Transcriptomic analysis of Verbena bonariensis roots in response to cadmium stress

Meng-qi Wang
Sichuan Agricultural University
Zhen-yu Bai
Sichuan Agricultural University
Ya-fang Xiao
Sichuan Agricultural University
Yan Li
Guizhou University
Qing-lin Liu (qinglinliu@126.com)
Sichuan Agricultural University
Lei Zhang
Sichuan Agricultural University
Yuan-zhi Pan
Sichuan Agricultural University
Bei-bei Jiang
Sichuan Agricultural University
Fan Zhang
Sichuan Agricultural University

Research article

Keywords: Verbena bonariensis, Cadmium stress, RNA-seq, Physiological changes, Molecular mechanisms.

Posted Date: June 21st, 2019

DOI: https://doi.org/10.21203/rs.2.10519/v1

Version of Record: A version of this preprint was published on November 20th, 2019. See the published version at https://doi.org/10.1186/s12864-019-6152-9.

Abstract

Background: Cadmium (Cd) has caused serious heavy metal (HM) pollution in the soil and finding suitable plants to remediate HM pollution is an environmentally friendly approach. Verbena bonariensis is a kind of garden plant with excellent ornamental, good environmental adaptability, which has great potential for future development. This study firstly reported Cd tolerance and an overall analysis of transcriptome in V. bonariensis. Results: In this study, the tolerance of V. bonariensis to Cd stress was investigated in four ways, including germination, growth response, physiological and molecular changes. The results showed that V. bonariensis is not a hyperaccumulator, but it is tolerant to Cd and has the ability to enrich Cd. In the transcriptome data of V. bonariensis roots under Cd stress, 237, 866 transcripts and 191, 370 unigenes were constructed. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that differentially expressed genes (DEGs) under Cd stress are predominately involved in cell structure, ROS scavenging system, chelating reaction and secondary metabolites, transpiration and photosynthesis. DEGs encoding lignin synthesis, chalcone synthase (CHS) and anthocyanidin synthase (ANS) were prominent in V. bonariensis response to Cd stress. The expression patterns of 10 DEGs, validated by quantitative real-time PCR (qRT-PCR), were highly accordant with the RNA-seq results. This study provided novel strategies for further studies on tolerance to Cd exposure and functional genomics in V. bonariensis which was useful information in improving molecular breeding to Cd and phytoremediation.

1. Background

In recent years, HM pollution in soil plant systems has become one of the most urgent problems in the world. Cd is a biologically non-essential and phytotoxic HM pollutant [1]. HMs are absorbed by roots from soil and transported to the aboveground parts of plants. They are accumulated in plants, not only seriously affect the growth and development of plants, but also through the food chain endangers animals and humans.

When the concentration of HMs exceeds the growth limit of plants, plasma membrane permeability, physiological and biochemical processes and nutritional status of plants have been damaged in varying degrees [2]. One of the main consequences is the increased production of reactive oxygen species (ROS), which damage cell membranes, nucleic acids and affect photosynthesis of plants [3-4]. The accumulation of excessive ROS will cause the balance between their production and the antioxidative system activity to be broken. Cd^{2+} enters the plant and disrupts the growth and development of the plant. The key to determining the ability of plants to accumulate and resist HMs lies in the chelation within the plants. There are four main chelating agents in plants, including phytochelatin (PC), metallothionein (MT), organic acid and amino acid [5]. PCs plays an important role in detoxification of excess metals and maintaining the internal balance of trace metal elements. It is synthesized non-translationally from reduced GSH in a transpeptidation reaction catalyzed by the enzyme PC synthase. The sensitivity of secondary metabolites to HM is species-specific [6]. The diversity and functions of soil microbial community structure were decided by generating root exudation of plants [7]. The ‘Plants call for support’

study hypothesizes that the pollution-induced root exudation changes caused by the pollution is beneficial to plant to select microbial communities that can reduce the stress of the pollution to the root system [8], Which means that plants adapt to HM stress by modifying their metabolism including the production of secondary metabolites in plant tissues [9].

The response of most plants to Cd stress has been analyzed by using the RNA-Seq platform. Gu et al. [10] proved that *Iris lactea var. chinensis* is tolerant to accumulations of Cd and lead (Pb) by investigating its transcriptome under Cd or Pb stresses. Yongkun et al. [11] conducted a transcriptome analysis of Cd responses in *Phytolacca americana L.* Gao et al. [12] demonstrated that several genes involved in modifying cell wall and translocating metal ion had higher expressed levels in *S. alfredii Hance* shoots than that in non-hyperaccumulating ecotype shoots under exposing Cd stress. Similar results by using transcriptome analysis were also reported in *Populus × canescens* [13], Noccaea caerulescens [14], *N. caerulescens* [15], *Viola yedoensis Makino* [16] and *Arabidopsis thaliana* [17].

Due to strong adaptability, vigorous growth and highly ornamental value of *V. bonariensis*. especially with the popularity of sightseeing farms, ithas great potential to promote planting. Therefore, it was worth to study about the ability that *V. bonariensis* rehabilitates HM contaminated soil. In this study, *V. bonariensis* was subjected to investigate its germination, morphologic and physiologic response and its ability to accumulate Cd^{2+}. In addition, a high-throughput sequencing technique was used to construct the transcriptome database of *V. bonariensis* under Cd stress, and the molecular mechanism of transport and detoxification to Cd was analyzed based on sequence annotation, which will provide a valuable reference for the discovery of potential Cd defense strategies in *V. bonariensis*.

