MicroRNAs-mRNAs Expression Profile and Their Potential Role in Cadmium Stress Response in *Brassica napus*

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Abstract

Background: Oilseed rape is an excellent candidate for phytoremediation of cadmium-contaminated soils given its advantages of high biomass, fast growth, moderate metal accumulation, ease of harvesting, and metal tolerance, but the cadmium response pathways in this species (*Brassica napus*) have yet to be fully elucidated. A combined analysis of miRNA and mRNA expression data to infer Cd-induced transcriptional regulation has not been previously been reported in *B. napus*. Results: In the current study, we characterized concurrent changes in miRNA and mRNA profiles in the roots and shoots of *B. napus* seedlings after 10 days of 10 mg/L Cd²⁺ treatment. In total, 39 miRNAs belonging to 14 families were identified in the root, and 53 miRNAs belonging 16 miRNA families were identified in the shoot, of which the miR397, miR395, miR408, miR398 and miR858 families showed the most significant expression changes in response to Cd stress. A further 399 in the root and 389 differentially expressed genes (DEGs) in the shoot were identified, with very little overlap between tissue types. A total of 24 root and 16 shoot anti-regulation miRNA-mRNA interaction pairs (up-regulation of miRNA and down-regulation of mRNA or vice-versa) were identified in response to Cd, which involved in the key pathways of plant stress response, e.g. four genes targeted by one miRNA were involved in a pathway for detoxification of superoxide radicals. Cd stress significantly impacted the photosynthetic pathway. We identified transcription factor activation, antioxidant response pathways and secondary metabolic processes such as glutathione (GSH) and phenylpropanoid metabolism as major components in the response to Cd-induced stress in both roots and shoots. Conclusions: miRNAs and mRNAs showed concurrent expression changes for cadmium response in *Brassica napus*. The leaves and roots showed several overlap of differentially expressed miRNAs but very little overlap of differentially expressed mRNAs for Cd response. Several anti-regulation miRNA-mRNA interaction pairs were identified, which involved in the key pathways of plant stress response like detoxification of superoxide radicals. Photosynthetic pathway, transcription factor activation, antioxidant response and secondary metabolic processes were major components for cadmium response.

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types. A total of 24 root and 16 shoot anti-regulation miRNA-mRNA interaction pairs (up-regulation of miRNA and down-regulation of mRNA or vice-versa) were identified in response to Cd, which involved in the key pathways of plant stress response, e.g. four genes targeted by one miRNA were involved in a pathway for detoxification of superoxide radicals. Cd stress significantly impacted the photosynthetic pathway. We identified transcription factor activation, antioxidant response pathways and secondary metabolic processes such as glutathione (GSH) and phenylpropanoid metabolism as major components in the response to Cd-induced stress in both roots and shoots.

Conclusions: miRNAs and mRNAs showed concurrent expression changes for cadmium response in *Brassica napus*. The leaves and roots showed several overlap of differentially expressed miRNAs but very little overlap of differentially expressed mRNAs for Cd response. Several anti-regulation miRNA-mRNA interaction pairs were identified, which involved in the key pathways of plant stress response like detoxification of superoxide radicals. Photosynthetic pathway, transcription factor activation, antioxidant response and secondary metabolic processes were major components for cadmium response.

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Background

Cadmium (Cd) is one of the most serious major soil contaminants, released in significant quantities into the environment through burning and mining of fossil fuels, run-off of trace metal-containing fertilizers, and through its presence in sewage sludge and fungicides [1]. Because of the mobility of Cd in soils and the ready accumulation of Cd by crops, soil Cd contamination has drawn worldwide attention [2]. Cd is not an essential element for plants, but it can be readily absorbed by roots and transported to the above-ground organs [3], subsequently affecting crop quality and food safety at low concentrations. 40mg/L Cd in soil was already found to affect yield in some crops, while 200mg/L had serious effects [4]. High Cd concentrations damage plant growth and reproduction through decreasing nutrient uptake, inhibiting photosynthetic activity [5], distorting membranes [6], and stunting plant growth [7-8], resulting in overall decreases in crop yield [3, 9]. Cd accumulation in the human body over time poses serious health risks, including a risk of chronic toxicity in kidney tubules, bones, lungs and other organs [10-11]. Thus, Cd contamination in soil affects not only crop productivity, but also causes risks to food safety [12].

Phytoremediation, where plants are used to remove heavy metals from soils, is an environmentally friendly, cost-effective technique for remediation of heavy metal-contaminated soils [13-15]. This technique relies on plant hyper-accumulation of heavy metals from soils, subsequently reducing the level of heavy metals in the soil [16]. An alternative technique to minimize heavy metals entering the food chain without treating contaminated soils has also been proposed, which is to breed cultivars or genotypes (or genetically modify plant traits) to produce crops which take up very low amounts of heavy metals [10, 17]. For both phytoremediation or hyperaccumulation and heavy metal exclusion strategies, understanding the mechanisms by which plants respond to Cd stress is desirable.

Plants have evolved a range of mechanisms for heavy metal detoxification, including pumping heavy metals out of the plasma membrane, chelation, and binding to various thiol compounds in the cytosol following by sequestration into vacuoles [18-20]. Antioxidant and signaling mechanisms also participate in the process [20-22]. Glutathione (GSH) is a well documented and also highly essential component for Cd detoxification [23-30], and GSH- or phytochelatin (PC)-conjugated vacuolar sequestration is one of the most important mechanisms of Cd accumulation and tolerance in plants [23, 31-34]. Regulation of GSH and GSH-related gene expression is fundamental to plant Cd detoxification [35-37]. In addition, many other key genes have been also shown to be involved in Cd detoxification and tolerance in plants, such as *ZNT* [38], *HMA* [34], *NRAMP3*, 4, 5 [34, 39-40], *ZntA* [41], *CAD2* [42], *PDR8* [43-44], *PDR12* [44], *ATM3* [45], *ACBP* [46], *EIN2* [47], *ABCC1*, 2 [34], *ZIF1* [34], *MTP1*, 3 [34], *PCR1*, 2 [34], and *MAN3* [34].

MicroRNAs (miRNAs) are a class of small non-coding RNAs (sRNAs) that regulate gene expression transcriptionally by guiding mRNA cleavage of target genes and post-transcriptionally by inhibiting translation [49]. MiRNAs play important roles in biological processes such as organ development and signal transduction, as well as in biotic and abiotic stress responses [50-57]. In particular, miRNAs are considered key factors in heavy metal regulatory networks. Cd-responsive miRNAs have been identified in rice (*Oryza* spp.) [58-59], narrowleaf cattail (*Typha angustifolia*) [60], *Medicago truncatula* [61], and in radish (*Raphanus* spp.) [62], strongly supporting miRNAs as key post-transcriptional regulators of heavy metal stress responses in plants [63].

Oilseed rape (*Brassica napus*) is one of the most important oil crops worldwide, and an excellent candidate for phytoremediation given its advantages of high biomass, fast growth, moderate metal accumulation, ease of harvesting, and metal tolerance [21]. A number of studies have elucidated the physiological responses of *B. napus* to Cd stress [64-70], with identification of key Cd-stress-responsive genes *BnPCS1*, *BnGSTU12*, *BnGSTU5*, and *BnHMAs*. Three major studies to date have attempted to identify Cd-responsive miRNAs through genome-wide studies in *B. napus* [71-73]. Huang et al. (2010) found 58 miRNAs after small RNA sequencing of root, stem and leaf tissue after treatment with 80 μ M Cd (about 15 mg/L) for 12, 24, 48 and 72 h, of which 9 miRNAs were differentially expressed as a result of Cd stress in at least one tissue type relative to the control [71]. Zhou et al. (2012) found 84 miRNAs in root and shoot tissue of young seedlings after Cd treatment (four libraries, Cd-treated pools derived from treatments of 40 and 80 μ M for 6–48 h) of which 13 miRNA families and five novel miRNAs were differentially expressed in the shoot and 8 miRNA families were differentially expressed in the root; at least 43 high-confidence gene targets of differentially expressed miRNAs in the roots and 126 convincing gene targets in the shoots were identified through degradome analysis [73]. Jian et al. (2018) looked at miRNA and mRNA expression in response to 1000 μ M CdCl₂ treatment (approximately 180 mg/L) in whole *B. napus* seedlings 0, 1 and 3 days after treatment (three libraries total), and identified 147 miRNAs, of which 39 were differentially expressed in response to Cd treatment, but for which target genes were only computationally predicted from the reference genome [72]. To date, studies have rarely profiled genome-wide miRNA and mRNA (gene expression) simultaneously under Cd stress. The optimal Cd concentration and treatment time with which to assess stress response is also still unclear. To acquire a

deeper understanding of how miRNA might interact with mRNA to regulate response to Cd stress in *B. napus*, we sequenced miRNA and mRNA in root and shoot tissues of oilseed rape seedlings after an optimized Cd-stress treatment of 10 days at 10 mg/L and in controls, in order to help elucidate the molecular genetic mechanisms underlying oilseed rape response to Cd stress.

Results

Changes of Leaf Chlorophyll Content response to Cd stress in B. napus

Leaf chlorophyll content clearly indicated response to Cd stress (Figure 1a). Across all Cd treatment concentrations leaf chlorophyll content generally decreased until about day 10, then started to increase again (Figure 1b), such that **minimum** chlorophyll content occurred at 10 days after initiation of the treatment. Of the different Cd treatments, 10 mg/L Cd²⁺ for 10 days resulted in the maximum decrease in chlorophyll content. Thus, the seedlings treated with 10 mg/L Cd²⁺ for 10 days were used for analysis of miRNA and mRNA expression profiles in response to Cd stress.

Construction and sequencing of small RNA libraries

The 12 small RNA libraries (root control, root Cd treatment, shoot control, shoot Cd treatment; three biological replicates for each sample) yielded 284M raw reads total, resulting in 239M clean reads (average 20M clean reads per library), of which 74M clean reads could be mapped to the reference genome (Table S2). A total of 265 miRNAs were identified in the 12 libraries, of which the most abundant were 21 nt miRNAs, followed by 24 nt, 22 nt and 20 nt miRNAs (Figure 2a). Mature 20 – 22 bp mostly started with 'U' as the first base (62 - 100%), while 24 bp miRNAs mostly started with 'A' (77%) (Figure 2b). Of the 265 miRNAs identified 79 were previously known (30%). Known miRNAs belonged to 26 families: 11 families contained one miRNA, three families contained two miRNAs and 12 families contained three or more miRNAs. Of the 186 newly predicted miRNAs (sequences shown in Table S3), 52 families were identified: 27 contained one miRNA, 11 contained two miRNAs and 14 families contained three or more miRNAs.

