *BjCET* and *BjMTPs* genes under 1 and 5 μM Cd treatment condition by qRT-PCR assays. The result revealed that *BjNramp1;4*, *BjNramp3;5*, *BjNramp3;10*, *BjNramp4;4*, *BjHMA3*, and *BjHMA4* might be involved in the uptake of Cd, Cu, Zn and Mn by the roots under low (1 μM) Cd treatment. The present study is important to understand the relation between micronutrient and heavy metal uptake, and provides essential information for the phytoremediation on soil heavy metal contamination to coordinate the detoxification of heavy metal and micronutrient accumulation. © 2018 Friends Science Publishers

Keywords: *Brassica juncea*; Cadmium; Phytoremediation; Phylogenetic tree; qRT-PCR

Introduction

Cadmium (Cd) is a nonessential and toxic heavy metal for humans. Cd has been detected in the majority of plant-derived food samples. Therefore, the Cd contaminated crops are becoming the major source of the Cd exposure to human body. Normally, Cd accumulation in plants is mostly due to the uptake of soil Cd. The Cd levels in plants are significantly influenced by soil parameters, such as pH, and exogenous chemicals, such as abscisic acid (Clemens *et al.*, 2013; Masood *et al.*, 2016; Kaur *et al.*, 2017; Shen *et al.*, 2017). At the same time, different cultivars of plant species accumulated a nearly 100-fold greater Cd concentration in the shoots (Uraguchi and Fujiwara, 2013; Ahmad *et al.*, 2015; Luo *et al.*, 2016; Yu *et al.*, 2017). Therefore, to understand the Cd uptake and accumulation pathways in plants, it is necessary to clarify the Cd transport processes and the relation between Cd and normal micronutrient, which is essential elements for plant growth (Schroeder *et al.*, 2013; Uraguchi and Fujiwara, 2013; Choppala *et al.*, 2014).

Transport proteins, which embedded within cell membranes, are a key channel for nutrient and heavy metal import and translocation in plants. With the development of genome sequencing technology and experimental systems for the transporter function, many of the major families of metal transporters have been well characterized in model plants and crops (Gallego *et al.*, 2012; Schroeder *et al.*, 2013). The cation diffusion facilitators (CDF) have been implicated in metal tolerance mechanisms in many organisms. The subgroup of CDF, named MTP, was reported to be involved in Manganese (Mn) transport (Delhaize *et al.*, 2003; Fujiwara *et al.*, 2015). In *Arabidopsis*, the identified 12 *AtMTP* genes were classified into three subgroups depending on their phylogenetic relationships. *AtMTP1*, *AtMTP2*, *AtMTP3* and *AtMTP4* were thought to be Zn transporters (Maser *et al.*, 2001; Kobae *et al.*, 2004). The *AtMTP3* was characterized to mediate Zn exclusion from shoot under Fe deficiency and Zn oversupply (Arrivault *et al.*, 2006). The proteins *AtMTP8* to *AtMTP11*, which are related to the Mn transporter *ShMTP1* in *Stylosanthes hamate*, were

suggested to be involved primarily in Mn transport (Delhaize *et al.*, 2003). The *Arabidopsis* AtHMA proteins belong to the P_{1B}-adenosine triphosphatase (ATPase) transporter family, and are implicated in heavy metal transport (Gravot *et al.*, 2004). The eight AtHMA members, named AtHMA1-AtHMA8, are classified into two subgroups: the AtHMA1-4 exhibit high similarity with Zn/Cd/Pb/Co ATPases characterized in prokaryotes, and the AtHMA5-8 are involved in Cu trafficking in plants (Williams and Mills, 2005; Sasaki *et al.*, 2014; Siemianowski *et al.*, 2014).

The range of metals transported by AtHMAs has been confirmed by expressing them in *E. coli* and yeast. The *hma2hma4* double mutant in *A. thaliana* displayed a chlorotic and stunted phenotypes which could be rescued solely by excess Zn supplying to the soil (Hussain *et al.*, 2004). This phenotype in the double mutant, not in individual mutants, revealed that there were overlapping functions for AtHMA2 and AtHMA4 transporters (Siemianowski *et al.*, 2014). The AtHMA3 was reported to contribute to metal detoxification, sequestering Cd and Pb in the vacuole (Gravot *et al.*, 2004). Phylogenetic analysis of the HMA family revealed that this family includes eight genes in *A. thaliana*, nine in the rice (*OsHMA1-OsHMA9*) and ten in barley (*HvHMA1-HvHMA10*) (Satoh-Nagasawa *et al.*, 2012, 2013; Kumagai *et al.*, 2014; Sasaki *et al.*, 2014). Obviously, the HMA family is very important for the micronutrient and heavy metal translocation in plants. The *Nramp* gene family was implicated in heavy metal transport according to the *Nramp* homologue in yeast, *SMF1*, which encodes the Mn transporter (Thomine *et al.*, 2000; Sasaki *et al.*, 2012; Milner *et al.*, 2014; Takahashi *et al.*, 2014). In *A. thaliana*, six *AtNramp* genes were identified and characterized. *AtNramp3* and *AtNramp4* can complement the Cd and Fe uptake mutants in yeast (Thomine *et al.*, 2000). *OsNramp1* in rice can only complement the Cd-deficient phenotype of the yeast mutant, not Mn or Fe (Takahashi *et al.*, 2011). However, the previous study revealed that *OsNramp5* contributed to Mn, Cd, and Fe transport in rice (Ishimaru *et al.*, 2012; Sasaki *et al.*, 2012). In *B. juncea*, four novel cation-efflux family transporters (*BjCET1-BjCET4*) were identified and recombinantly expressed in Cd treated seedlings of *B. juncea*. *BjCET2* was confirmed to be involved in Cd and Zn transport (Xu *et al.*, 2009). Expression of *BjCET3* and *BjCET4* in yeast revealed these two *BjCETs* contributed to Zn efflux, and possibly Cd, Co, and Ni transport (Lang *et al.*, 2011).