2. Materials And Methods

2.1 Material and design of germination experiment

V. bonariensis seeds were purchased from Germany Benary seed company. The treatment solution was prepared with deionized water and $CdCl_2$•$2.5H_2O$, the concentrations were 5 mg/L, 10 mg/L, 20 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, the RO water was used as control. The two sheets of filter paper were placed in a culture dish of 9 cm in diameter, add 10 ml of treatment solution to filter paper saturated, as a bed of germination. 30 seeds were placed in each dish, with a total of three replicates. All experiments were performed in three biological repetitions. The dishes were placed in the incubator (16 h photoperiod, 25℃/16℃ day/night temperature). The culture dish is sealed with a sealing film to keep the humidity constant. The germination condition was observed regularly every day until the germination of the control group was no longer changed, and the incubation time was about 2 weeks.

Germination rate (%) = number of germinated seeds within 7 days/ total seed * 100%;

GI = $\sum$Gt /Dt (Gt for germination number of t days, Dt for corresponding days);

VI = GI * biomass (the biomass was the fresh weight of individual seedling) [18].

2.2 Cd treatment

The robust plants with consistent growth were selected for soil Cd stress treatment. Seedling age is 30 days. Each plastic flowerpot was planted with one seedling, 15 pots per treatment. Mix perlite and peat soil were mixed evenly at 1:1 (v: v) and sterilized with proper amount of carbendazim. After 15 days of air drying, the mixed soil put into the circular plastic flowerpot (d=12cm) according to the standard of 1 kg per pot. The soil moisture content was controlled at 70% of the saturated soil moisture content by using the RO water (about 180 ml).

(1) Cd treatment with different concentration. In order to obtain more detailed and accurate data, the content of Cd was measured in *V. bonariensis*. A total of 50 mg/kg (T1), 100 mg/kg (T2), 200 mg/kg (T3), 300 mg/kg (T4), and 400mg/kg (T5) were set up for 5 Cd concentrations. The control group（CK）did not add Cd solution. Each pot was planted with three seedlings, each treatment with 10 pots. During the experiment, plants and soil samples were collected every 10 days for 4 times.

(2) Cd treatment for RNA-seq. The experiment was repeated three times with a total of 90 pots. The control group did not add Cd solution, the concentration of Cd in the experimental group was 100 mg/kg. $CdCl_2 \cdot 2_1/2H_2O$ and RO water were mixed to form 150 ml Cd solution with different concentrations. The mix solution was applied evenly into the corresponding flowerpot on the first day. 20 days under Cd stress, the roots were harvested. The root samples were immediately frozen in liquid nitrogen and stored at -80°C. In this experiment, the seedings were grown in ambient conditions with a photoperiod of 14 h at 25℃ and a relative humidity of 75%.

2.2.1 Determination of Cd in plants and soils

Plant (root, stem and leaf) and soil samples were collected and dried. Then these samples were crushed and per 0.5 g samples were digested by HNO_3-$HCLO_4$, Cd content determination using atomic absorption flame spectrophotometer. All of the above experiments were repeated three times.

$BCF = C_{plant} / C_{soil}$;

$BTF = C_{overground\ part} / C_{subterranean\ part}$;

In the formula, C_{plant} is the concentration of HM in a part (root, stem and leaf) of a plant (mg/kg); C_{soil} is the concentration of corresponding HM element in soil (mg/kg); $C_{overground\ part}$ is the concentration of HM in the upper part of the plant (mg/kg); $C_{subterranean\ part}$ is the concentration of HM in lower parts of plant (mg/kg) [19].

2.2.2 Determination of morphological characteristics and physiological indexes

The leaf and root samples were collected. The part for morphological measurement, and other part immediately frozen in liquid nitrogen and stored at -80°C for physiological measurements. Morphological

features were measured according to Bai et al.[20]. Histochemical staining of ROS (H_2O_2, O_2^-) methods referred to Wang et al. [21]. The content of MDA was determined by thiobarbituric acid colorimetric assay proposed by Cakmak and Marschner [22]. Nitro-blue tetrazolium photoreduction method, Guaiacol method and Ultraviolet absorption method were used to determine the activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), respectively [23]. The ascorbate peroxidase (APX) activity was determined by reference to Nakano et al. and the OD_{290} changes were measured every minute [24]. The activity of GSH and the content of proline (PRO) were determined according to Quessada et al. [25] and Bates et al. [26], respectively. Blade gas exchange parameter was measured at nine o'clock in the morning and the endogenous light intensity was 800μmol quanta $m^{-2}s^{-1}$ [27].

2.3 RNA isolation, library construction and RNA-seq

Roots of two different treatments were collected from *V. bonariensis*. We performed RNA-seq analysis with three replicates. Total RNA isolation was performed with Trizol Reagent (Invitrogen) according to the manufacturer's protocol. RNA purity and concentration were checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA), respectively. In brief, mRNA was enriched from total RNA using poly-T-oligo-attached magnetic beads. The next, it was used as template to synthesize double stranded cDNA and purified with AMPure XP beads (Beckman Coulter, Beverly, USA). Once more, the purified double stranded cDNA was subjected to terminal repair and supplemented with A tail and connect sequencing connector. In order to select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system. At last, PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. And qualifed libraries which assessed on the Agilent Bioanalyzer 2100 system were sequenced on an Illumina Hiseq2500 platform.

2.4 Raw sequence processing, assembling and functional annotation

After filtered, clean data was obtained. Transcriptome de novo assembly was accomplished using Trinity with min-kmer-cov set to 2 by default. A BLASTx search was used for further functional annotation of the unigenes against the NCBI non-redundant protein sequences (Nr), NCBI nucleotide sequences (Nt) and Swiss-prot with an E-value of $\leq 10^{-5}$, while compared with euKaryotic Ortholog Groups (KOG) with an E-value of $\leq 10^{-3}$. The HMMER3 program was used to assign Protein family (Pfam) with an E-value = 0.01. GO using Blast2GO v2.5 program with an E-value = $1e^{-6}$. According to the KEGG database, pathway assignments were carried out using BLASTx with E value = $1e^{-10}$.