MicroRNA profiling of oilseed rape under Cd stress

Of the 265 miRNAs, 53 (20%) were identified as differentially expressed under Cd stress relative to the control (Figure 3a). Of these differentially expressed miRNAs, 20 (12 novel and 8 previously known) showed the same relative expression in both the root and shoot, with 19/20 showing up-regulation under Cd stress (Figure 3b).

Of the 25 miRNA families identified, 14 included miRNA differentially expressed in the root (33 miRNAs) and 16 included miRNA differentially expressed in the shoot (46 miRNAs) in response to Cd stress (Table

1). The remaining six differentially expressed miRNAs in the root and seven in the shoot were independent of the known miRNA families (Table 1). Of the 25 miRNA families, five families were up-regulated in both root and shoot under Cd stress, nine families were differentially expressed only in the root (one family up-regulated and eight families down-regulated) and 11 miRNA families were differentially expressed only in the shoot (seven families up-regulated, three families down-regulated, and one family with both up- and down-regulated miRNAs) (Table 1). Of the remaining differentially expressed miRNAs independent of the known miRNA families, two miRNAs were common to both root and shoot, four were only differentially expressed in the root, and five were only differentially expressed in the shoot.

Global mRNA expression profiles in the oilseed rape root and shoot in response to Cd stress

The same 12 samples used for miRNA sequencing were also used to generate global mRNA expression profiles of oilseed rape seedlings in response to Cd stress: 44 – 64 M clean reads were generated, with 61 – 70% read mapping to the reference genome (Table S4). In all, 103 816 unigenes consisting of 2,776 new genes and 101 040 reference genes were obtained, of which 98.6% (102 392) could be annotated (including 102 351, 65 343, 77 895, 29 203, 32 074 and 92 782 unigenes in the Nr, Swiss-Prot, GO, COG, KEGG, eggNOG databases, respectively).

Transcripts detected with at least two-fold differences ($|\log_2\text{Ratio}| \geq 1$ and $\text{FDR} < 0.001$) in the Cd treatment libraries compared with the control samples were included in our analysis to identify differentially expressed genes. There were 103 up-regulated and 296 down-regulated genes in the root, and 148 up-regulated and 241 down-regulated genes in the shoot (Figure 4). Eight genes were commonly differentially expressed in both root and shoot tissues. Except for one differentially expressed gene (DEG) which was up-regulated in the shoot but down-regulated in the root, the remaining seven DEGs showed the same expression patterns in both root and shoot, with six genes up-regulated and one gene down-regulated.

Gene ontology (GO) and pathway enrichment analysis for genes differentially expressed in response to Cd stress

In the root, 68, 38 and 13 GO terms were significantly enriched for biological processes, molecular function and cellular component. In the shoot, 52, 33 and 8 GO terms were significantly enriched for biological processes, molecular function and cellular component. Biological processes related to secondary metabolic processes were predominantly enriched in both root and shoot, while processes related to camalexin biosynthesis and transition metal ion transport were especially predominant in the root, and processes related to glucosinolate and indoleacetic acid biosynthesis were especially predominant in the shoot. The molecular functions related to superoxide dismutase were especially

predominant in the root, while molecular functions related to transcription factor were especially predominant in the shoot.

Pathway enrichment analysis revealed a few important pathways that were significantly enriched in response to Cd stress (Figure 5). Pathways related to secondary metabolism processes, such as phenylalanine metabolism, phenylpropanoid biosynthesis, were the top significantly enriched pathway in the root (Figure 5). Pathways involved in amino acid biosynthesis, starch and sucrose metabolism, and other similar and additional secondary metabolism pathways, such as the 2-oxocarboxylic acid metabolism, phenylalanine metabolism, phenylpropanoid biosynthesis were the top significantly enriched pathways in the shoot.

DEGs related to key biological processes

DEGs involved in Cd transport

Metal transporters have been revealed to mediate heavy metal uptake and translocation in plants [74-78]. In the present study, 18 and 12 known heavy metal transporters were identified in the root and shoot, including one ZIP (ZRT/IRT-like Proteins), three YSL (yellow Stripe 1-like transporters), four Ppase (pyrophosphatase), six NRT (nitrate transporters), two COPT (copper transport protein family), three CML (calcium-binding proteins), two CAM (calmodulin), five ABC (ATP Binding Cassette) transporter, one CAX (vacuolar cation/proton exchanger), and one ATP synthase gene (Table 2, Table S5). DEGs encoding YSL, PPase, NRT, and ABC transporter were common to both the root and shoot, while DEGs encoding ZIP, COPT, CML and CAM were specific to the root, and DEGs encoding CAX and ATP synthase were specific to the shoot. All genes encoding ZIPs, YSL, COPT, CAM, ABC transporter, and CAX tended to be up-regulated, while all genes encoding PPase, NRT, and ATP synthase were down-regulated.

DEGs involved in regulation of transcription

Of the 780 genes differentially expressed in the root and shoot of oilseed rape seedlings in response to Cd, 67 genes (8.6%) were involved in transcriptional regulation (Table S6). Interestingly, more than twice as many differentially expressed transcription factors (TFs) were found in the shoot (46 DEGs belonging to 18 TF families) than in the root (22 DEGs belonging to 7 TF families) (Table 2, Table S6). Five TF families were differentially expressed in both shoot and root (Table S6). Of the differentially expressed TFs, more were down-regulated (29 and 16 in root and shoot respectively) than up-regulated (17 and 6 in root and shoot respectively) (Table 2, Table S6).

DEGs involved in secondary metabolic process

A total of 53 DEGs in the root and 58 DEGs in the shoot were found to be involved in secondary metabolism processes (Table 2, Table S7). Eight secondary metabolic pathways were identified in both the root and shoot: glucosinolate biosynthesis and catabolism, lignin biosynthesis and catabolism, phenylpropanoid [metabolism](#), toxin catabolism, unsaturated fatty acid biosynthesis, flavonoid/flavonol/anthocyanin biosynthesis, and coumarin biosynthesis. DEGs involved in lignin biosynthesis and cutin biosynthesis were especially prominent in the shoot and root, respectively (Table 2). The highest numbers of DEGs involved in secondary [metabolism](#) processes were related to glucosinolates in both the root (17 DEGs; 14 involved in biosynthesis and 3 involved in catabolism) and shoot (37 DEGs; 36 involved in biosynthesis and 1 in catabolism) (Table 2). All 14 DEGs in the root and 33/36 of the DEGs in the shoot involved in glucosinolate biosynthesis were down-regulated (Table 2). Similarly, DEGs related to lignin biosynthesis, phenylpropanoid [metabolism](#), toxin catabolism, and coumarin biosynthesis also showed a trend of down-regulation in both root and shoot (Table 2). By contrast, DEGs related to lignan biosynthesis, unsaturated fatty acid biosynthesis and flavonoid/flavonol/anthocyanin biosynthesis tended to be down-regulated in the root, but up-regulated in the shoot (Table 2).

DEGs involved in antioxidant response

A total of 58 DEGs in the root and 46 DEGs in the shoot encoded different antioxidant families, putatively in response to Cd-induced oxidative stress (Table 2, Table S8). Half of the DEGs (52%) in the shoot were up-regulated, while most DEGs (85%) in the root were down-regulated. The antioxidant families peroxidase (POD), 2-oxoglutarate (2-OG) dioxygenase, laccase, and monooxygenase (MO) were induced in both the root and shoot, but the expression trend was opposite between the two tissues, with most DEGs down-regulated in the root but up-regulated in the shoot. Many of the antioxidant families were specific between tissues for Cd response, such as the copper/zinc superoxide dismutase (SOD), Fe-SOD, ferritin, NAD(P)-binding Rossmann-fold protein, and zinc-binding dehydrogenase (ZBDH) families, which were specifically induced in the root, and the lipoxygenase, copper amine oxidase (CuAO), ferredoxin:NADP(H) oxidoreductase (FNR), and nitrite reductase (NIR) families which were specifically induced in the shoot. These findings suggested that antioxidant-encoding genes may play important roles in reducing Cd toxicity in both root and shoot in *B. napus*, but that the mechanism of protection may be distinct between tissues.

DEGs involved in chlorophyll synthesis and photosynthesis

25 DEGs related to chlorophyll synthesis and photosynthesis were identified, and all of these DEGs were down-regulated in response to Cd treatment (Table 2, Table S9). These genes were mainly involved in chlorophyll synthesis, photosystem subunit-related, chlorophyll-protein complex subunit encoding, rubisco small subunit encoding, electron transport, carbon reduction, and other photosynthesis processes.

Combined expression analysis of microRNAs and their target mRNAs during Cd treatment

A total of 37 differentially expressed miRNAs were identified in the root. These miRNAs had 816 predicted miRNA-mRNA interaction pairs with 407 predicted mRNA targets (one of which was identified to be up-regulated, five of which were identified to be down-regulated and 401 of which had no significant expression change) (Table S10). A further 52 differentially expressed miRNAs were identified in the shoot. These formed 1629 predicted miRNA-mRNA pairs with 731 predicted mRNA targets (44 of which were up-regulated, 10 of which were down-regulated and 717 of which showed no significant expression change) (Table S10).