It is known that Cd shares chemical similarity to Zn, Fe, and Mn and can thus replace them in the iron-related groups of many proteins in plant cells (D'Alessandro *et al.*, 2013). *OsIRT1* (iron regulated transporters1, *OsIRT2*, and *OsNramp1* are Fe transporters with limited specificity and are induced under Fe deficiency condition. Overexpression of *OsNramp1* increased Cd sensitivity and Cd accumulation in leaves. *OsNramp3* was shown to play an important role in the accumulation of Cd under different Fe supply

conditions. Many metal transporters share the same transport pathways with heavy metal (Ahmad *et al.*, 2015; Wu *et al.*, 2016). Therefore, the research to clarify the connection between micronutrient and toxic metals are very important to understand the influence of heavy metal stress to micronutrient uptake and plant growth.

Among the members of *Brassicaceae* family, *Brassica juncea* is a potential Cd hyperaccumulator species. Especially, the Indian mustard (*B. juncea*) represented the model cultivar of *B. juncea* to investigate the Cd uptake and accumulation mechanism (D'Alessandro *et al.*, 2013). In this study, the Indian mustard were treated with different Cd concentration from 0 to 25 μM . The dry weight, Cd content and Cu, Zn, Mn, Fe content in root and shoot of *B. juncea* were detected respectively. Furthermore, the metal transport genes from *Nramp*, *HMA*, *CET* and *MTP* families were identified and the gene expression levels in roots, stems and leaves of *B. juncea* under 0, 1 and 5 μM were analyzed by qRT-PCR assays. The present study is necessary to reveal the effect on micronutrient uptake and translocation in *B. juncea* under Cd stress. Furthermore, this study provides important information for in-depth studies on the molecular mechanism of the micronutrient and Cd accumulation in the Cd hyperaccumulator Indian mustard.

Material and Methods

Plant Growth Condition and Cd Treatment

Seeds of Indian mustard (*Brassica juncea* cultivar with no.426308) were obtained from the North Central Regional Plant Introduction Station (D'Alessandro *et al.*, 2013). Seeds were surface sterilized by 6% (w/v) NaClO (including 0.05% Twain20) for 3 min, rinsed completely using sterile water and then vernalized in Petri dishes at 4°C in the dark for 2 days. The seeds were cultivated on filter paper in cultur room (25°C, 80 $\mu\text{mol}/\text{m}^2/\text{slight}$ 16 h/dark 8 h). Seedlings were transferred to plastic pots filled with 4 L of nutrient solution 6-day after germination, and solutions were renewed 3 days. The nutrient solution contain 2.0 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1.0 mM KH_2PO_4 , 3.0 mM KNO_3 , 0.6 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 60 μM H_3BO_3 , 20 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.0 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.4 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.6 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 40 μM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 40 μM EDTA-2Na.

After growing for 14 days, the seedlings were transplanted to other nutrient solutions with different concentrations of Cd i.e., 0, 1, 5, 10, 15 and 25 μM . We harvested the seedlings grown 12-day under Cd stress. After getting the total fresh weight of seedling, the shoot and root were separately held for measuring shoot and root fresh and dry weight by drying in an oven, and the content of metal elements.

Metal Element Contents

In order to measure the content of metal elements (Cd, Ca, Mg, Cu, Zn, Fe and Mn) in plant tissues, the Flame Atomic

Absorption Spectrometry (FAAS) was used after the digestion of plant samples with diacid (3 HNO₃: 1 HClO₄) and LaCl₃, a release agent to eliminate interference of phosphate (PO₄³⁻) and sulfate (SO₄²⁻), was used to dispose the solution.

RNA Isolation, cDNA Synthesis and Real-time Quantitative-PCR

Total RNAs in the *B. juncea* samples were extracted from frozen roots, stems and leaves using the EZ-10 DNA away RNA Mini-prep Kit (Sangon, Shanghai) according to the manufacturer's protocol. NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used to measure the concentration and quality of RNA samples. The synthesis of cDNA was relied on PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Tapan).

The relative expression of genes about ion transport was measured by Real-time quantitative-PCR. The sequences were obtained and downloaded from NCBI. All primers for RT-qPCR were designed based on Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>) and synthesized commercially (BGI, Beijing, China). All the expression data were obtained from two individual biological replicates and two independent technical replicates. *BjCAC* was treated as internal control gene.

Statistical Analysis

All data were from at least three replicates and were expressed as the mean ± standard error (SE). One way ANOVA was carried out with multiple comparisons using Duncan's test to compare the means of different treatments at $P \leq 0.05$. All statistical analyzes were performed using the SPSS 17.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Physiological Changes of *B. juncea* After Cd Treatment

To investigate the physiological and biochemical changes of *B. juncea* after Cd treatment, we detected the biomass of root and shoot of *B. juncea* grown in 1, 5, 10, 15 and 25 μM Cd concentration for 12 days. The result showed that the biomass of *B. juncea* was severely affected with increased concentrations of Cd. The leaves showed significantly chlorosis phenotype due to Cd toxicity (Fig. 1). The root morphology showed bending phenotype. Furthermore, we also measured the fresh weight of *B. juncea* under Cd treatment. The result showed that the biomass of root was significantly increased after 1 μM Cd treatment, but quickly decreased under 5 μM or higher Cd concentration treatment (Fig. 2A). This result revealed that *B. juncea* has strong ability against the Cd stress under lower Cd concentration.

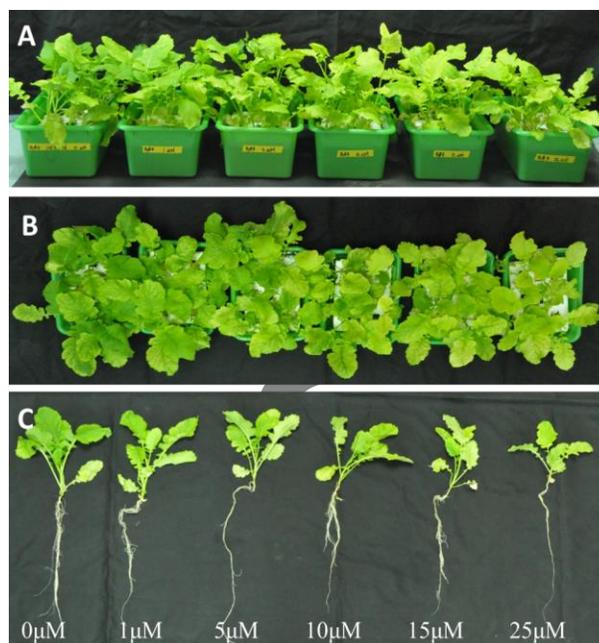


Fig. 1: The physiology phenotype of *B. juncea* after gradient Cd concentration treatment. After growing 14 days, the seedlings were transplanted to other nutrient solutions with different concentrations of Cd, and the Cd concentrations were 0, 1, 5, 10, 15 and 25 μM, respectively. We harvested the seedlings grown 12-day under Cd treatment