2.5 Differential expression analysis

The input data of gene differential expression is the read count data obtained in the analysis of gene expression level. The read count data were standardized by trimmed mean of M-values (TMM), and then DEGseq was used to carry out the difference analysis. In order to control the false positive rate, qvalue combined with foldchange to filter is needed, DEGs screening condition was padj < 0.05.

2.6 qRT-PCR validation

To further validate the DEGs identified in analysis of the RNA-seq data, 10 DEGs were selected randomly to perform qRT-PCR analysis with three replicates. The RNA from the isolated RNA sequencing samples mentioned above. The gene-specific primers were designed by Primer 5. The *Actin* gene (F: GAAAGATGGCTGGAAGAGGG; R: GCTATGAACTCCCTGATGGTC) was served as a reference control to detect expression level of 10 DEGs. The primer sequences were shown in Table S1 (Additional file 1). The data was analyzed by using the $2^{-\Delta\Delta CT}$ method.

2.7 Statistical analysis

The experimental data were statistically analyzed by SPSS17.0 (SPSS Inc., Chicago, USA) and the significance test of difference was made by the LSD method, significance level setting P = 0.05.

3. Results

3.1 The germinated situation and Cd accumulation of V. bonariensis under different Cd concentration stress

Table 1 showed that different concentrations of Cd^{2+} have different effects on the germination of plant seeds. Compared with the control, Germination rate and Germination index (GI) was higher at 20 mg/L than that of other groups. The concentration of Cd in 20 mg/L and below promoted vigor index (VI) and fresh weight in different degrees. At 14 d, all the seedlings treated with more than 50 mg/L concentrations of Cd were death.

The contents of Cd in the shoots and roots increased with the increase of Cd concentration and time, and the Cd contents of roots were significantly higher than those in the shoots (Fig.1 a, b). When the Cd concentration in the soil increased to 400 mg/kg (T5) for 30 d, Cd content of whole plants reached the maximum that is 133.11 mg/kg (Fig.1c). According to Fig.2a, the minimum bioaccumulation factor (BCF) (at the root of the plant) increased with increase of Cd stress duration and concentration, and the range of variation is 0.309 to 0.9990. According to Fig. 2b, translocation factor (BTF) reached to the maximum (0.3344) at the 50 mg/kg Cd concentration. The absorption of HMs is one of the important indexes to evaluate the extraction of HM contaminated soils by hyperaccumulator. It can be found from Fig. 2c that Cd absorption reached to peak value under all concentration at 30 d, the maximum is 31.66 ug/pot in the 300 mg/kg (T4).

3.2 The morphological and physiological changes of V. bonariensis under 100mg/Kg Cd stress

Through the measurement of various morphological (Additional file 2: Figure S2; Additional file 3: Figure S3) and physiological (Additional file 4: Figure S4) indexes in the prophase, 100 mg/kg was selected for RNA-Seq. The morphological and physiological changes of the plants treated respectively under CK and 100 mg/kg Cd concentration for 20 d were compared.

The plants dwarfing, yellowing of leaves, slight darkening of roots on Fig.3a and a large amount of H_2O_2 and O_2^- produced in leaves were intuitively observed on Fig. 3b. After further quantifying, petiole length (PL), the root length (RL), number (RN) and dry to fresh ratio (Dw/Fw) were significantly reduced by 17.39%, 31.87%, 35.29% and 27.92%, respectively, the height of upper part (HP) and leaf area (LA) declined slightly. The indexes of morphological characteristics as a whole were down (Fig. 3c). Cd^{2+} increased the content of MDA and PRO, and activity of GSH in leaves and roots. SOD, POD, CAT and APX were elevated in leaves and decreased in roots under Cd stress (Fig. 4). Net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), Chlorophyll a (Chla) and chlorophyll b (Chlb) decreased in different degrees, only CO_2 concentration (Ci) slightly increased (Fig. 5).

3.3 Sequence analysis and assembly

Large amounts of data were produced with the Illumina HiSeq 2500 sequencing the two libraries (CK and Cd) of *V. bonariensis*. After data filtering, a total of 55, 962, 351 and 61, 462, 567 clean reads with 93.33% and 93.36% Q30 bases were selected for the CK and Cd libraries, respectively. With the Trinity program, in total, 237, 866 transcripts and 191, 370 unigenes with an average length of 1,103 bp and 1,298 bp were constructed, respectively. Data files obtained by Illumina HiSeqTM have been submitted to the NCBI database with accession number GSE113569.

3.4 Sequence annotation and classification

After comparing with the public seven databases, a total of 153, 895 (80.41%) annotative unigenes were obtained. The success rate of the functional annotation in the seven databases is shown in Figure S5 (Additional file 5). The species that offered the most matches was *Sesamum indicum* (97,567 unigenes) and the next was *Erythranthe guttata* (20,692).

Using the GO annotation database, a total of 101,415(52.99%) unigenes were annotated and there were 50.98% in Biological process (BP), 35.44% in Cellular component (CC), and 13.57% in Molecular function (MF) (Additional file 6: Figure S6). In all three data sets, ‘cellular process’, ‘metabolic process’ and ‘single-organism process’ were the most highly represented under BP; ‘cell’, ‘cell part’ and ‘organelle’ terms were dominant in CC, and ‘binding’ and ‘catalytic activity’ were the most significant terms in the MF. Using the KEGG database, a total of 57,061 unigenes were grouped into five branches. Among these pathways, ‘Carbohydrate metabolism’ was the group with the greatest number of genes (5,164, 9.06%), followed by ‘Translation’ (4,284, 7.50%) and ‘Folding, sorting and degradation’ (3,767, 6.60%).