The [theory](#) that a miRNA-mRNA interaction pair means anti-regulation of a miRNA and a corresponding mRNA suggests that if a target gene is down-regulated, the effective activity of the putatively regulatory miRNA should be enhanced, and vice versa. A total of 24 anti-regulation miRNA-mRNA interaction pairs were identified in the root (Table 3). This included 23 pairs with up-regulated miRNAs and down-regulated mRNAs, and only one pair with down-regulated miRNA and up-regulated mRNA (Table 3). Three miRNA families (miR395, miR398 and miR169_2) and six target mRNAs were involved in these 24 anti-regulation miRNA-mRNA interaction pairs (Table 3). Of the target genes, one down-regulated target (*BnaA03g04440D*) of miR395 comprised an ABA-binding protein encoding magnesium chelatase which is involved in plastid-to-nucleus signal transduction and is related to [chlorophyll biosynthetic process](#) and [photosynthesis](#). Four targets of miR398 showed high [homology](#) (*e* value = 0.0) and were also down-regulated. These were *BnaA04g16310D*, *BnaC04g39580D*, *BnaC08g42970D*, and *BnaCnng33420D*. *BnaA04g16310D* and *BnaC04g39580D* encode chloroplastic copper/zinc superoxide dismutase 2 (CSD2) that can detoxify superoxide radicals; *BnaC08g42970D* encodes a cytosolic copper/zinc superoxide dismutase (CSD1) that can detoxify superoxide radicals; and *BnaCnng33420D* encodes copper-zinc superoxide dismutase copper chaperone (CCS). Thus, the four targets of miR398 were highly correlated and all involved in the CCS-dependent detoxification of superoxide radicals pathway, which is regulated by biotic and abiotic stress ([Beyer et al., 1991](#)). In the shoot, 16 anti-regulation miRNA-mRNA interaction pairs were identified, including 15 pairs with up-regulated miRNAs and down-regulated mRNAs, and only one pair with down-regulated miRNA and up-regulated mRNA (Table 3). Five miRNA families (miR2111, miR395, miR397, miR408 and miR398), one miRNA (*bnamiR6029*) independent of the known miRNA families, and seven target mRNAs were involved (Table 3). The seven target mRNAs were mainly involved in [oxidation-reduction process](#) and plant organ development. For instance, *BnaA09g44060D* encoding a 1-aminocyclopropane-1-carboxylate oxidase contributes to [oxidation-reduction process](#); *BnaC04g07220D* and *BnaA07g26900D* encoding a laccase-like multicopper oxidase 4 and transcription factor which contributes to cell wall biosynthesis and [transcription regulation](#); *BnaA04g11970D* encoding a proline-rich protein involved in the [plant-type cell wall organization](#); *BnaA02g18700D* encoding a FCS like zinc finger 6 involved in [cell growth](#) and [seedling development](#). *BnaA03g11200D* encoding a inner centromere protein, which is involved in female gametophytic cell specification and seed development.

Biological reproducibility analyses and real-time RT-PCR validation

To validate the expression data, the relative expression levels of 14 randomly selected differentially expressed mRNAs and nine miRNAs from our sequencing data were investigated with qRT-PCR. Our results showed that the qRT-PCR analysis for 12 of the 14 the differentially expressed mRNAs and seven of the nine differentially expressed miRNAs displayed similar expression patterns to those generated from high-throughput sequencing (Figure S1), thus confirming the reliability of the data provided by RNA-Seq.

Discussion

miRNA regulation of genes in response to Cd stress in oilseed rape

Patterns of “anti-regulation” between miRNA and target mRNA, where one is up-regulated and the other is down-regulated in response to the same treatment, are thought to provide strong evidence for regulatory interactions between miRNA and mRNA [79-80]. Co-analysis of the expression of miRNA and their putative mRNA targets in response to Cd stress revealed 23/24 anti-regulation pairs in the root (three miRNA families targeting six mRNAs) and 15/16 anti-regulation pairs in the shoot (five miRNA families targeting seven mRNAs) where miRNA up-regulation was coupled with down-regulation of the mRNA, with the remaining two pairs showing miRNA down-regulation coupled with mRNA up-regulation. Several of these targeted genes were involved in processes expected to be initiated in response to Cd stress. In the shoot, four related genes, an A4 and a C4 copy of CSD2, a CSD1 and a CCS gene, targeted by a single miRNA, belong to the copper/zinc superoxide dismutase genes, which were involved in the CCS-dependent detoxification of superoxide radicals pathway, and were regulated by biotic and abiotic stress [81-82], while another gene in the root was also involved [oxidation-reduction process](#) and [defense response](#) (an *ACO1* gene homolog). Other genes were putatively involved in chlorophyll biosynthesis/photosynthesis pathways, sulfate transport, cell wall biosynthesis and organization, cell growth and transcriptional regulation, similar to the categories which we also found to be enriched for differentially expressed genes in response to Cd stress. Our genome-wide study highlights both miRNAs and genes which would make excellent targets for detailed experimental investigation of Cd response in oilseed rape in future.

The expression of a range of miRNAs were changed under Cd stress in oilseed rape

Zhou et al. (2012) previously identified eight miRNA families differentially expressed in response to Cd stress in *B. napus* roots, and 13 miRNA families differentially expressed in response to Cd stress in *B. napus* shoots [73]. In the present study, a total of 14 and 16 miRNA families were identified to be responsive to Cd stress in roots and shoots, respectively (Table 1). As expected, several of the Cd-

responsive miRNAs previously identified by Zhou et al. (2012) also showed differential regulation under Cd stress in our study [73]. For instance, miR319, miR395, miR398, and miR2111, were up-regulated under Cd stress, miR158, miR159, miR172 and miR162 were down-regulated, whereas miR585 were down-regulated in root and up-regulated in shoot. However, the miR161, miR164, miR171, miR319, miR394, miR400, miR857 and miR1885 families detected in Zhou et al. 2012 were not significantly differentially regulated under Cd stress in our study, while the miR166, miR169, miR390, miR391, miR397, miR399, miR408, miR818, miR827, miR845, miR860, miR1140, miR1511, and miR5270 families detected in our study were not detected by Zhou et al. (2012) [73]. These differences might be caused by the *B. napus* genotype used or by the longer length of our Cd treatment. Thus, further functional studies are needed to validate the precise regulatory roles of these miRNAs in response to Cd stress in *B. napus*.

Cd stress significantly influences the photosynthetic pathway in oilseed rape seedlings

Cd can influence photosynthetic pigments, causing a decrease in total chlorophyll content and thus affecting photosynthetic function [83-84]. In our study, the total chlorophyll content showed a very significant decrease five days after treatment under 5 mg/L, 10 mg/L and 20 mg/L Cd²⁺ (Figure 1b), and the [minimum](#) chlorophyll content occurred after 10 days of treatment when compared with the control. The decrease in chlorophyll content in our study was similar to that observed by Ali et al. (2013b)[65] and Ding et al. (2018) [58], who showed that chlorophyll content significantly decreased with increasing Cd treatment. We found that the chlorophyll decrease occurred only for younger leaves, and did not affect the chlorophyll content of older leaf tissue (Figure 1a). However, unlike the results reported by Ali et al. (2013b)[65], who showed that chlorophyll content significantly decreased as Cd concentration increased (0, 100, and 500 µM), we found that higher Cd concentration arrested the development of younger leaves, causing stability and even a gradual increase in chlorophyll content in older leaves after a long time in high Cd concentration treatment.

Previous studies suggested that decreases in chlorophyll content may be due to Cd accumulation on the thylakoid membrane and binding to membrane proteins, causing the destruction of the chloroplast enzyme system and chlorophyll synthesis system [85]. We found five key genes involved in chlorophyll synthesis were significantly down-regulated (four in the root and one in the shoot) in response to Cd stress. Cd stress has been found to negatively affect chloroplast structure, photosystem I and photosystem II (PSII), as well as overall plant photosynthesis processes [86]. We also observed six genes encoding PSII subunits and five genes involved in the photosynthesis process to be significantly down-regulated in the root in response to Cd treatment, while in the shoot three genes involved in PSI cyclic electron transport, two genes encoding small rubisco subunits, one gene encoding rubisco activase, one gene involved in PSII repair and one gene involved in carbon reduction processes were significantly down-regulated in response to Cd. Our results highlight trends of decreasing chlorophyll during Cd treatment, and we identified a set of down-regulated photosynthesis-related genes explaining the leaf chlorosis phenomenon.

Although roots do not make chlorophyll or photosynthesise, 16 genes involved in plant chlorophyll synthesis or in the photosynthetic process were identified in response to Cd in the root. Thus, despite the gene annotation predictions, these genes must have functions other than photosynthesis. For instance, it has been reported that the overexpression of light-dependent protochlorophyllide oxidoreductase PORA or PORB provides protection against photooxidative damage by overexpression of light-dependent protochlorophyllide oxidoreductase PORA or PORB [87]. As well, the magnesium-chelatase H subunit functions in abscisic acid signaling to regulate plant adaptation to environmental stress challenges, by regulating stomatal aperture and expression of stress-responsive genes [88]. The pyridine nucleotide-disulphide oxidoreductases motifs are highly conserved in the deduced amino acid with sequences fromof glutathione reductase, which plays an essential role in cell defense against reactive oxygen metabolites [89]. Thus, we speculate that the differential expression of chlorophyll- or photosynthesis-related genes in the roots involves cellular protection mechanisms in the against the environmental stress caused by Cd.

Glutathione (GSH) metabolism is involved in Cd response in both roots and shoots of oilseed rape seedlings

In the present study, 24 DEGs (9 DEGs from the root and 15 DEGs from the shoot) were found to be located in glucosinolate and GSH biosynthesis pathways. Figure 6 presents the transcriptional regulation of glucosinolate and GSH biosynthesis in response to Cd. Interestingly, Cd treatment induced down-regulation of almost all of the DEGs involved in the glucosinolate biosynthesis pathway in both the root and the shoot (except for one gene encoding sulfotransferase, which was up-regulated in the root) (Figure 6). Glucosinolates are nitrogen- and sulfur-containing secondary metabolites synthesized from amino acids [90]. Although glucosinolates play an important role in defense against herbivores and pathogens [91-92], they are also a rich source of sulfur. Indeed, sulfur deficiency has been shown to down-regulate the CYP79 B3 gene involved in the first step of the biosynthetic pathway [93], and to decrease glucosinolate levels [94]. Our results showing that Cd down-regulates many of the genes involved in glucosinolate biosynthesis suggests that the synthesis of glucosinolate is limited in order to support the sulfate demand for biosynthesis of PCs. In agreement with this hypothesis, we also observed down-regulation of three genes encoding adenosine phosphosulfate (APS) kinases and four genes encoding sulfotransferases (Figure 6).