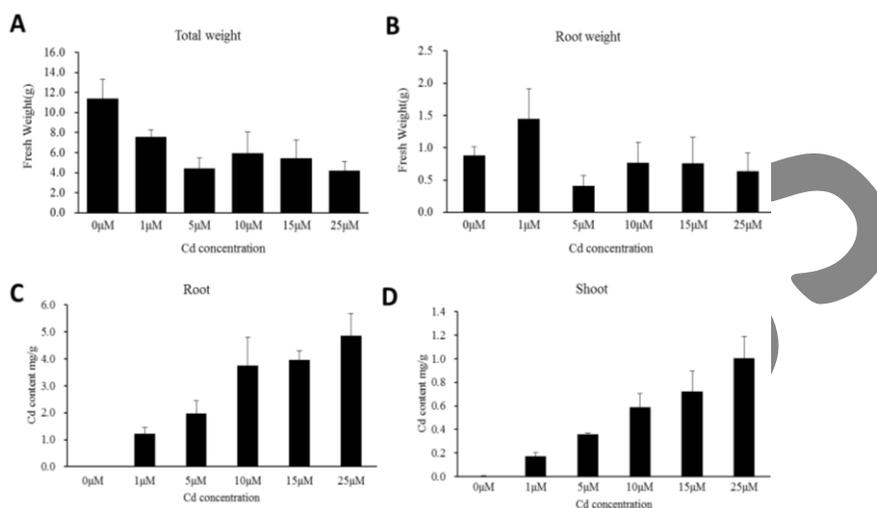
We further detected the Cd concentration in root and shoot of *B. juncea* under gradient Cd concentration stress. The result also showed that the root accumulated 5 times more Cd comparing with the shoot (Fig. 2B). To evaluate the translocation efficiency of Cd from nutrient to root or shoot, the translocation factors (TF) and Bioaccumulation factors (BCF) were investigated, respectively (Table 1). The result showed that both $BCF_{\text{root/solution}}$ and $BCF_{\text{shoot/solution}}$ were decreased with the gradient increased Cd concentration treatment. The TF of all detected micronutrients and Cd content showed no significant difference under different Cd treatment conditions.

Cd Stress Affected the Accumulation of Cu, Zn, Mn, and Fe in *B. juncea*

It is known that *B. juncea* were significantly affected by Cd stress. And previous studies revealed that light-independent carbon fixation reactions, ATP synthesis, photosynthesis efficiency etc. were significantly affected by Cd stress using proteomics and metabolomics analysis (Mohamed *et al.*, 2012; D'Alessandro *et al.*, 2013; Irfan *et al.*, 2014; Kapoor *et al.*, 2014; Wu *et al.*, 2015; Benakova *et al.*, 2017). However, the research on the effect of other metal accumulation and translocation in *B. juncea* under Cd stress remains unknown.

Table 1: BCF and TF for Cd and four mineral nutritions of different tested

Cd treatment / μ M	Cd			TF			
	BCF _{Root/Solution}	BCF _{Shoot/Solution}	Cd	Zn	Mn	Fe	Cu
0	NS	NS	NS	0.401 \pm 0.078 a	0.114 \pm 0.004 cd	0.061 \pm 0.008 ab	0.118 \pm 0.019 ab
1	1.226 \pm 0.229 a	0.171 \pm 0.034 a	0.141 \pm 0.030 b	0.291 \pm 0.050 bc	0.080 \pm 0.030 d	0.070 \pm 0.030 ab	0.127 \pm 0.042 ab
5	0.394 \pm 0.093 b	0.072 \pm 0.003 b	0.196 \pm 0.060 ab	0.293 \pm 0.037 bc	0.114 \pm 0.056 cd	0.080 \pm 0.023 a	0.159 \pm 0.024 a
10	0.375 \pm 0.106 bc	0.059 \pm 0.012 bc	0.162 \pm 0.030 ab	0.257 \pm 0.036 c	0.290 \pm 0.089 a	0.059 \pm 0.003 ab	0.120 \pm 0.031 ab
15	0.264 \pm 0.024 bc	0.048 \pm 0.012 bc	0.183 \pm 0.049 ab	0.317 \pm 0.092 abc	0.198 \pm 0.041 bc	0.077 \pm 0.035 ab	0.093 \pm 0.023 b
25	0.195 \pm 0.033 c	0.040 \pm 0.007 c	0.208 \pm 0.026 a	0.365 \pm 0.048 ab	0.282 \pm 0.077 ab	0.043 \pm 0.009 b	0.096 \pm 0.015 b

**Fig. 2:** The biomass of *B. juncea* and Cd accumulation in root and shoot of *B. juncea* after Cd treatment. (A, B) the total weight and root weight of *B. juncea* after Cd treatment. (C, D) the Cd content in root and shoot of *B. juncea* after Cd treatment

Therefore, we investigated the affection of Cd on the accumulation of Copper (Cu), Zipper (Zn), Manganese (Mn) and Iron (Fe) in root and shoot of *B. juncea*. The result showed that the Cu content in root was increased with the gradient increase of Cd treatment concentration from 0 until 25 μ M (Fig. 3A). The Zn content was also increased under all Cd treatment condition comparing with normal condition. However, the Zn content in root was up to the peak amount under 5 μ M Cd treatment condition and was stable or even decrease under 10, 15 or 25 μ M Cd treatment condition (Fig. 3B). Interestingly, the Mn content in root was quickly increased under 1 μ M Cd treatment condition and then decreased with the gradient increase of Cd treatment (Fig. 3C). The Fe content in root was not significantly affected by Cd stress (Fig. 3D). The Cu content, Zn content and Mn content in shoot were all increased with the gradient increased Cd treatment. However the Fe content in shoot was also not significant affected by Cd stress (Fig. 4). The result revealed that Cd stress mainly affected the accumulation of Cu, Zn, and Mn, but not Fe. And the affection was not constantly increase with the gradient increase of Cd treatment. The upper limit of the Cd concentration, which caused affection on Cu, Zn and Mn was between 1-5 μ M Cd treatment.