3.5 Analysis of GO term and KEGG pathway involving DEGs

In order to further understand the alteration in gene expression of *V. bonariensis* responding to Cd stress, differential expression analysis with DEGseq was performed. 23, 424 DEGs were obtained, of which 12,558 were up-regulated and 10,866 were down-regulated under Cd treatment.

A total of 16,580 DEGs in *V. bonariensis* were enriched in 60 GO terms. BP, CC and MF accounted for 55.28%, 12.83% and 28.65%, respectively. Among the top 15 significantly enriched GO terms for DEGs, 7 GO terms were related to cell wall (Table 2).

8,600 DEGs were assigned to 124 KEGG pathways. Table 3 showed the top-ten significant up- and down-regulation pathways involving DEGs, respectively. In top-ten up-regulated pathways, the 'glutathione metabolism' was the top-one significantly up-regulated pathway. 133 DEGs were up-regulated and accounted for 76% of all DEGs of this pathway. There are three pathways relating to organic acid metabolism in top-ten up-regulated pathway, including 'Citrate cycle (TCA cycle)' (88 up- and 10 down-regulated DEGs), 'Glyoxylate and dicarboxylate metabolism' (82 and 40) and 'alpha-Linolenic acid metabolism' (60 and 23). The 'Photosynthesis-antenna proteins' and 'photosynthesis' were the first two significantly down-regulated pathways. In 'Photosynthesis-antenna proteins' pathway, all 76 DEGs (75 down- and 1 up-regulated DEGs) were related to the light-harvesting chlorophyll protein complex (LHC). Among which, 18 DEGs were related to Lhca, while 58 DEGs were involved in Lhcb. In 'Photosynthesis', only 9 genes in all 78 DEGs were up-regulated. In addition, the secondary metabolism pathway is worth mentioning. In 'phenylpropanoid biosynthesis', the number of DEGs associated with lignin synthesis was 18 and all was up-regulated (Additional file 7: Table S7). CHS (5 DEGs) and ANS (9) were related to flavonoid biosynthesis (Additional file 8: Table S8).

3.6 qRT-PCR

To confirm the reliability of high-throughput sequencing results, ten DEGs were selected and analyzed for qRT-PCR. It can be concluded that the fold variation between RNA-seq expression and qRT-PCR analyses is almost the same (Fig. 6).

4. Discussion

4.1 The changes of germination and morphological characteristics of V. bonariensis under Cd different concentration stress

The period of seed germination and seedling growth is a sensitive period for plants to environmental stress [28]. Therefore, the study of seed germination and seedling growth under stress can reflect the tolerance of plants to stress to some extent. Previous studies have shown that 10 mg/L Cd stress severely affects the germination of *Medicago Sativa* [29], *Coreopsis drummondii* and *Impatiens walleriana Hook. f.* seeds, and their germination rate was reduced by about 50% compared with the control [30]. Our results showed that the critical concentration of tolerance to *V. bonariensis* germination was about 50 mg/L of Cd. Cd concentration not greater than 20 mg/L can promote the seedlings germination and growth.

The adaptive characteristics of plant growth and morphology are usually the most basic mechanisms for their adaptation to the environment. The roots of the plant are the first to suffer from HMs of soil sites, mainly visible in reduced growth, pigmentation, lateral root numbers, root activity and disturbed

absorption of water and nutrient utilization [31]. With the HM ions transported to shoot, the symptoms of toxicity further showed plant dwarfism, leaf chlorosis, reduced biomass, inhibited photosynthesis and eventually can lead to death [32]. These changes were present in *V. bonariensis* under Cd stress (Fig.3). The roots elongation was inhibited more than shoots under Cd stress in *V. bonariensis*, it is consistent with studies of *Pinus sylvestris L* and hyperaccumulator *S. nigrum* [33, 34]. Petiole, as an important biological linker, is the channel of water and nutrient between leaf and stem of plant [35]. The shortening petiole of *V. bonariensis* can speed up the transportation of water and nutrients, and improve the resistance to stress. For leaf chlorosis, there are two possible reasons: one is the accumulation of Cd in the leaves to a certain amount, chlorophyll destruction and leaves chlorosis; and the other is the serious damage to the root system, the loss of water transport function, resulting in water shortage of leaves. Above points of view were also supported by the decrease of chlorophyll content, petiole length, leaf area, root length and number in *V. bonariensis* (Fig.3c).

4.2 Cd accumulation and transport capacity of V. bonariensis

Typically, most positively charged HM ions bind readily to compounds with a negative charge in tissues of plants and accumulate in roots [36]. In our results, Cd accumulation in roots was significantly higher than that in aboveground parts, which was the retention effect of root system on Cd^{2+}. This behavior of enriching Cd at the root, thereby hindering Cd^{2+} from damaging photosynthesis and metabolism, is a survival strategy for plants in response to stress. The biomass of *V. bonariensis* were significantly reduced under 100 mg/kg Cd stress that was significantly higher than critical concentration of *S. nigrum* that more than 25 mg/kg Cd inhibited its growth and decreased biomass (Additional file 2: Figure S2) [37]. BCF is an indicator of the degree of difficulty in transferring HM elements in soil plant system [37]. The BTF is used to assess the transport and accumulation of HMs from plant roots to the upper part of the plant. As a hyperaccumulator, the BCF and BTF should be greater than 1(Fig.2a, b). The results proved that *V. bonariensis* did not have the characteristics of hyperaccumulator. Cd absorption amount of *V. bonariensis* was 31.66 ug/pot (Fig.2c), which of Cd hyperaccumulator *Bidens pilosa L.* was only 17.92 ug/pot [37].