Four genes (glutathione S-conjugate transporter and γ -glutamyl transpeptidase) located closely in the downstream of tripeptide glutathione (GSH) was significantly differentially expressed in our study. GSH is proposed to play a significant role in stress response [95-100] (Figure 6). When plants respond to heavy metals, GSH is the substrate for biosynthesis of the heavy metal-binding peptides phytochelatins (PCs), which form metal-thiolate bonds and PC-metal complexes and are then sequestered to the vacuoles [101]. Thus, it is not surprising that GSH plays a very important role in Cd tolerance [102-103]. In *Arabidopsis thaliana*, GSH-deficient mutants with 15–30% of wild-type levels of GSH show Cd sensitivity [104], providing further experimental support for the importance of this gene in Cd stress response. Three of these four DEGs were up-regulated and were found only in the shoot, suggesting tissue-specific

regulation of gene expression. We failed to observe transcriptional regulation of sulfite reductase gene and downstream genes involved in cysteine and GSH biosyntheses in response to Cd treatment (Figure 6), despite the fact that most of these genes play a key role in Cd sensitivity (e.g. gamma-glutamylcysteine synthase (GSH1) [29]. Similar results also found in *Arabidopsis thaliana*: although GSH1 enzyme activity increases rapidly in response to Cd in a dosage-dependent manner[105] and glutamylcysteine levels also increase in response to Cd [106], the expression level of these genes shows no significant change. Our results confirm that sulfite reductase and other downstream genes involved in cysteine and GSH biosynthesis are post-transcriptionally regulated in response to Cd in *B. napus*, as already demonstrated for other stresses by May et al. (1998a)[97]. Also, in our analyses, genes encoding PC synthases showed no expression change after Cd treatment (Figure 6). In previous studies, PC synthases were found to be constitutively expressed and post-transcriptionally regulated by activation of the enzyme in the presence of the metal [35].

Glutathione S-transferase (GST) and glutathione S-conjugate transporters are a group of multifunctional enzymes which catalyze conjugation of GSH with xenobiotic compounds for plant detoxification. In our analysis, two of three DEGs encoding glutathione S-transferase and one glutathione S-conjugate transporter were up-regulated in the shoot in response to Cd (Figure 6). Research in yeast cells has found that GST is involved in GSH-Cd formation to decrease Cd uptake [107]. GST also functions as glutathione peroxidase (GPOX) and participates in plant defense against oxidative stress and toxicity generated from heavy metals: Moons et al. (2003) found that Cd, Co, Ni and Zn significantly enhance the expression of GST genes in rice [108]. Thus, the up-regulation of GST and glutathione S-conjugate transporter in Cd response in oilseed rape seedlings suggests similar regulatory mechanisms to those in rice, such that the up-regulation of GST is associated with plant Cd detoxification.

Phenylpropanoid metabolism was notably involved in Cd response in both roots and shoots of oilseed rape seedling

Cd accumulation in plants causes the accumulation of reactive oxygen species (ROS) [109-110]. ROS can directly damage a wide range of cellular components, such as mitochondria [111], chloroplasts [112], and microtubules [113]. Enhancing the ROS-scavenging is an important plant Cd response mechanism. Phenylpropanoids and its derivatives are well-known ROS scavengers. Several reports have demonstrated that diverse forms of biotic and abiotic stress result in the accumulation of one or more phenolic compounds [114-117]. In the present study, 12 and 23 DEGs in roots and shoots were directly involved in pathways of phenylpropanoids and its derivatives (Figure 7). According to the gene expression pattern in this metabolism pathway, several conclusions might be deduced from the above. Firstly, phenylpropanoid products and the phenylpropanoid derivatives e.g. sinapate ester, lignin, flavonols, and anthocyanin, appear to be involved in Cd response in roots and shoots of oilseed rape seedlings. Secondly, all 12 DEGs in the root were down-regulated and 21/23 DEGs in the shoot were up-regulated, suggesting different regulation of root and shoot tissues. Thirdly, 13/20 DEGs in the shoot were involved in the anthocyanin biosynthesis and modification pathways, but only one gene in these two pathways was found in root, suggesting that the phenylpropanoid-related Cd response for the shoot was mostly anthocyanin-related,

root anthocyanins are not involved. These findings in oilseed rape are similar in part to those of Herbette et al. (2006), who demonstrated that genes involved in phenylpropanoid and anthocyanin production are differentially expressed in *Arabidopsis thaliana* exposed to Cd treatment [105]. However, Herbette et al. (2006) found up-regulated expression of genes involved in phenylpropanoid and anthocyanin production only in leaves of Cd-treated plants, in contrast to our data [105]. This difference may correspond to transitory effects at different time points under Cd stress.

Antioxidant response was a crucial Cd-induced response in both roots and shoots of oilseed rape seedlings

The oxidative stress induced by Cd has been shown to play an important role in the plant toxicity [118]. Under normal conditions, the level of reactive oxygen species (ROS) remains low and does not pose a hazard to plants. However, ROS metabolism is blocked after Cd stress, and excess ROS, such as superoxide anions, hydroxyl ions, and hydrogen peroxide, accumulate in the cell, cause membrane lipid peroxidation, disrupt the electron transport chain, and lead to oxidative damage in plants [119]. Several studies have found that proline and malondialdehyde content were increased after Cd stress, and that antioxidants such as peroxidase, superoxide dismutase, hydrogen peroxidase, GSH and GST also increased [58, 120], suggesting the stimulation of the antioxidant system. In agreement with the above physiological results, our data found that a set of genes encoding antioxidants such as POD, SOD, Fe-SOD, FNR and lipoxygenase were induced by Cd stress. However, opposite expression trends and tissue-specific antioxidant families were detected between the tissues of the root and shoot, which suggests that the Cd-induced stress response was distinct between tissues, as also found by Zhou et al. (2012)[61] in *B. napus* and Herbette et al. (2006) in *A. thaliana* [104].

TFs play a crucial role in Cd response in both roots and shoots of oilseed rape seedling

TFs are known to play crucial roles in response to abiotic stresses in plants. In our study, the majority of differentially expressed TFs belonged to the TF families (bHLH) DNA-binding proteins, bZIP, zinc finger, ERF and MYB, and WRKY. In *Arabidopsis* and soybean, bHLH transcription factors like bHLH38, bHLH39, bHLH29, ORG3-Like, have been identified to enhance Cd tolerance via decreased cadmium transfer from roots to shoots, and to improve the iron homeostasis and concentration of shoots [121-123]. In *Arabidopsis*, the zinc-finger protein AtZAT6 showed significant correlation with Cd tolerance [121], while the TFs families of WRKY, bZIP, ERF, and MYB, are associated with Cd stress in species such as tobacco, maize, walnut and creep bentgrass [124-127]. We also observed several other TF families expressed in response to Cd treatment (Table 2). Briefly, the evidence for direct or indirect roles of TFs in response to Cd is quite limited. Our study may provide some suggestions regarding the mechanisms of Cd tolerance at the transcriptional level for future research.

Conclusions

The cadmium stress induced concurrent expression changes of miRNAs and mRNAs in *B. napus* seedlings. The leaves and roots showed several overlap of differentially expressed miRNAs but very little overlap of differentially expressed mRNAs for Cd response. Several anti-regulation miRNA-mRNA interaction pairs were identified in response to Cd, which involved in the key pathways of plant stress response like detoxification of superoxide radicals. Photosynthetic pathway, transcription factor activation, antioxidant response and secondary metabolic processes were identified as major components for cadmium response of *B. napus* seedlings. Our results identify key genes and processes for further investigation of Cd stress responses in *B. napus*.

Methods

Plant materials, Cd treatment and chlorophyll content measurements

Seeds of *B. napus* cultivar 'Zheyongyou 29' were collected from the Key Laboratory of Crop Physiology, Ecology and Genetic Breeding, Ministry of Education, Agronomy College, Jiangxi Agricultural University, and used in this study. No other permissions were necessary to collect samples. The seeds of 'Zheyongyou 29' were surface sterilized and germinated on petri dishes with deionized water at 25 °C for 3 days in the dark. Germinated seeds were grown in plastic pots in growth chambers under 14 h light (25 °C) and 10 h dark (18 °C) conditions for three weeks. Seedlings were transplanted into plastic containers containing half-strength Hoagland nutrient solution for one week. 28-day-old seedlings were exposed to treatment with CdCl₂ at six concentrations: 0 mg/L (control), 5 mg/L, 10 mg/L, 20 mg/L, 30 mg/L, and 40 mg/L of Cd²⁺. New unfolding leaves were measured for total chlorophyll content according to the methods reported in Yin et al. (2008)[128]. About 0.1 g of leaf tissue was homogenized in 5 mL of mixture with acetone : ethanol : water at a 9 : 9 : 2 (v / v / v) ratio, incubated in the dark for 8 - 12 h, and then centrifuged at 3500 r/min for 10 minutes. The absorbance of the supernatant was then measured at 652 nm. Eight measurements of chlorophyll content were taken at two-day intervals. Based on these results [maximum](#) decreases in chlorophyll content occurred at 10 days post-treatment under 10 mg/L Cd²⁺ concentration, so the roots and shoots under this condition were then collected separately for high-throughput sequencing and qRT-PCR. The collected samples were frozen in liquid nitrogen immediately and stored at -80°C.

Total RNA was extracted from roots and shoots of oilseed rape seedlings after 10 days of 10 mg/L Cd²⁺ treatment and for the control treatment using EZ-10 RNA Miniprep Kits (Sangon Biotech Co., Ltd., Shanghai, China), with three biological replicates for each sample. RNA purity was checked using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using a Qubit RNA Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Small RNA and transcriptome sequencing

Twelve small RNA sequencing libraries were generated using NEBNext®Ultra™ small RNA Sample Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. Polyacrylamide gel electrophoresis (PAGE) was used to screen and isolate RNA fragments in order to produce small RNA libraries. PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The library preparations were sequenced on an Illumina HiSeq X Ten platform by Biomarker Technologies Co., Ltd. (Beijing, China) and single-end reads were generated.

Twelve sequencing libraries for transcriptome analysis were prepared according to manufacturer's instructions (Illumina kit). mRNA with Poly (A) was purified from the total RNA, using oligo(dT) magnetic beads, and then fragmented with an RNA fragmentation kit. First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Following the isolation of suitable cDNA fragments by gel electrophoresis analysis, and PCR enrichment, the library preparations were sequenced on an Illumina HiSeq 2500 platform by Biomarker Technologies Co., Ltd. (Beijing, China), for RNA sequencing, and paired-end reads were generated.