Identification of BjNramp Family in *B. juncea* under Cd Treatment

To further investigate the regulation of iron uptake and translocation under Cd treatment, we further analyzed the change of gene expression levels in some iron transporter families. NRAMPs were a large family of metal ion transporters. The previous studies revealed that NRAMPs are a major route of Cd transporters in plants (Takahashi et al., 2014). The Nramp3 in *A. thaliana* and *Thlaspi caerulescens* transported Fe, Mn and Cd, but not Zn. The Nramp4 in *Thlaspi caerulescens* can transport Fe, Mn, Cd and Zn (Oomen et al., 2009). The phylogenetic tree of NRAMP families in *A. thaliana*, *Brassica napus*, and *Brassica juncea* were constructed according to the related NRAMP protein sequences (Supplementary Fig. S1). The result revealed that 18 homologous of NRAMP members were identified in *B. juncea*. We then detected the changes of *BjNramp* genes in root, leaf and shoot under gradient Cd treatment conditions. The result showed that the expression levels of *BjNramp1;4*, *BjNramp3;5*, *BjNramp3;10*, *BjNramp4;4* in root were increased under 1 μ M Cd treatment condition comparing with normal condition.

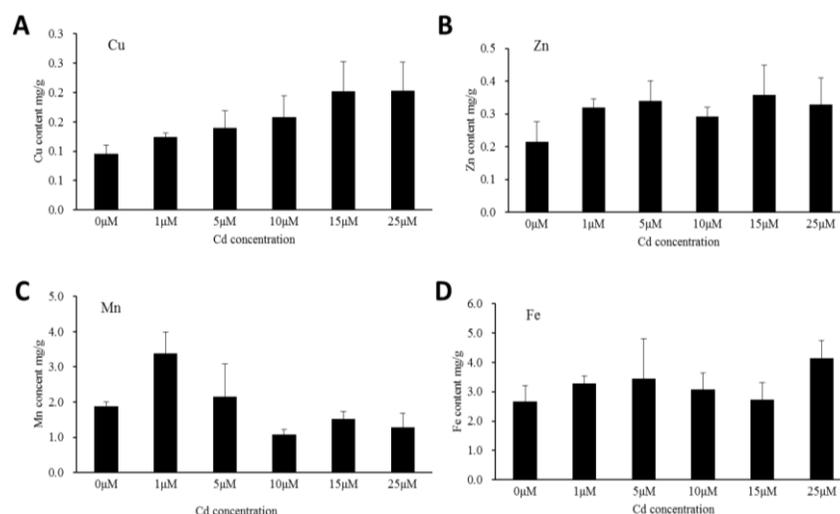


Fig. 3: The accumulation of Cu, Zn, Mn and Fe in the root of *B. juncea* after Cd treatment.

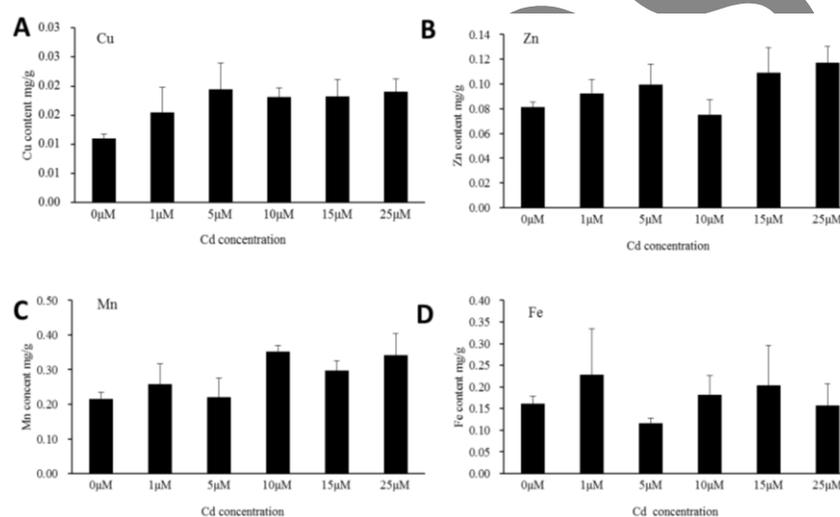


Fig. 4: The accumulation of Cu, Zn, Mn and Fe in the shoot of *B. juncea* after Cd treatment

However, the expression levels of *BjNramp3;1*, *BjNramp3;2*, *BjNramp3;3*, *BjNramp3;4*, *BjNramp3;9*, *BjNramp4;1*, and *BjNramp4;3* in root were decreased under 1 μM Cd treatment condition comparing with normal condition. In the shoot tissues, the expression levels of almost all *BjNramp* genes were down regulated in stems under 1 μM Cd treatment condition comparing with normal condition, except the levels of *BjNramp6;3* and *BjNramp6;4*. Furthermore, the expression of 7 *BjNramp* genes (*BjNramp1;4*, *BjNramp3;4*, *BjNramp3;10*, *BjNramp4;1*, *BjNramp4;3*, *BjNramp6;3* and *BjNramp6;4*) were up regulated in leaves under 1 μM Cd treatment condition comparing with normal condition. To be mentioned, almost all genes in all tissues were down regulated under 5 μM Cd treatment condition comparing with normal or 1 μM Cd treatment conditions, except *BjNramp1;4*, *BjNramp6;3* and *BjNramp6;4* in leaves (Fig. 5).