Through the study, *V. bonariensis* did not meet the standard of Cd hyperaccumulator, it is a Cd tolerant plant that had strong tolerance or strong absorption ability to Cd. *V. bonariensis* accumulates most of Cd in roots and reduces the amount of Cd in leaves and other sensitive organs, thus reducing the toxic effects of Cd on plants. This is consistent with the results of the study that *Lonicera Japanica Thunb* [38]and *Helianthus annuus* [39] also has strong absorbability to Cd. In brief, *V. bonariensis* has rapid growth, large biomass, strong Cd tolerance and absorption ability, and has potential application value in the remediation of Cd pollution.

4.3 Effects of Cd stress on cell wall and cell membrane of V. bonariensis

The plant cell wall played an important role for HM defense and detoxification [40]. The appearance of Cd^{2+} will firstly affect the structure of cell wall. It is the first barrier for HMs to enter the body, Cd of roots

is firstly bound to the pectin site by the root cell wall and carbohydrates, which prevents HM ions from entering the protoplasm of the cell and protecting it from harm [41]. When exposed to HMs, the cell wall could activate hundreds of specific stress-responsive signaling proteins to protect the cell from susceptible sites into the protoplast. In our results, there are 7 GO entries with cell wall tissue correlation, which suggests that *V. bonariensis* may increase its tolerance to HMs by combining the root cell wall with Cd^{2+}. The lignin relating to phenylpropanoid pathway can reinforce specialized cell walls [42]. The number of DEGs associated with lignin synthesis was 18 and all was up-regulated. This indicated that the cell wall of *V. bonariensis* may be reinforce under Cd stress.

The membrane is the second barrier for HMs to enter the body. Cd is an important mutagen of plasma membrane peroxidation. Excessive Cd^{2+} makes plants produce more ROS, induces more MDA, causes membrane lipid peroxidation and destroys membrane ion channel structure [43]. MDA content increased significantly in leaves and roots under Cd stress, which demonstrated that the cell of *V. bonariensis* has occurred membrane lipid peroxidation.

4.4 Effects of Cd stress on ROS scavenging system of V. bonariensis

Previous studies have shown that there are two possible biological pathways for the toxicity of HMs to plants [44, 45]. One is the HM stress oxidation can inhibit the activity of protective enzyme, the resulting free radicals can damage the main biological macromolecules such as proteins and nucleic acids. Two is the HM ion into the plant, not only in combination with nucleic acids, proteins, enzymes and other substances, but also can replace some specific elements must exercise its function of enzymes and proteins, making them denatured or reduced their activity. Under Cd stress, the ROS scavenging system of plants played a vital role in plants. SOD, as the primary defense enzyme in cell scavenging ROS, can convert O_2^- disproportionation into H_2O_2 and eliminate -OH by catalyzing the Fenton reaction [46]. Cd stress is thought to elevate SOD activity in plants, but this promotion and its duration vary with treatment concentration, plant species, and plant size [47]. In our study, the SOD in leaves and roots decreased under Cd treatment, it was speculated that excessive Cd^{2+} or stress time could inhibit the activity of SOD. The activities of POD, CAT and APX increased at the leaves and decreased in the roots under Cd stress in *V. bonariensis*. The roots contacted directly with soil, it may be the primary site of HM toxicity and the stress level at the roots is higher than that at the leaves. Therefore, when antioxidant enzyme activities of the root were inhibited, antioxidant enzyme activities of the leaves could still cope with Cd stress. In the up-regulated GO enrichment categories relating to oxidative reactions, the enrichment degree of ‘oxidation-reduction process’, ‘oxidoreductase activity’ and ‘catalytic activity’ were high. The result showed that the plants may provoke oxidative reactions in response to Cd stress. *V. bonariens* refrained from HM damage by increasing the antioxidant system.

4.5 Effects of Cd stress onChelating reactionof V. bonariensis

Upon exposure to HMs, plants often synthesize diverse metabolites, particularly specific amino acids, such as PRO and histidine, peptides such as glutathione and phytochelatins (PC), and organic acids [48].

They can interact with Cd^{2+} to form chelates, reduce the concentration of Cd^{2+} in soil, and avoid direct contact between Cd^{2+} and organelles, thereby reducing the toxicity of Cd.

Amino acid, as part of the plant's primary metabolites, play an important role in the alleviation of HM stress, which is an integral part of the coenzyme and ligand involved in the metal complexation [49]. Cd stress resulted in a significant increase in the content of some amino acids, which might be a plant specific genetic trait. PRO can regulate plant osmotic/redox reactions and participate in the metal complexation. In our study, Cd stress increased PRO accumulation of aboveground by 29.76%, whereas roots only increased 4.68%. Similarly, the amount of PRO in leaves under Cd stress is higher than that of roots in *Bacopamonnieri* [50].

PCs has great affinity for HMs, and can chelate various HMs and make it lose its activity [51]. When the Cd^{2+} entered the cytoplasm through the cell wall and cell membrane, it combined with PC to form LMW complex, which was transferred into vacuole under the action of htm1 membrane transport protein. Then HMW complexes were synthesized by LMW and Cd in vacuoles and immobilized in vacuole. The HMW complexes were less toxic to plants. PCs is a sulfhydryl polypeptide composed of cysteine, glutamic acid and glycine. GSH is the precursor of PC synthesis and it can use some sulfur-containing compounds in root cells to combine with Cd^{2+} to form stable chelates [52]. In our study, 76% of the DEGs involved in the 'glutathione metabolism' pathway was up-regulated, and GSH content increased (Fig.4). From this result, the increase of PC content can be predicted.