Sequencing data analysis

For small RNA sequencing data, clean reads were firstly obtained by removing contaminants, adaptors, low-quality reads, and reads either shorter than 15 nt or longer than 30 nt. The remaining unique RNAs were mapped to the *B. napus* reference genome (<http://www.genoscope.cns.fr/brassicnapus/>) using the SOAP2 program [129]. Sequences with a perfect match were retained for further analysis. Using Bowtie tools "soft", the clean reads were aligned respectively with the Silva database (<http://www.arb-silva.de/>), the GtRNadb database (<http://lowelab.ucsc.edu/GtRNadb/>), the Rfam database (<http://rfam.xfam.org/>), and the Repbase database (<http://www.girinst.org/repbase/>) in order to filter out ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and other ncRNA and repeats. The remaining reads were used to detect known miRNA and novel miRNA predicted by comparison to the genome reference and with known miRNAs from miRBase (<http://www.mirbase.org/index.shtml>). Mireap software (<https://sourceforge.net/projects/mireap/>) was used for novel miRNA secondary structure prediction. The miRNA expression was normalized using the formula: normalized expression = actual miRNA count/total count of clean reads × 1,000,000. The fold change was calculated as: fold change = log₂ (Cd/CK). Differential expression analysis was performed using the DESeq R package (1.10.1). DESeq provides statistical routines for determining differential expression in digital miRNA expression data using a model based on the negative binomial distribution. The resulting *P* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Fold change, *P* values and false discovery rate (FDR) values were used together to screen for differentially expressed miRNAs. The website psRNATarget 2011 (<http://plantgrn.noble.org/psRNATarget/>) was used to predict target genes of miRNAs according to

default parameters. The GOseq R package[130] was used to perform the GO enrichment analysis of the target genes, and DEG enrichment in KEGG pathways was assessed using the software KOBAS [131].

For transcriptome data, clean reads were screened from raw data by removing contaminants, adaptors, and low-quality reads. The software “TopHat2” was used to map reads to the reference genome (*Brassica_napus* v4.1). Gene expression quantification was estimated by fragments per kilobase of transcript per million fragments mapped. The DEGseq (2010) R package was used to analyze the differentially expressed genes (DEGs) with a FDR < 0.001 and $|\log_2(\text{fold change})| \geq 1$. The GOseq R package[130] was used to perform the GO enrichment analysis of the DEGs, and DEG enrichment in KEGG pathways was assessed using the software KOBAS [131].

qRT-PCR validation

20 differentially-expressed mRNAs and 10 miRNAs were randomly chosen for quantitative real-time RT-PCR (qRT-PCR) validation. The total RNA extraction procedure for these was as described above. For mRNA quantification, SuperScript™II Reverse Transcriptase (Invitrogen, USA) and Oligo(dT) primers were used to synthesise first-strand cDNA. Gene-specific primers were designed (Table S1). A Bio-Rad CFX96 Real-time System was used for qRT-PCR. Three technical replicates were performed, and the BnActin7 gene was used as a control. SYBR® Green PCR Supermix (CA, USA) was chosen for qRT-PCR, the reactions contained 2 µL of cDNA, 10 µL of SYBR Supermix, 7.2 µL of H₂O, and 0.4 µL primer (10 µM) in a volume of 20 µL. The reaction program was as follows: 98 °C for 30 s, followed by 40 cycles of 98 °C for 10 s and 60 °C for 30 s. For mature miRNA quantification, the One Step Primer Script® miRNA cDNA Synthesis Kit (Takara) and SuperScript III Reverse Transcriptase (Invitrogen) were used. U6 snRNA of *B. napus* was used as a control, and three technical replicates were performed for each reaction. 20 µL reaction consisting of 2 µL cDNA, 0.2 µM forward and reverse primer, and 10 µL of 2× SYBR Green PCR Master Mix, were carried out on an iCycler iQ real-time PCR detection system (BIO-RAD) with program as follows: 95 °C for 30 s, and 45 cycles of 95 °C for 5 s, 58 °C for 15 s, and 72 °C for 20 s.

Abbreviations

2-OG dioxygenase: 2-oxoglutarate dioxygenase; ABC: ATP Binding Cassette; APS: Adenosine phosphosulfate; CAM: Calmodulin; CAX: Cation/proton exchanger; CCS: Copper-zinc superoxide dismutase copper chaperone; Cd: Cadmium; CML: Calcium-binding proteins; COPT: Copper transport protein; CSD: Copper/zinc superoxide dismutase; CuAO: Copper amine oxidase; DEG: Differentially expressed gene; FDR: False discovery rate; FNR: Ferredoxin:NADP(H) oxidoreductase; GO: Gene ontology; GPOX: Glutathione peroxidase; GSH: Glutathione; GST: Glutathione S-transferase; KEGG: Kyoto Encyclopedia of Genes and Genomes; MiRNAs: MicroRNAs; MO: Monooxygenase; NIR: Nitrite reductase; NRT: Nitrate transporters; PAGE: Polyacrylamide gel electrophoresis; PC: Phytochelatin; POD: Peroxidase; Ppase: Pyrophosphatase; RNA: Ribonucleic acid; ROS: Reactive oxygen species; SOD: Superoxide

dismutase; TFs: transcription factors; YSL: Yellow Stripe 1-like transporters; ZBDH: Zinc-binding dehydrogenase; ZIP: ZRT/IRT-like Proteins

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publish

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are presented within the manuscript and supplementary files.

Competing Interests

The authors declare that they have no conflict of interest.

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Authors' contributions

YF and DF designed research; YF, YZ, BL, and HY analyzed data; YF wrote the paper, ASM assisted with interpretation of results, manuscript writing and revision. All authors read and approved the final manuscript.

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References

References

1. Alloway BJ, Steinnes E. Anthropogenic additions of cadmium to soils. *Cadmium in Soils and Plants*. 1999; 97-123.
2. Tanhuanpaa P, Kalendar R, Schulman AH, Kiviharju E A major gene for grain cadmium accumulation in oat *Avena sativa* L. *Genome*. 2007; 50: 588-594.
3. Shamsi IH, Wei K, Zhang GP, Jilani GH, Hassan MJ. Interactive effects of cadmium and aluminum on growth and antioxidative enzymes in soybean. *Biologia Plantarum*. 2008; 52: 165-169.
4. John MK. Cadmium uptake by eight food crops as influenced by various soil levels of cadmium. *Environmental Pollution*. 1973; 4: 7-15.
5. Patra M, Bhowmik N, Bandopadhyay B, Sharma A. Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. *Environmental and Experimental Botany*. 2004; 52: 199-223.
6. DalCorso G, Farinati S, Furini A. Regulatory networks of cadmium stress in plants. *Plant Signaling & Behavior*. 2010; 5:663-667.
7. Martinka M, Vaculik M, Lux A. Plant Cell Responses to Cadmium and Zinc. *Applied Plant Cell Biology*. 2014; 22: 209-246.
8. Montero-Palmero MB, Martin-Barranco A, Escobar C, Hernandez LE. Early transcriptional responses to mercury: A role for ethylene in mercury-induced stress. *New Phytologist*. 2014; 201: 116-130.
9. Hassan MJ, Shao GS, Zhang GP. Influence of cadmium toxicity on growth and antioxidant enzyme activity in rice cultivars with different grain cadmium accumulation. *Journal of Plant Nutrition*. 2005; 28: 1259-1270.

10. Grant CA, Clarke JM, Duguid S, Chaney RL. Selection and breeding of plant cultivars to minimize cadmium accumulation. *Science of the Total Environment*. 2008; 390: 301-310.
11. Ishikawaa S, Ishimaru Y, Igura M, et al. Healthy and unhealthy plants: the effect of stress on the metabolism of Brassicaceae. *Environmental and Experimental Botany*. 2009; 67: 23-33.
12. Mclaughlin MJ, Maier NA, Correll RL, Smart MK, Sparrow LA, Mckay A. Prediction of cadmium concentrations in potato tubers *Solanum tuberosum* L. by pre-plant soil and irrigation water analyses. *Australian Journal of Soil Research*. 1999; 37:191-207.
13. Ebbs SD, Lasat MM, Brady DJ, Cornish J, Gordon R, Kochian LV. Phytoextraction of cadmium and zinc from a contaminated soil. *Journal of Environmental Quality*. 1997; 26: 1424-1430.
14. Chen J, Yang ZM, Su Y, Han FX, Monts DL. Phytoremediation of heavy metal/metalloid-contaminated soils, In: Steinberg RV, editors. Contaminated soils, environmental impact, disposal and treatment New York, USA, Nova Science Publishers. 2009.
15. Pilon-Smits E, Pilon, M. Phytoremediation of metals using transgenic plants. *Critical Reviews in Plant Sciences*. 2002; 21: 439-456.
16. McGrath SP, Zhao FJ, Lombi E. Phytoremediation of metals, metalloids, and radionuclides. *Advances in Agronomy*. 2002; 75: 1e56.
17. Liu W, Zhou Q, Sun Y, Liu, R. Identification of Chinese cabbage genotypes with low cadmium accumulation for food safety. *Environmental Pollution*. 2009; 157: 1961-1967.
18. Clemens S, Kim EJ, Neumann D, Schroeder JI. Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *European Molecular Biology Organization Journal*. 1999; 18: 3325-3333.
19. Gallego SM, Pena LB, Barcia RA, et al. Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. *Environmental & Experimental Botany*. 2012; 835: 33-46.
20. Hall MJ, Brown MT. Copper and manganese influence the uptake of cadmium in marine macroalgae. *Bulletin of Environmental Contamination and Toxicology*. 2002; 68: 49-55.
21. Clemens S. Developing tools for phytoremediation: towards a molecular understanding of plant metal tolerance and accumulation. *International Journal of Occupational Medicine and Environmental Health*. 2001; 14: 235-239.
- 22 Gill SS, Anjum NA, Gill R, Hasanuzzaman M, Sharma P, Tuteja N Mechanism of cadmium toxicity and tolerance in crop plants. *Crop Improvement Under Adverse Conditions*. 2013; 361-385.