Identification of *BjHMA* and *BjCET* Family in *B. juncea* under Cd Treatment

The PIB-ATPase subfamily HMA (for Heavy Metal Associated) plays an important role in the process of micronutrient translocation or heavy metal detoxification (Morel *et al.*, 2009). The eight *HMA* genes in *A. thaliana* cluster in two subgroups depending on heavy metal specificity. *AtHMA1-4s* belong to Zn/Co/Cd/Pb subgroup and *AtHMA5-8s* belong to Cu/Ag subgroup (Williams and Mills, 2005). The phylogenetic analysis revealed that three *HMA* genes exist in *B. juncea* (Supplementary Fig. S2). We further investigated the expression levels of *BjHMA* genes, which were homologues of *AtHMA3* and *AtHMA4*. The result showed that both the expression levels of *BjHMA3* and *BjHMA4* in root were obviously higher than that in stem and leaf, and both were up

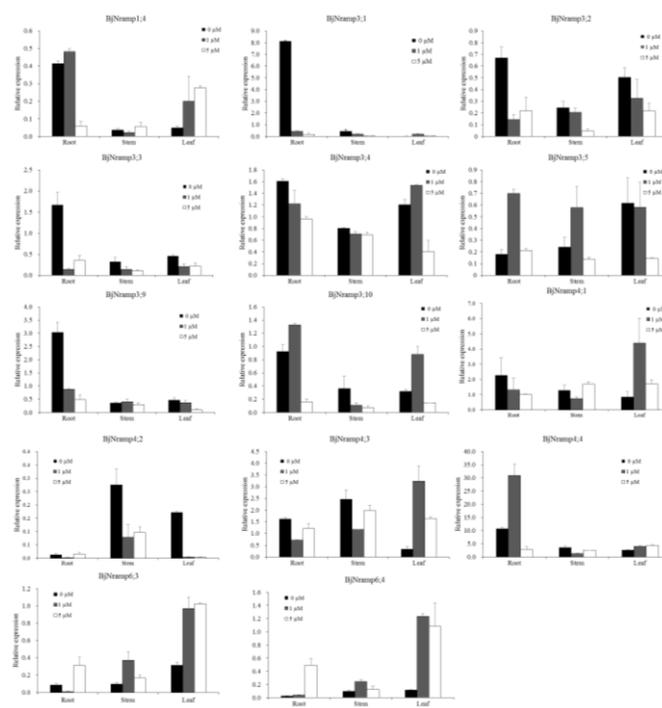


Fig. 5: The expression regulation of *BjNramps* in the roots, stems and leaves of *B. juncea* after Cd treatment

regulated under 1 μM Cd treatment condition comparing with normal condition. However, both were then significantly decreased under 5 μM Cd treatment condition comparing with 1 μM Cd treatment condition and normal condition (Fig. 6). The result revealed that the detoxification system of *BjHMA3* and *BjHMA4* were positively regulated under lower (1 μM) Cd treatment condition.

The cation-efflux family transporters (CET) was previously identified from Cd treated seedlings of *B. juncea*. *BjCET2* was characterized in mediating Zn and Cd accumulation in *B. juncea* (Xu et al., 2009). And heterologous expression of *BjCET3* and *BjCET4* in yeast revealed their functions in transport Zn, and possibly Cd, Co, and Ni (Lang et al., 2011). To investigate the relation between the Cd/Zn accumulation in *B. juncea* and the expression of *BjCET* family, we further measured the expression of all 4*BjCET* genes in *B. juncea* (Supplementary Fig. S3). The result showed that the *BjCET1-4* was mainly expressed in leaves. And the expression levels of *BjCET1-4* in leaves were all down regulated under 1 μM and 5 μM Cd treatment condition comparing with normal condition (Fig. 6).

Identification of *BjMTP* Family in *B. juncea* under Cd Treatment

Another cation-efflux protein family, named MTP was shown to be specific for Mn and Zn export from the cytoplasm (Peiter et al., 2007). In *A. thaliana*, the 12 MTP proteins can be divided into three distinct groups. AtMTP1-4 form a phylogenetic subgroup. AtMTP1 and AtMTP3 are

two Zn transporter, which are localized to the vacuolar membrane. AtMTP8-11 form another subgroup according to the phylogenetic tree. AtMTP11 encoded a Mn transporter (Delhaize et al., 2007). AtMTP12 formed a functional complex with AtMTP5 for Zn transport into the Golgi (Fujiwara et al., 2015). To investigate the role of MTP for the Zn/Mn accumulation in root or shoot of *B. juncea*, the *MTP* genes were identified and the related expression levels were analyzed under gradient Cd concentration treatment. The phylogenetic tree revealed that nine *BjMTP* genes exist in *B. juncea* (Supplementary Fig. S4). We then chose 5 *BjMTP* genes for qRT-PCR assay. The expression levels of *BjMTP3* and *BjMTP5* in leaves were significantly up regulated under 1 μM Cd treatment condition comparing with normal condition. *BjMTP4* was mainly expressed in leaves of *B. juncea*. However, the expression of *BjMTP4* was significantly decreased in both roots and leaves under 1 or 5 μM Cd treatment condition (Fig. 7). Both *BjMTP11;1* and *BjMTP11;4* in leaves was up regulated under 1 μM Cd and down regulated later under 5 μM Cd treatment condition.

Discussion

As already known, the agricultural soil Cd contamination concentration is lower or near 1 μM . Therefore, it is important to know the key genes regulate the Cd and other metal uptake into root of *B. juncea*. Cd is not only toxic to animals, humans, and some microbes, but also unnecessary to plant growth (Uraguchi and Fujiwara, 2013).