The organic acids of plants, such as oxalic acid, malic acid and citric acid, can be transformed the toxic Cd into low toxic or non-toxic form by chelating, and thus increase the tolerance of plants[53]. The pathways of organic acids in *V. bonariensis* were significant up-regulated in our results. It can be speculated that the efficiency of organic acid synthesis may be elevated, which promoted the binding of Cd^{2+} to organic acids in the cytoplasm or vacuoles, and alleviated the damage of HMs to *V. bonariensis*. organic acids secretion capacity in Cd tolerant plants such as *Rorippaglobosa* is far greater than that in non-tolerant plants *Rorippa* [54]. The increase of organic acid can also increase the soil acidity of the rhizosphere and reduce the uptake of Cd by plants. *Bechmerianivea* exposed to low concentration of Cd, can secrete organic acids in its rhizosphere, with Cd chelating, Cd^{2+} concentration around the rhizosphere of *Bechmerianivea* decreased [55]. In transcriptome data of *V. bonariensis* under Cd stress, there were three pathways relating to organic acid metabolism in top-ten up-regulated pathway, including 'Citrate cycle', 'Glyoxylate and dicarboxylate metabolism' and 'alpha-Linolenic acid metabolism'. The result can prove the important function of organic acid metabolism in *V. bonariensis* response to Cd stress.

4.6 Effects of Cd stress on Secondary metabolites of V. bonariensis

Although secondary metabolites are not essential for plant growth and development, they are often involved in environmental stress [56]. The phenolic metabolism is an important process of plants' secondary metabolism. Abiotic stresses induce a large number of phenolic compounds, forming mechanical barriers that prevent osmotic stress, or remove excessive amounts of ROS in cells [57]. The

phenolic compounds are mainly composed of flavonoids, simple phenols and quinones. The flavonoids, as an important antioxidant in plants, play a key role in resisting the damage caused by stress [58]. The synthesis efficiency of flavonoids can be improved due to the activation of peroxidase under Cd stress [59]. CHS and ANS relating to flavonoid biosynthesis belong to the family of oxidoreductases. CHS is the first enzyme to lead phenylpropane metabolic pathway to flavonoids synthesis. It is a natural defense enzyme and a synthetic intermediate in plants [60]. Anthocyanins is a strong antioxidant, which can scavenge oxygen free radicals in plant cells and alleviate the toxicity of oxygen free radicals. In our results, only one gene down-regulated in 5 CHS and 9 ANS genes, respectively. The activities of CHS and ANS is highly likely to be improved in *V. bonariensis*in response to Cd stress.

The phenylpropanoid biosynthesis has been demonstrated to contribute to various aspects of plant biotic and abiotic responses [61]. Increases in phenolic compound content under abiotic stress, particularly with respect to phenylpropanoid, have been extensively described [62]. In *Lupinus luteus L*., the phenylpropanoid pathway metabolites elevated Pb tolerance of its roots [63]. The 'Phenylpropanoid biosynthesis' occupied the third place in up-regulated pathway (Table 2), which was sufficient to show the importance it played in *V. bonariensis* response to Cd stress.

4.7 Effects of Cd stress on transpiration and photosynthesisof V. bonariensis

Under Cd stress, the Tr of *V. bonariensis* decreases. The results may be due to Cd^{2+} enters guard cells through Ca^{2+} ion channel, which may induce stomatal closure through ABA pathway and inhibit transpiration in plants. These elements disturb stomatal opening. In addition, Cd stress reduced the length and number of roots, limiting plant uptake of water (Fig.3a). Therefore, the leaf area of *V. bonariensis* decreases, which makes plants cell maintain water. Similarly, Tr and leaf area of *Brassica juncea* were inhibited under Cd stress [64].

Cd^{2+} damaged nucleoli of root tip, inhibited the synthesis of RNA and the activities of RNAase, ribonuclease and proton pump. It inhibited nitrate reductase activity, reduced the root uptake of nitrate and transport to the aboveground part. Along with the transport of HM ions to the upper part of plants, the toxicity symptoms are characterized by dwarfism and decreased biomass. Obviously, these factors affected the upward transport of nutrients, thus inhibited the photosynthesis and delayed the growth of the plant. The photosynthesis is mainly reflected in decreased photosynthetic rate, destroyed photosynthetic organs, damaged photosynthetic systems, disturbed carbon dioxide fixation, and even leading to death when effects are severe [65].

Pn and Gs decreased and CO_2 concentration (Ci) increased in our experiment (Fig.5). Stomatal and non-stomatal components are closely related to the decrease in Pn [66]. Besides, chlorophyll decomposition as a non-stomatal limitation is one of the reasons for the decline in Pn. The results illustrated that the decrease of photosynthesis in *V. bonariensis* leaves under Cd stress was not only due to the decrease of CO_2 supply as a result of Gs decline, but also to the non-stomatal factors that hindered the utilization of

CO_2, resulting in the accumulation of intercellular CO_2. Non-stomatal factors may injure the chloroplast of *V. bonariensis* under stress and decrease the photosynthetic cell activity.

In 'Photosynthesis-antenna proteins' pathway, only one gene encoding LHC was up-regulated. As a peripheral antenna system, antenna proteins in LHC play an important role in the efficient absorption of light energy [67]. Most of the DEGs associated with the 'Photosynthesis' were down regulated, indicating that Cd stress has led to disorders in photosynthetic responses. Cd stress affects light harvesting, electron transport and carbon assimilation efficiency during photosynthesis in *V. bonariensis*. This was consistent with previous studies on the response of Maize to Pb [68]. These physiological and molecular changes suggested that down-regulation of the photosynthetic pathway may be a response of *V. bonariensis* to Cd stress.