- 23 Cobbett C, Goldsbrough P. PHYTOCHELATINS AND METALLOTHIONEINS: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology*. 2002; 53: 159-82.
- 24 Florescáceres ML, Hattab S, Hattab S, Boussetta H, Banni M, Hernandez, LE. Specific mechanisms of tolerance to copper and cadmium are compromised by a limited concentration of glutathione in alfalfa plants. *Plant Science*. 2015; 233: 165-173.
- 25 Hernández LE, Sobrinoplata J, Monteropalmero MB, Carrasco-Gil S, Flores-CaceresML, Ortega-Villasante C. Contribution of glutathione to the control of cellular redox homeostasis under toxic metal and metalloid stress. *Journal of Experimental Botany*. 2015; 66: 2901-2911.
- 27 Jozefczak M, Bohler S, Schat H, et al. Both the concentration and redox state of glutathione and ascorbate influence the sensitivity of Arabidopsis to cadmium. *Annals of Botany*. 2015; 116: 601-612.
- 28 Sobrino-Plata J, Meyssen D, Cuypers A, Escobar C, Hernández LE. Glutathione is a key antioxidant metabolite to cope with mercury and cadmium stress. *Plant & Soil*. 2014; 377: 369-381.
- 29 Xiang C, Werner BL, Christensen EM, Oliver DJ. The biological functions of glutathione revisited in Arabidopsis transgenic plants with altered glutathione levels. *Plant Physiology*. 2001; 126: 564-74
- 30 Zhu LY, [Pilon-Smits EA](#), [Jouanin L](#) [Terry N](#). Overexpression of glutathione synthetase in indian mustard enhances cadmium accumulation and tolerance. *Plant Physiology*. 1999; 119: 73-80.
- 31 Grill E, Löffler S, Winnacker EL, Zenk MH. Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific gamma-glutamylcysteine dipeptidyl transpeptidase phytochelatase. *Proceedings of the National Academy of Sciences. USA*, 1989; 86: 6838-6842.
- 32 Jozefczak M, Remans T, Vangronsveld J, Cuypers A. Glutathione is a key player in metal-induced oxidative stress defenses. *International Journal of Molecular Sciences*. 2012; 13, 3145-3175.
- 33 Li ZS, Alfenito M Rea PA, Walbot V, Dixon RA. Vacuolar uptake of the phytoalexin medicarpin by the glutathione conjugate pump. *Phytochemistry*. 1997;45, 689-93.
- 34 Lin, Y F and Aarts, M G M The molecular mechanism of zinc and cadmium stress response in plants. *Cellular & Molecular Life Sciences*. 2012; 69: 3187-3206.
- 35 Cobbett C. Phytochelatins and their roles in heavy metal detoxification. *Plant Physiology*. 2000;123, 825-832.
- 36 Cobbett CS, May MJ, Howden R, Rolls, B. The glutathione-deficient, cadmium-sensitive mutant, cad2-1, of *Arabidopsis thaliana* is deficient in γ -glutamylcysteine synthetase. *Plant Journal for Cell & Molecular Biology*. 2010; 16: 73-78.

- 37 Lee S, Moon J S, Ko TS, Petros D, Goldsbrough PB, Korban SS. Overexpression of Arabidopsis phytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. *Plant Physiology*. 2003; 13: 656-663.
- 38 Pence NS, Larsen PB, Ebbs SD, et al. The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *Proceedings of the National Academy of Sciences, USA*. 2000; 97: 4956-4960.
- 39 Sasaki A, Yamaji N, Yokosho K, Ma JF. Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice, *Plant Cell*. 2012; 24: 2155-2167.
- 40 Ishikawa S, Ishimaru Y, Igura M, et al. Ion-beam irradiation, gene identification, and marker-assisted breeding in the development of low-cadmium rice. *Proceedings of the National Academy of Sciences, USA*. 2012; 109:19166–19171.
- 41 Noll M, Lutsenko S. Expression of *ZntA*, a zinc-transporting P1-type ATPase, is specifically regulated by zinc and cadmium. *Journal of Molecular Biology*. 2010; 49: 297-302.
- 42 Shiraishi E, Inoue M, Joho M, Tohyama H. The cadmium-resistant gene, *CAD2*, which is a mutated putative copper-transporter gene *PCA1*, controls the intracellular cadmium-level in the yeast *S cerevisiae*. *Current Genetics*. 2000; 37: 79-86.
- 43 Kim DY, Bovet L, Maeshima M, Martinoia E, Lee Y. The ABC transporter *AtPDR8* is a cadmium extrusion pump conferring heavy metal resistance. *The Plant Journal*. 2007; 50: 207-218.
- 44 Migocka M, Papierniak A, Rajs A. Cucumber PDR8/ABCG36 and PDR12/ABCG40 plasma membrane proteins and their up-regulation under abiotic stresses. *Biologia Plantarum*. 2017; 61: 115-126.
- 45 Kim DY, Bovet L, Kushnir S, Noh EW, Martinoia E, Lee Y. *AtATM3* is involved in heavy metal resistance in Arabidopsis. *Plant Physiology*. 2006; 140: 922-932.
- 46 Xiao S, Chye ML. Arabidopsis *ACBP1* overexpressors are PbII-tolerant and accumulate PbII. *Plant Signaling & Behavior*. 2008; 39: 693-694.
- 47 Cao S, Chen Z, Liu G, et al. The Arabidopsis Ethylene-Insensitive 2 gene is required for lead resistance. *Plant Physiology & Biochemistry*. 2009; 47, 308-312.
- 48 Chen J, Yang L, Gu J, Bai X, Cao S. MAN3 gene regulates cadmium tolerance through the glutathione-dependent pathway in *Arabidopsis thaliana*. *New Phytologist*. 2014; 205: 570-582.
- 49 Voinnet O. Origin, biogenesis, and activity of plant microRNAs. *Cell*. 2009; 136: 669–687.
- 50 Abdel-Ghany SE, Pilon M. MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in *Arabidopsis*. *Journal of Biological Chemistry*. 2008; 283: 15932-

15945.

51 Aukerman MJ, Sakai H. Regulation of flowering time and floral organ identity by a microRNA and its *APETALA2-like* target genes. *THE PLANT CELL ONLINE*. 2003; 15: 2730-2741.

52 Fujii H, Chiou TJ, Lin SI, Aung K, Zhu JK. A miRNA involved in phosphate starvation response in *Arabidopsis*. *Current Biology*. 2005; 15: 2038-2043.

53 Guo HS, Xie Q, Fei JF, Chua NH. MicroRNA directs mRNA cleavage of the transcription factor *NAC1* to downregulate auxin signals for *Arabidopsis* lateral root development. *Plant Cell*. 2005; 17: 1376-1386.

54 Kawashima CG, Matthewman CA, Huang S, et al. Interplay of *SLIM1* and *miR395* in the regulation of sulfate assimilation in *Arabidopsis*. *The Plant Journal*. 2011; 66: 863-876.

55 Liu HH, Tian X, Li YJ, Wu CA, Zheng CC Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA*. 2008; 14:836-843.

56 Palatnik F, Allen E, Wu X, et al. Control of leaf morphogenesis by microRNAs. *Nature*. 2003; 425: 257-263.

57 Sunkar R, Kapoor A, Zhu JK. Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of *miR398* and important for oxidative stress tolerance. *Plant Cell*. 2006; 18: 2051-2065.

58 Ding Y, Chen Z, Zhu, C. Microarray-based analysis of cadmium-responsive microRNAs in rice *Oryza sativa*. *Journal of Experimental Botany*. 2011; 62: 3563-3573.

59 Tang MF, Mao DH, Xu LW, et al. Integrated analysis of miRNA and mRNA expression profiles in response to Cd exposure in rice seedlings. *BMC Genomics*. 2014; 15: 835.

60 Xu YC, Chu LL, Jin QJ, et al. Transcriptome-Wide Identification of miRNAs and Their Targets from *Typha angustifolia* by RNA-Seq and Their Response to Cadmium Stress. *PLoS ONE*. 2015; 10: e0125462.

61 Zhou ZS, Huang SQ, Yang ZM. Bioinformatic identification and expression analysis of new microRNAs from *Medicago truncatula*. *Biochemical and Biophysical Research Communications*. 2008; 374: 538-542.

62 Xu L, Wang Y, Zhai LL, et al. Genome-wide identification and characterization of cadmium-responsive microRNAs and their target genes in radish (*Raphanus sativus* L) roots. *Journal of Experimental Botany*. 2013; 64: 4271-4287.

63 Khraiweh B, Zhu JK, Zhu JH. Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochimica Et Biophysica Acta*. 2012; 1819: 137-148.

- 64 Ali B, Huang CR, Qi ZY, et al. 5-aminolevulinic acid ameliorates cadmium-induced morphological, biochemical, and ultrastructural changes in seedlings of oilseed rape. *Environmental Science and Pollution Research*. 2013a; 20: 7256-7267.
- 65 Ali B, Wang B, Ali S, et al. 5-aminolevulinic acid ameliorates the growth, photosynthetic gas exchange capacity, and ultrastructural changes under cadmium stress in *Brassica napus* L. *Plant Growth Regulation*. 2013b; 32: 604-614.
- 66 Ali B, Gill RA, Yang S, et al. Hydrogen sulfide alleviates cadmium-induced morpho-physiological and ultrastructural changes in *Brassica napus*. *Ecotoxicology and Environmental Safety*. 2014a; 110: 197-207.
- 67 Ali B, Qian P, Jin R, et al. Physiological and ultra-structural changes in *Brassica napus* seedlings induced by cadmium stress, *Biologia Plantarum*. 2014b; 58: 131-138.
- 68 Ali B, Song WJ, Hu WZ, et al. Hydrogen sulfide alleviates lead-induced photosynthetic and ultrastructural changes in oilseed rape. *Ecotoxicology and Environmental Safety*. 2014c; 102: 25-33.
- 69 Meng HB, Hua SJ, Shamsi IH, Jilani G, Li YL, Jiang LX. Cadmium-induced stress on the seed germination and seedling growth of *Brassica napus* L and its alleviation through exogenous plant growth regulators. *Plant Growth Regulation*. 2009; 58: 47-59.
- 70 Yan H, Filardo F, Hu X, Zhao X, Fu DH. Cadmium stress alters the redox reaction and hormone balance in oilseed rape *Brassica napus* L leaves. *Environmental Science and Pollution Research*. 2015; 23: 3758-69.
- 71 Huang SQ, Xiang AL, Che LL, et al. A set of miRNAs from *Brassica napus* in response to sulphate deficiency and cadmium stress. *Plant Biotechnology Journal*. 2010; 8: 887-899.
- 72 Jian H, Yang B, Zhang A, et al. Genome-wide identification of micromnas in response to cadmium stress in oilseed rape (*Brassica napus* L) using high-throughput sequencing. *International Journal of Molecular Sciences*. 2018; 19: 1431.
- 73 Zhou ZS, Song JB, Yang ZM. Genome-wide identification of *Brassica napus* microRNAs and their targets in response to cadmium. *Journal of Experimental Botany*. 2012; 63: 4597-4613.
- 74 Arrivault S, Senger T, Krämer, U. The Arabidopsis metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. *The Plant Journal*. 2006; 46, 861-79.
- 75 Eide D, Broderius M, Fett J, Guerinot ML. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proceedings of the National Academy of Sciences*. 1996; 93: 5624-5628.
- 76 Kramer U, Talke IN, Hanikenn M. Transition metal transport. *Febs Letters*. 2007; 581: 2263-2272.