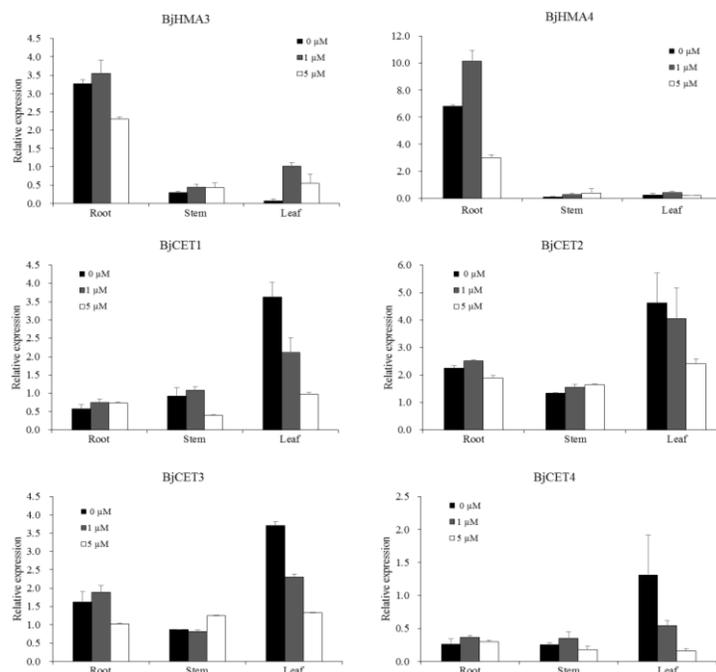


Fig. 6: The expression regulation of *BjHMAs* and *BjCETs* in the roots, stems and leaves of *B. juncea* after Cd treatment

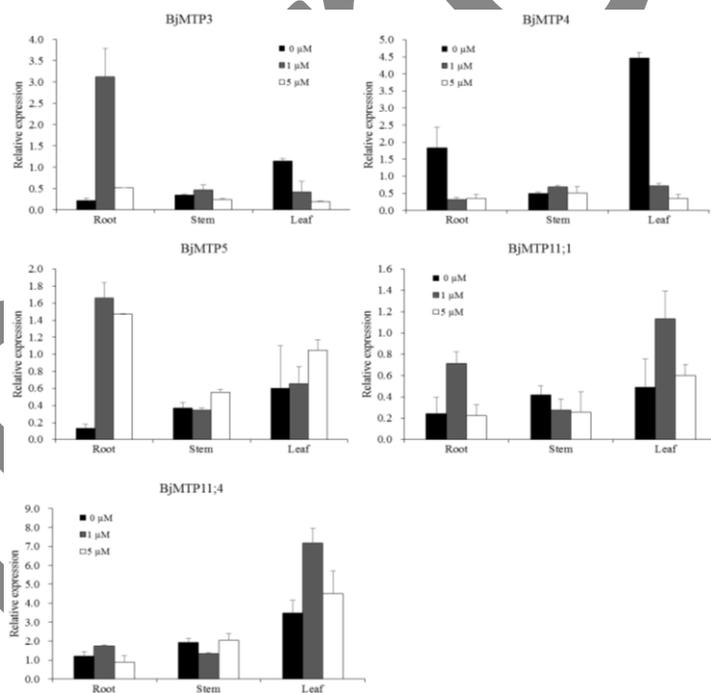


Fig. 7: The expression regulation of *BjMTPs* in the roots, stems and leaves of *B. juncea* after Cd treatment

However, Cd shares chemical similarity to Zn, Fe, and Mn and can thus replace them in the ionic related groups of many proteins in plant cells (D'Alessandro *et al.*, 2013). Therefore, Cd can selectively share different micronutrient uptake and translocation pathway in plants, and further affect the plant growth. As shown in

Fig. 1 and Fig. 2, the *B. juncea* biomass, especially the shoot biomass, was severely affected with the gradient increased Cd treatment. Furthermore, the BCF and TF result revealed that the transfer efficiency of Cd was increased with the increase of Cd treatment (Table 1).

Considering that Cd may compete with many micronutrients for translocation in plants, it is necessary to know the detail affection to each micronutrient. In the present study, we detected the Zn, Fe, Mn and Cu contents of root and shoot under 0 to 25 μM Cd treatment. The result revealed that Cd significantly improved the accumulation of Cu in roots, and weakly increased Zn accumulation in roots. Mn content was firstly increased under 1 μM Cd treatment comparing with normal condition, but then decreased under gradient increased Cd treatment from 5 μM to 25 μM (Fig. 3). It is known that mitigation of Cd accumulation in rice affected by Fe fertilization (Chen *et al.*, 2017). However, Cd weakly decreased the Fe accumulation in roots. Interestingly, Cd treatment very weakly affected the micronutrient content in shoot (Fig. 4). The detection of Cd in shoot revealed that only 20% Cd accumulated in shoot when comparing with the Cd content in root. Therefore, the Cd content in shoot is too low to make significant affection to micronutrient Zn, Fe, Mn and Cu.

Many studies have shown that some micronutrient and heavy metal uptake and accumulation is closely related to the characters of Nramp, HMA, and MTPs in other plants. However, the related research in the hyperaccumulator *B. juncea* remains unknown. In the present study, the phylogenetic analysis of Nramp, HMA, CET and MTPs in *A. thaliana*, *B. napus*, *B. juncea* identified 18 *BjNramp*, 4 *BjHMA*, 3 *BjCET*, and 9 *BjMTP* genes in *B. juncea* (Supplementary Fig. S1-S4 and Supplementary Table S1). In *A. thaliana*, AtNramps were implicated in the Mn and Cd transport. AtHMAs were mainly involved in Zn, Cu and Cd transport. As shown in previous studies, BjCETs were characterized as Fe and Cd transport. AtMTPs were related to Zn and Fe transport. qRT-PCR analysis showed that the expression levels of *BjNramp1;4*, *BjNramp3;5*, *BjNramp3;10*, *BjNramp4;4*, *BjHMA3*, and *BjHMA4* in root were increased under 1 μM Cd treatment condition comparing with normal condition. Oppositely, neither *BjCETs* nor *BjMTPs* in roots was increased under Cd treatment condition. This result revealed that the candidates from *BjNramp1;4*, *BjNramp3;5*, *BjNramp3;10*, *BjNramp4;4*, *BjHMA3*, and *BjHMA4* are involved in the uptake of Cd, Cu, Zn and Mn into roots under low (1 μM) Cd treatment condition (Fig. 5, 6 and 7). To be mentioned, there should also be many other enzymes and proteins, which are related to Cd and other micronutrients uptake and translocation, and these proteins were not analyzed here (Uraguchi *et al.*, 2014; Chen *et al.*, 2015).