5. Conclusions

In this study, the Cd tolerance of *V. bonariensis* was comprehensively analyzed from physiological and molecular aspects. It first identified *V. bonariensis* as a HM tolerant plant and provided its first large-scale transcriptional data set in response to Cd stress. ROS system, transpiration and photosynthetic, secondary metabolism and Chelating reaction in *V. bonariensis* under Cd stress were understood in detail by using transcriptional data, and found some promising DEGs that can improve the tolerance to Cd in plants. In conclusion, our research will help to understand the mechanism of Cd resistance in *V. bonariensis* and provide clues for further studies on the relationships between plants and HMs in other *Verbena* plants.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

The raw sequencing data have been submitted to the NCBI Sequence Read Archive database with accession number GSE113569.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This research was supported by National Natural Science Foundation of China (31770742), Sichuan Science and Technology Program (2019YJ0512), Natural Science Foundation of Guizhou (20181044), The introduction of talent project of Guizhou University (201756) and Guizhou University cultivation project (20175788-33).

Author Contributions

M.Q.W., Z.Y.B. L.Y. and Q.L.L. conceived and designed the experiments. M.Q.W., Z.Y.B., and Y.F.X. performed the experiments. M.Q.W., L.Z., Y.-Z.P., B.-B.J. and F.Z. analyzed the data. M.Q.W. and Z.Y.B. wrote the paper. All authors read and approved the final manuscript. M.Q.W and Z.Y.B contributed equally to this work and should be considered co-first authors.

Acknowledgements

All authors declare that they have no conflict of interest.

Additional File Legends

Table S1 The primers of 10 DEGs and different parameters derived from qRT-PCR analysis.

Figure S2 Changes of *Verbena bonariensis* biomass under Cd different concentration stress.

Figure S3 Changes of *Verbena bonariensis* morphological indexes under Cd different concentration stress.(a) Leaf area; (b) petiole long; (c) Plant height; (d) Root length; (e) Number of lateral roots.

Figure S4 Changes of *Verbena bonariensis* physiological indexes under Cd different concentration stress. (a) POD activity; (b) SOD activity; (c) APX activity (d) Soluble sugar content; (e) Soluble protein content; (f) PRO content; (g) GSH activity; (h) MDA activity.

Figure S5 Unigenes notes success statistics in each database.

Figure S6 Unigenes classified statistics based on GO annotations.

Table S7 DEGs encoding lignin synthesis in ‘phenylpropanoid biosynthesis’ pathway

Table S8 DEGs encoding chalcone synthase (CHS) and anthocyanidin synthase (ANS)

References

1. Thakur, S., Choudhary, S., & Bhardwaj, P. Comparative transcriptome profiling under cadmium stress reveals the uptake and tolerance mechanism in brassica juncea. Journal of Plant Growth Regulation(8). 2019;1-12. DOI:10.1007/s00344-019-09919-8

2. Liu S.L., Pan Y.Z., Yang R.J., Ding J.J., Yang H.E., Wang L., Ming-Dong M.A. Effects of exogenous NO on mineral nutrition absorption,lipid peroxidation and ATPase of plasma membrane in Catharanthus roseus tissues under cadmium stress. Journal of Plant Nutrition & Fertilizer. 2014;20:445-458. DOI: 10.11674/zwyf.2014.0222.

3. Ma, X., Yu, D., Shao, W., Min, X., Zuo, Z., & Wang, H., et al. Transcriptome-wide identification and characterization of the copper and cadmium stress-responsive small rnas and their targets in *arabidopsis thaliana*. Plant & Soil. 2018;391–405. DOI:10.1007/s11104-018-3697-3

4. Tewari, R.K., Kumar, P., Sharma, P.N., Bisht, S.S. Modulation of oxidative stress responsive enzymes by excess cobalt. Plant Sci. 2002;162, 381–388. DOI:10.1016/s0168-9452(01)00578-7

5. Chen A.K., Gong Y.L., Zhou X.J., Liu X.H. Accumulation of Cd, As and their interaction with mineral elements in different rice (*Oryza sativa L.*) cultivars grown in Gejiu mine, Yunnan Province. Ecological Science. 2013;32:668-672. DOI:10.1016/j.pnpbp.2007.11.016

6. Molnár, K., Farkas, E. Current results on biological activities of lichen secondary metabolites: a review. Z. für Naturforschung C. 2010;65, 157–173. DOI:10.1515/znc-2010-3-401

7. Michalet S, Rohr J, Warshan D et al. Phytochemical analysis of mature tree root exudates in situ and their role in shaping soil microbial communities in relation to tree N-acquisition strategy. Plant Physiol Biochem. 2013;72:169–177. DOI:10.1016/j. plaphy.2013.05.003.

8. Thijs S, Sillen W, Rineau F et al. Towards an enhanced understanding of plant–microbiome interactions to improve phytoremediation: engineering the metaorganism. Front Microbiol. 2016;DOI: doi:10.3389/fmicb.2016.00341.

9. Singh S, Parihar P, Singh R et al. Heavy metal tolerance in plants: role of transcriptomics, proteomics, metabolomics, and ionomics. Front Plant Sci. 2016;6:1143. DOI:10.3389/fpls.2015.01143.