- 77 Ueno D, Milner MJ, Yamaji N, et al. Origin, biogenesis, and activity of plant microRNAs. *Cell*. 2009; 136: 669-687.
- 78 Wang T, Hua Y, Chen M, Zhang J, Guan C, Zhang Z. Mechanism enhancing Arabidopsis resistance to cadmium: the role of *NRT15* and proton pump. *Frontiers in Plant Science*. 2018; 19: 1892.
- 79 Cheng C, Li LM. Inferring microRNA activities by combining gene expression with microRNA target prediction. *PloS One*. 2008; 34, e1989.
- 80 Wang YP, Li KB. Correlation of expression profiles between microRNAs and mRNA targets using NCI-60 data. *BMC Genomics*. 2009; 101: 218.
- 81 Herouart D, Van Montagu M, Inze D. Redox-activated expression of the cytosolic copper/zinc superoxide dismutase gene in Nicotiana. *Proceedings of the National Academy of Sciences*. 1993; 90: 3108-3112.
- 82 Chu CC, Lee WC, Guo WY, et al. A copper chaperone for superoxide dismutase that confers three types of copper/zinc superoxide dismutase activity in Arabidopsis. *Plant Physiology*. 2005; 139: 425-436.
- 83 Chandra R, Kang H. Mixed heavy metal stress on photosynthesis, transpiration rate, and chlorophyll content in poplar hybrids. *Forest Science and Technology*. 2016; 12: 55-61.
- 84 Hattab S, Dridi B, Chouba L, Ben Kheder M, Bousetta H. Photosynthesis and growth responses of pea *Pisumsativum* L under heavy metals stress. *Journal of Environmental Sciences*. 2009; 21: 1552-1556.
- 85 Stobart AK, Griffiths WT, Ameenbukhari I, Sherwood RP. The effect of Cd^{2+} on the biosynthesis of chlorophyll in leaves of barley. *Physiologia Plantarum*. 1985; 63: 293-298.
- 86 Didur O, Juneau P, Popovic R. Effect of cadmium on photosystem II activity in *Chlamydomonas reinhardtii*: alteration of O-J-I-P fluorescence transients indicating the change of apparent activation energies within photosystem II. *Photosynthesis Research*. 2011; 107: 151-157.
- 87 Sperling U, Van Cleve B, Frick G, Apel K, Armstrong GA. Overexpression of light-dependent pora or porb in plants depleted of endogenous por by far-red light enhances seedling survival in white light and protects against photooxidative damage. *Plant Journal*. 2010; 12: 649-658.
- 88 Wu FQ, Xin Q, Cao Z, et al. The magnesium-chelatase H subunit binds abscisic acid and functions in abscisic acid signaling: new evidence in Arabidopsis. *Plant Physiology*. 2009; 150: 1940-1954.
- 89 Seo JS, Lee KW, Rhee JS, et al. Environmental stressors (salinity, heavy metals, H_2O_2) modulate expression of glutathione reductase (GR) gene from the intertidal copepod *tigriopus japonicus*. *Aquatic Toxicology (Amsterdam)*. 2006; 80: 0-289.

- 90 Pietrowski M, Schemenewitz A, Lopukhina A, et al. Desulfoglucosinolate sulfotransferases from *Arabidopsis thaliana* catalyze the final step in the biosynthesis of the glucosinolate core structure, *Journal of Biological Chemistry*, 2004; 279: 50717-50725.
- 91 Wittstock U, Gherghenzon J. Constitutive plant toxins and their role in defense against herbivores and pathogens. *Current Opinion in Plant Biology*. 2002a; 5: 300-307.
- 92 Wittstock U, Halkier B. Glucosinolate research in the Arabidopsis era. *Trends in Plant Science*. 2002b; 7: 263-270.
- 93 Maruyama-Nakashita A, Inoue E, Watanabe-Takahashi A, Yamaya T, Takahashi H. Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effects of sulfur nutrition on multiple metabolic pathways. *Plant Physiology*. 2003; 132, 597-605.
- 94 Zhao FJ, Evans EJ, Bilsborrow PE, Syers JK. Influence of nitrogen and sulphur on the glucosinolate profile of rapeseed *Brassica napus* L. *Journal of the Science of Food & Agriculture*. 1994; 64: 295-304.
- 95 Alscher RG. Biosynthesis and antioxidant function of glutathione in plants. *Physiologia Plantarum*. 1989; 77: 457-464.
- 96 Marrs KA. The functions and regulation of glutathione S-transferases in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*. 1996; 47: 127-157.
- 97 May M, Vernoux T, Sánchez-Fernández R, Van Montagu M, Inzé D. Evidence for posttranscriptional activation of gamma-glutamylcysteine synthetase during plant stress responses. *Proceedings of the National Academy of Sciences, USA*. 1998a; 95: 12049-12054.
- 98 May MJ, Vernoux T, Leaver C, Van Montague M, Inze, D. Glutathione homeostasis in plants: implications for environmental sensing and plant development. *Journal of Experimental Botany*. 1998b; 49: 649-667.
- 99 Noctor G, Foyer CH. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology*. 1998; 49: 249–279
- 100 Rea PA, Li ZS, Lu YP, Drozdowicz YM, Matinoia E. From vacuolar GS-X pumps to multispecific ABC transporters, *Annual Review of Plant Physiology and Plant Molecular Biology*. 1998; 49: 727-760.
- 101 Rauser WE. Phytochelatins and related peptides Structure, biosynthesis, and function. *Plant Physiology*. 1995; 109: 1141-1149.
- 102 Clemens, S 2006; Evolution and function of phytochelatins synthases, *Plant Physiology*, 163, 319-332
- 103 Cobbett CS. Phytochelatins and their roles in heavy metal detoxification. *Plant Physiology*. 2000; 123: 825-832.

- 104 Howden R, Andersen CR, Goldsbrough PB, Cobbett CS. A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. *Plant Physiology*. 1995; 107: 1067-1073.
- 105 Herbette S, Taconnat L, Hugouvieux V, et al. Genome-wide transcriptome profiling of the early cadmium response of *Arabidopsis* roots and shoots. *Biochimie Paris*. 2006; 88: 0-1765.
- 106 Sarry JE, Kuhn L, Ducruix C, et al. Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice. *Plant Cell*. 2012; 24: 2155-2167.
- 107 Adamis PDB, Gomes DS, Pinto MLCC, Panek AD, Eleutherio EA. The role of glutathione transferases in cadmium stress. *Toxicology Letters*. 2004; 154: 81-88.
- 108 Moons A. Osgstu3 and osgstu4, encoding tau class glutathione S-transferases, are heavy metal- and hypoxic stress-induced and differentially salt stress-responsive in rice roots. *Febs Letters*. 2003; 553: 0-432.
- 109 Romero-Puertas M, Rodriguez-serrano M, Corpas F, Gomez M, Del Rio L. Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. *Plant Cell Environ*. 2004; 27: 1122-1134.
- 110 Sandalio L, Dalurzo H, Gomez M, Romero-Puertas M, Del Rio L. Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *Journal of Experimental Botany*. 2001; 52: 2115-2126.
- 111 Taylor NL, Millar AH. Oxidative stress and plant mitochondria. *Methods in Molecular Biology*. 2007; 372: 389-403.
- 112 Khanna-Chopra R. Leaf senescence and abiotic stresses share reactive oxygen species-mediated chloroplast degradation. *Protoplasma*. 2012; 249: 469-481.
- 113 Livanos P, Galatis B, Apostolakos P. The interplay between ROS and tubulin cytoskeleton in plants. *Plant Signaling & Behavior*. 2014; 9: e28069.
- 114 Ferreres F, Figueiredo R, Bettencourt S, et al. Identification of phenolic compounds in isolated vacuoles of the medicinal plant *Catharanthus roseus* and their interaction with vacuolar class III peroxidase: an H_2O_2 affair?. *Journal of Experimental Botany*. 2011; 62: 2841-2854.
- 115 Nakabayashi R, Yonekura-Sakakibara K, Urano K, et al. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology*. 1998; 49: 249-279.
- 116 Mierziak J, Kostyn K, Kulma A. Flavonoids as important molecules of plant interactions with the environment. *Molecules*. 2014; 19: 16240-16265.
- 117 Jahangir M, Abdel-Farid IB, Kim HK, Choi YH, Verpoorte R. Healthy and unhealthy plants: the effect of stress on the metabolism of Brassicaceae, *Environmental and Experimental Botany*. 2009; 67: 23–33.