Conclusion

The biomass of *B. juncea* was severely decreased with Cd stress. Furthermore, the accumulation of the plant essential nutrients Zn, Mn, Fe and Cu were affected in varying of degrees. Cd treatment significantly improved the uptake of Cu, and maybe the Zn, but negatively affected the uptake of Mn (>1 μM Cd condition). The qRT-PCR assays of

BjNramp, *BjHMA*, *BjCET* and *BjMTP* showed that low concentrations of Cd (1 μM) can promote the Mn, Zn and Cu accumulation system, which improve the increase of root biomass. The result revealed that *BjNramp1;4*, *BjNramp3;5*, *BjNramp3;10*, *BjNramp4;4*, *BjHMA3*, and *BjHMA4* may play a key role for the uptake and accumulation of Cd into roots. The In-depth studies on the molecular mechanism of the micronutrient and Cd accumulation for developing safe plant-derived food are imperative in future.

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References

- Ahmad, P., M. Sarwat, N.A. Bhat, M.R. Wani, A.G. Kazi and L.S. Tran, 2015. Alleviation of cadmium toxicity in *Brassica juncea* L. (Czern. & Coss.) by calcium application involves various physiological and biochemical strategies. *PLoS One*, 10: 1-17
- Arrivault, S., T. Senger and U. Kramer, 2006. The Arabidopsis metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. *Plant J.*, 46: 861-879
- Benáková, M., H. Ahmadi, Z. Ducaiova, E. Tylova, S. Clemens and J. Tuma, 2017. Effects of Cd and Zn on physiological and anatomical properties of hydroponically grown *Brassica napus* plants. *Environ. Sci. Pollut. Res.*, 24: 20705-20716
- Chen, J., L. Yang, J. Gu, X. Bai, Y. Ren, T. Fan, Y. Han, L. Jiang, F. Xiao, Y. Liu and S. Cao, 2015. MAN3 gene regulates cadmium tolerance through the glutathione-dependent pathway in *Arabidopsis thaliana*. *New Phytol.*, 205: 570-582
- Chen, Z., Y.T. Tang, A.J. Yao, J. Cao, Z.H. Wu, Z.R. Peng, S.Z. Wang, S. Xiao, A.J.M. Baker and R.L. Qiu, 2017. Mitigation of Cd accumulation in paddy rice (*Oryza sativa* L.) by Fe fertilization. *Environ. Pollut.*, 231: 549-559
- Choppala, G., N. Saifullah, S. Bibi, M. Iqbal, Z. Rengel, A. Kunhikrishnan, N. Ashwath and Y.S. Ok, 2014. Cellular Mechanisms in Higher Plants Governing Tolerance to Cadmium Toxicity. *Crit. Rev. Plant Sci.*, 33: 374-391
- Clemens, S., M.G. Aarts, S. Thomine and N. Verbruggen, 2013. Plant science: the key to preventing slow cadmium poisoning. *Trends Plant Sci.*, 18: 92-99
- D'Alessandro, A., M. Taamalli, F. Gevi, A.M. Timperio, L. Zolla and T. Ghnaya, 2013. Cadmium stress responses in *Brassica juncea*: hints from proteomics and metabolomics. *J. Proteom. Res.*, 12: 4979-4997
- Delhaize, E., B.D. Gruber, J.K. Pittman, R.G. White, H. Leung, Y. Miao, L. Jiang, P.R. Ryan and A.E. Richardson, 2007. A role for the AtMTP11 gene of *Arabidopsis* in manganese transport and tolerance. *Plant J.*, 51: 198-210
- Delhaize, E., T. Kataoka, D.M. Hebb, R.G. White and P.R. Ryan, 2003. Genes encoding proteins of the cation diffusion facilitator family that confer manganese tolerance. *Plant Cell*, 15: 1131-1142
- Fujiwara, T., M. Kawachi, Y. Sato, H. Mori, N. Kutsuna, S. Hasezawa and M. Maeshima, 2015. A high molecular mass zinc transporter MTP12 forms a functional heteromeric complex with MTP5 in the Golgi in *Arabidopsis thaliana*. *FEBS J.*, 282: 1965-1979

- Gallego, S.M., L.B. Pena, R.A. Barcia, C.E. Azpilicueta, M.F. Iannone, E.P. Rosales, M.S. Zawoznik, M.D. Groppa and M.P. Benavides, 2012. Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. *Environ. Exp. Bot.*, 83: 33-46
- Gravot, A., A. Lieutaud, F. Verret, P. Auroy, A. Vavasseur and P. Richaud, 2004. AtHMA3, a plant P1B-ATPase, functions as a Cd/Pb transporter in yeast. *FEBS Lett.*, 561: 22-28
- Hussain, D., M.J. Haydon, Y. Wang, E. Wong, S.M. Sherson, J. Young, J. Camakaris, J.F. Harper and C.S. Cobbett, 2004. P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in Arabidopsis. *Plant Cell*, 16: 1327-1339
- Irfan, M., A. Ahmad and S. Hayat, 2014. Effect of cadmium on the growth and antioxidant enzymes in two varieties of *Brassica juncea*. *Saudi J. Biol. Sci.*, 21: 125-131
- Ishimaru, Y., R. Takahashi, K. Bashir, H. Shimo, T. Senoura, K. Sugimoto, K. Ono, M. Yano, S. Ishikawa, T. Arai, H. Nakanishi and N.K. Nishizawa, 2012. Characterizing the role of rice NRAMP5 in Manganese, Iron and Cadmium Transport. *Sci. Rep.*, 2: 286-294
- Kapoor, D., S. Kaur and R. Bhardwaj, 2014. Physiological and biochemical changes in *Brassica juncea* plants under Cd-induced stress. *Biomed. Res. Int.*, 2014: 726070-79
- Kaur, R., P. Yadav, A. Sharma, A. Kumar Thukral, V. Kumar, S. Kaur Kohli and R. Bhardwaj, 2017. Castasterone and citric acid treatment restores photosynthetic attributes in *Brassica juncea* L. under Cd(II) toxicity. *Ecotoxicol. Environ. Saf.*, 145: 466-475
- Kobae, Y., T. Uemura, M.H. Sato, M. Ohnishi, T. Mimura, T. Nakagawa and M. Maeshima, 2004. Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. *Plant Cell Physiol.*, 45: 1749-1758
- Lang, M., M. Hao, Q. Fan, W. Wang, S. Mo, W. Zhao and J. Zhou, 2011. Functional characterization of BjCET3 and BjCET4, two new cation-efflux transporters from *Brassica juncea* L. *J. Exp. Bot.*, 62: 4467-4480
- Luo, Z.B., J. He, A. Polle and H. Rennenberg, 2016. Heavy metal accumulation and signal transduction in herbaceous and woody plants: Paving the way for enhancing phytoremediation efficiency. *Biotechnol. Adv.*, 34: 1131-1148
- Maser, P., S. Thomine, J.I. Schroeder, J.M. Ward, K. Hirschi, H. Sze, I.N. Talke, A. Amtmann, F.J. Maathuis, D. Sanders, J.F. Harper, J. Tchieu, M. Gribskov, M.W. Persans, D.E. Salt, S.A. Kim and M.L. Gueriot, 2001. Phylogenetic relationships within cation transporter families of Arabidopsis. *Plant Physiol.*, 126: 1646-1667
- Masood, A., M.I. Khan, M. Fatma, M. Asgher, T.S. Per and N.A. Khan, 2016. Involvement of ethylene in gibberellic acid-induced sulfur assimilation, photosynthetic responses, and alleviation of cadmium stress in mustard. *Plant Physiol. Biochem.*, 104: 1-10
- Mohamed, A.A., A. Castagna, A. Ranieri and L. Sanita di Toppi, 2012. Cadmium tolerance in *Brassica juncea* roots and shoots is affected by antioxidant status and phytochelatin biosynthesis. *Plant Physiol. Biochem.*, 57: 15-22
- Morel, M., J. Crouzet, A. Gravot, P. Auroy, N. Leonhardt, A. Vavasseur and P. Richaud, 2009. AtHMA3, a P1B-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in Arabidopsis. *Plant Physiol.*, 149: 894-904
- Oomen, R.J., J. Wu, F. Lelievre, S. Blanchet, P. Richaud, H. Barbier-Brygoo, M.G. Aarts and S. Thomine, 2009. Functional characterization of NRAMP3 and NRAMP4 from the metal hyperaccumulator *Thlaspi caerulescens*. *New Phytol.*, 181: 637-650
- Peiter, E., B. Montanini, A. Gobert, P. Pedas, S. Husted, F.J. Maathuis, D. Blaudez, M. Chalot and D. Sanders, 2007. A secretory pathway-localized cation diffusion facilitator confers plant manganese tolerance. *Proc. Natl. Acad. Sci. USA*, 104: 8532-8537
- Sasaki, A., N. Yamaji and J.F. Ma, 2014. Overexpression of OsHMA3 enhances Cd tolerance and expression of Zn transporter genes in rice. *J. Exp. Bot.*, 65: 6013-6021
- Sasaki, A., N. Yamaji, K. Yokosho and J.F. Ma, 2012. Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice. *Plant Cell*, 24: 2155-2167
- Sato-Nagasawa, N., M. Mori, K. Sakurai, H. Takahashi, A. Watanabe and H. Akagi, 2013. Functional relationship heavy metal P-type ATPases (OsHMA2 and OsHMA3) of rice (*Oryza sativa*) using RNAi. *Plant Biotechnol.*, 30: 511-515
- Shen, G., J. Niu and Z. Deng, 2017. Abscisic acid treatment alleviates cadmium toxicity in purple flowering stalk (*Brassica campestris* L. ssp. chinensis var. purpurea Hort.) seedlings. *Plant Physiol. Biochem.*, 118: 471-478
- Siemianowski, O., A. Barabas, M. Kendziorek, A. Ruszczynska, E. Bulska, L.E. Williams and D.M. Antosiewicz, 2014. HMA4 expression in tobacco reduces Cd accumulation due to the induction of the apoplastic barrier. *J. Exp. Bot.*, 65: 1125-1139
- Takahashi, R., Y. Ishimaru, T. Senoura, H. Shimo, S. Ishikawa, T. Arai, H. Nakanishi and N.K. Nishizawa, 2011. The OsNRAMP1 iron transporter is involved in Cd accumulation in rice. *J. Exp. Bot.*, 62: 4843-4850
- Thomine, S., R. Wang, J.M. Ward, N.M. Crawford and J.I. Schroeder, 2000. Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes. *Proc. Natl. Acad. Sci. USA*, 97: 4991-4996
- Uraguchi, S. and T. Fujiwara, 2013. Rice breaks ground for cadmium-free cereals. *Curr. Opin. Plant Biol.*, 16: 328-334
- Uraguchi, S., T. Kamiya, S. Clemens and T. Fujiwara, 2014. Characterization of OsLCT1, a cadmium transporter from indica rice (*Oryza sativa*). *Physiol. Plant.*, 151: 339-347
- Williams, L.E. and R.F. Mills, 2005. P(1B)-ATPases--an ancient family of transition metal pumps with diverse functions in plants. *Trends Plant Sci.*, 10: 491-502
- Wu, Z., X. Yin, G.S. Banuelos, Z.Q. Lin, Y. Liu, M. Li and L. Yuan, 2016. Indications of Selenium Protection against Cadmium and Lead Toxicity in Oilseed Rape (*Brassica napus* L.). *Front Plant Sci.*, 7: 1875-1885
- Wu, Z., X. Zhao, X. Sun, Q. Tan, Y. Tang, Z. Nie, C. Qu, Z. Chen and C. Hu, 2015. Antioxidant enzyme systems and the ascorbate-glutathione cycle as contributing factors to cadmium accumulation and tolerance in two oilseed rape cultivars (*Brassica napus* L.) under moderate cadmium stress. *Chemosphere*, 138: 526-536
- Xu, J., T. Chai, Y. Zhang, M. Lang and L. Han, 2009. The cation-efflux transporter BjCET2 mediates zinc and cadmium accumulation in *Brassica juncea* L. leaves. *Plant Cell Rep.*, 28: 1235-1242
- Yu, R., D. Li, X. Du, S. Xia, C. Liu and G. Shi, 2017. Comparative transcriptome analysis reveals key cadmium transport-related genes in roots of two pak choi (*Brassica rapa* L. ssp. chinensis) cultivars. *BMC Genom.*, 18: 587-593

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