- 118 Bernard F, Dumez S, Brulle F, et al. Antioxidant defense gene analysis in *Brassica oleracea* and *Trifolium repens* exposed to Cd and/or Pb. *Environmental Science and Pollution Research*. 2016; 23: 3136-3151.
- 119 Hasanuzzaman M, Nahar K, Anee TI, Fujita M. Exogenous silicon attenuates cadmium-induced oxidative stress in *Brassica napus* L By modulating AsA-GSH pathway and glyoxalase system. *Frontiers in Plant Science*. 2017; 8: 1061.
- 120 Iannelli MA, Pietrini F, Fiore L, Petrilli L, Massacci A. Antioxidant response to cadmium in *Phragmites australis* plants. *Plant Physiology and Biochemistry (Paris)*. 2002; 40: 977-982.
- 121 Chen J, Yang L, Yan X, et al. Zinc-finger transcription factor ZAT6 positively regulates cadmium tolerance through the glutathione-dependent pathway in *Arabidopsis*. *Plant Physiology*. 2016; 171: 707-719.
- 122 Wu H, Chen C, Du J, et al. Co-overexpression *FIT* with *AtbHLH38* or *AtbHLH39* in *Arabidopsis*-enhanced cadmium tolerance via increased cadmium sequestration in roots and improved iron homeostasis of shoots. *Plant Physiology*. 2012; 158: 790-800.
- 123 Xu Z, Liu X, He X, et al. The soybean basic helix-loop-helix transcription factor *ORG3-Like* enhances cadmium tolerance via increased iron and reduced cadmium uptake and transport from roots to shoots. *Frontiers in Plant Science*. 2017; 8: 1098.
- 124 Guan C, Ji J, Li X, Jin C, Wang G. LcMKK, a MAPK kinase from *Lycium chinense*, confers cadmium tolerance in transgenic tobacco by transcriptional upregulation of ethylene responsive transcription factor gene, *Journal of Genetics*. 2016; 95: 875-885.
- 125 Li M, Hao P, Cao F. Glutathione-induced alleviation of cadmium toxicity in *Zea mays*. *Plant Physiol Biochem*. 2017; 119, 240-249.
- 126 Xu Z, Ge Y, Zhang W, Zhao Y, Yang G. The walnut *JrVHAG1* gene is involved in cadmium stress response through ABA-signal pathway and MYB transcription regulation. *BMC Plant Biology*. 2018; 18: 19.
- 127 Yuan J, Bai Y, Chao Y, et al. Genome-wide analysis reveals four key transcription factors associated with cadmium stress in creeping bentgrass (*Agrostis stolonifera* L). *PeerJ*. 2018; 6: e5191.
- 128 Yin XL, Jiang L, Song NH, Yang H. Toxic reactivity of wheat *Triticum aestivum* plants to herbicide isoproturon. *Journal of Agricultural and Food Chemistry*. 2008; 56. 4825-4831.
- 129 Li R, Yu, C, Li, Y, et al. SOAP2: an improved ultrafast tool for short read alignment. *Bioinformatics*. 2009; 25: 1966-1967.

130 Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome biology*. 2010; 11: R14.

131 Mao X, Cai T, Olyarchuk JG, Wei L. Automated genome annotation and pathway identification using the KEGG Orthology KO as a controlled vocabulary, *Bioinformatics*, 2005; 21: 3787-3793.

Figure Legends

Figure 1. The growth performance and total chlorophyll content changes of *B. napus* seedlings exposed to six Cd concentration gradients. a The leaf chlorosis performance of *B. napus* seedlings exposed to no stress and to Cd stress under different Cd concentrations. b The changes in total chlorophyll content of *B. napus* seedlings exposed to six Cd²⁺ concentrations over time: 0 mg/L (control), 5 mg/L, 10 mg/L, 20 mg/L, 30 mg/L, 40 mg/L.

Figure 2. Lengths of small RNAs (sRNAs) and first base preferences of mature miRNAs in 12 libraries (three biological replicates) constructed from shoot and root tissues of *Brassica napus* seedlings under 0 or 10 mg / mL cadmium stress for 10 days. a The proportion of sRNAs of different lengths in the 12 sequencing libraries. The Y-axis displays the number of sRNAs of a certain length, while the X-axis represents sRNAs of different lengths. b The first base preference of mature miRNAs in the 12 libraries. The Y-axis displays the proportion of mature miRNAs with a certain base type as the first base, and the X-axis represents the length classification of the sRNAs.

Figure 3. Differentially expressed miRNAs in the root and shoot of *Brassica napus* seedlings in response to cadmium stress. a The number of miRNAs up- or down-regulated by Cd treatment by > 2-fold in the root and shoot ($P < 0.05$); b A Venn diagrams showing the unique and shared differentially expressed miRNAs in the root and shoot under Cd stress; c Hierarchical cluster analysis of differentially expressed miRNAs in the root and shoot. CS1, CS2, CS3 indicate control samples for the shoot, TS1, TS2 and TS3 indicate treatment samples for the shoot, CR1, CR2 and CR3 indicate control samples for the root and TR1, TR2 and TR3 indicate treatment samples for the root. The fold-change ratios of the miRNAs are indicated by different colors.

Figure 4. Differentially expressed genes in the root and shoot of *Brassica napus* seedlings in response to cadmium stress. a The number of genes up- or down-regulated by Cd treatment by > 2-fold in root and shoot ($P < 0.05$); b A Venn diagrams showing the unique and shared differentially expressed genes in the root and shoot under Cd stress; c Hierarchical cluster analysis of differentially expressed genes in the root and shoot. CS1, CS2, CS3 indicate control samples for the shoot, TS1, TS2 and TS3 indicate treatment samples for the shoot, CR1, CR2 and CR3 indicate control samples for the root and TR1, TR2 and TR3 indicate treatment samples for the root. The fold-change ratios of the genes are indicated by different colors.

Figure 5. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes (DEGs) in the root and shoot of *Brassica napus* seedlings in response to cadmium

stress. a and b represented the top 20 enriched pathways in the root and shoot, respectively. Each circle represents a KEGG pathway, the Y-axis represents the pathway name, and the X-axis represents the enrichment factor, which compares the ratio of genes annotated to a pathway among the DEGs to the ratio of genes annotated to that pathway among all genes. The larger the enrichment factor, the more significant the enrichment of DEGs in the pathway.

Figure 6. Transcriptional changes of genes involved in sulfur and GSH metabolism in the root and shoot of cadmium-treated *Brassica napus* seedlings. The metabolites are represented in gray boxes, arrows represent enzymatic reactions, and names of the enzymes and tissues are given under or above the colored bars. Colored bars on the arrows indicate relative expression of the corresponding genes across the three different biological replicates for the control (C1, C2 and C3; left) and cadmium treatment (T1, T2 and T3; right).

Figure 7. Transcriptional changes of genes involved in phenylpropanoid metabolisms in the root and shoot of cadmium-treated *Brassica napus* seedlings. The metabolites are represented in gray boxes, arrows represent enzymatic reactions, and names of the enzymes and tissue type are given under or above the colored bars. Colored bars on the arrows indicate relative expression of the corresponding genes across the three different biological replicates for the control (C1, C2 and C3; left) and cadmium treatment (T1, T2 and T3; right).

Tables

Due to technical limitations, tables 1 through 3 are only available as a download in the supplemental files section.

Figures

Figure 1

The growth performance and total chlorophyll content changes of *B. napus* seedlings exposed to six Cd concentration gradients. a The leaf chlorosis performance of *B. napus* seedlings exposed to no stress and to Cd stress under different Cd concentrations. b The changes in total chlorophyll content of *B. napus* seedlings exposed to six Cd²⁺ concentrations over time: 0 mg/L (control), 5 mg/L, 10 mg/L, 20 mg/L, 30 mg/L, 40 mg/L.

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Lengths of small RNAs (sRNAs) and first base preferences of mature miRNAs in 12 libraries (three biological replicates) constructed from shoot and root tissues of *Brassica napus* seedlings under 0 or 10 mg / mL cadmium stress for 10 days. a The proportion of sRNAs of different lengths in the 12 sequencing libraries. The Y-axis displays the number of sRNAs of a certain length, while the X-axis represents sRNAs of different lengths. b The first base preference of mature miRNAs in the 12 libraries.

The Y-axis displays the proportion of mature miRNAs with a certain base type as the first base, and the X-axis represents the length classification of the sRNAs.

Figure 3

Differentially expressed miRNAs in the root and shoot of *Brassica napus* seedlings in response to cadmium stress. a The number of miRNAs up- or down-regulated by Cd treatment by > 2-fold in the root and shoot ($P < 0.05$); b A Venn diagrams showing the unique and shared differentially expressed miRNAs

in the root and shoot under Cd stress; c Hierarchical cluster analysis of differentially expressed miRNAs in the root and shoot. CS1, CS2, CS3 indicate control samples for the shoot, TS1, TS2 and TS3 indicate treatment samples for the shoot, CR1, CR2 and CR3 indicate control samples for the root and TR1, TR2 and TR3 indicate treatment samples for the root. The fold-change ratios of the miRNAs are indicated by different colors.

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Differentially expressed genes in the root and shoot of *Brassica napus* seedlings in response to cadmium stress. a The number of genes up- or down-regulated by Cd treatment by > 2-fold in root and shoot ($P < 0.05$); b A Venn diagrams showing the unique and shared differentially expressed genes in the root and shoot under Cd stress; c Hierarchical cluster analysis of differentially expressed genes in the root and shoot. CS1, CS2, CS3 indicate control samples for the shoot, TS1, TS2 and TS3 indicate treatment samples for the shoot, CR1, CR2 and CR3 indicate control samples for the root and TR1, TR2 and TR3 indicate treatment samples for the root. The fold-change ratios of the genes are indicated by different colors.

Figure 5

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes (DEGs) in the root and shoot of *Brassica napus* seedlings in response to cadmium stress. a and b represented the top 20 enriched pathways in the root and shoot, respectively. Each circle represents a KEGG pathway, the Y-axis represents the pathway name, and the X-axis represents the enrichment factor, which compares the ratio of genes annotated to a pathway among the DEGs to the ratio of genes

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Figure 6

Transcriptional changes of genes involved in sulfur and GSH metabolism in the root and shoot of cadmium-treated *Brassica napus* seedlings. The metabolites are represented in gray boxes, arrows represent enzymatic reactions, and names of the enzymes and tissues are given under or above the colored bars. Colored bars on the arrows indicate relative expression of the corresponding genes across the three different biological replicates for the control (C1, C2 and C3; left) and cadmium treatment (T1, T2 and T3; right).

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