10. Gu, C. S., Liu, L. Q., Deng, Y. M., Zhang, Y. X., Wang, Z. Q., & Yuan, H. Y., et al. De novo, characterization of the, iris lactea, var. chinensis, transcriptome and an analysis of genes under cadmium or lead exposure. Ecotoxicology and Environmental Safety. 2017;144, 507-513. DOI: 10.1016/j.ecoenv.2017.06.071

11. Yongkun, C., Junkai, Z., Hao, Z., Jian, L., Qihong, Z., & Jichen, X., et al. Transcriptome analysis of phytolacca americana l. in response to cadmium stress. PLOS ONE. 2017;12(9), e0184681-. DOI: 10.1371/journal.pone.0184681

12. Gao J., Sun L., Yang X., Liu J. X., Schiefelbein, J. Transcriptomic analysis of cadmium stress response in the heavy metal hyperaccumulator sedum alfredii hance. PLoS ONE. 2013;8(6), e64643. DOI 10.1371/journal.pone.0064643

13. He J, Li H, Luo J, Ma C, Li S, Qu L, et al. A transcriptomic network underlies microstructural and physiological responses to cadmium in Populus x canescens. Plant Physiol. 2013;162: 424–439. DOI: 10.1104/pp.113.215681

14. Milner MJ, Mitani-Ueno N, Yamaji N, Yokosho K, Craft E, Fei Z, et al. Root and shoot transcriptome analysis of two ecotypes of Noccaea caerulescens uncovers the role of NcNramp1 in Cd hyperaccumulation. Plant J. 2014;78: 398–410. DOI: 10.1111/tpj.12480

15. Lin YF, Severing EI, te Lintel Hekkert B, Schijlen E, Aarts MG. A comprehensive set of transcript sequences of the heavy metal hyperaccumulator *Noccaea caerulescens*. Front Plant Sci. 2014;5: 261 DOI: 10.3389/fpls.2014.00261

16. Gao J, Luo M, Zhu Y, He Y, Wang Q, Zhang C. Transcriptome sequencing and differential gene expression analysis in *Viola yedoensis Makino* (*Fam. Violaceae*) responsive to cadmium (Cd) pollution. Biochem Biophys Res Commun. 2015;459: 60–65. DOI: 10.1016/j.bbrc.2015.02.066

17. Weber M, Trampczynska A, Clemens S. Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd2+-hypertolerant facultative metallophyte Arabidopsis halleri. Plant Cell Environ. 2006;29: 950–963. DOI 10.1111/j.1365-3040.2005.01479.x

18 Dong W. GERMINATION TESTING METHODS ON ACHNATHERUM SPLENDENS SEEDS. Prataculturalence. 1998.

19. Pan Y.H., Wang H.B., Gu Z.P. Accumulation and translocation of heavy metals by macrophytes. Acta Ecologica Sinica. 2010;30:6430-6441. DOI doi:10.1016/S1872-5813(11)60001-7

20. Bai Z., Wang T., Wu Y., Wang K., Liang Q., Pan Y., Jiang B., Zhang L., Liu G., Jia Y. Whole-transcriptome sequence analysis of differentially expressed genes in Phormium tenax under drought stress. Sci Rep.2017 7:41700. DOI 10.1038/srep41700

21. Wang K., Zhong M., Wu Y.H., Bai Z.Y., Liang Q.Y., Liu Q.L., Pan Y.Z., Zhang L., et al. Overexpression of a chrysanthemum transcription factor gene DgNAC1 improves the salinity tolerance in chrysanthemum. Plant Cell Reports. 2017;36:1-11. DOI 10.1007/s00299-017-2103-6

22. Cakmak, I. , & Marschner, H. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. PLANT PHYSIOLOGY.1992 98(4), 1222-1227. DOI 10.1104/pp.98.4.1222

23. Beauchamp C., Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry.1971 44:276-287. DOI 10.1016/0003-2697(71)90370-8

24. Nakano Y., Asada K. Hydrogen Peroxide is Scavenged by Ascorbate-specific Peroxidase in Spinach Chloroplasts. Plant & Cell Physiology. 1981;22:867-880. DOI 10.1093/oxfordjournals.pcp.a076232

25. Quessada M.P., Macheix J.J. Caracterisation d'une peroxydase impliquee specifiquement dans la lignification, en relation avec l'incompatibilite au greffage chez l'abricotier. Physiologie Vegetale. 1984.

26. Irigoyen JJ, Emerich DW, Sanchez-Diaz M. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (Medicago sativa) plants. Physiol. Plant. 2010;84: 55-60. DOI 10.1111/j.1399-3054.1992.tb08764.x

27. Mguis K., Abassi M., Khadhri A., Ykoubi-Tej M., Mahjoub A., Brahim N.B., Ouerghi Z. Responses of leaf growth and gas exchanges to salt stress during reproductive stage in wild wheat relative Aegilopsgeniculata Roth. and wheat (Triticumdurum Desf.). Acta Physiologiae Plantarum. 2013;35:1453-1461. DOI 10.1007/s11738-012-1185-6

28. Min L.I., Luo Y.M., Song J., Zhang D.S. Ecotoxicological Effects of pH,Salinity and Heavy Metals on the Barley Root Elongation in Mixture of Copper Mine Tailings and Biosolids. Soils.2006;38:578-583. DOI 10.1016/S1872-2032(06)60052-8

29. JR P., JL G., KJ T., E G., S A., E R., JG P. Uptake and effects of five heavy metals on seed germination and plant growth in alfalfa (*Medicago sativa L.*). Bulletin of Environmental Contamination and Toxicology. 2001;66:727-734. DOI 10.1007/s001280069

30. Ma M., Gong H., Deng H. Effects of Heavy Metal Stress on Seed Germination and Seedling Growth of Eight Urban Plants. Chinese Agricultural Science Bulletin. 2012;28:206-211. DOI 10.1007/s11783-011-0280-z

31. SA H., Q F., B A., S H., A A. Cadmium: toxicity and tolerance in plants. J Environ Biol. Environmental Chemistry Letters. 2013;11:229-254.

32. Liu X.Q., Peng K.J., Wang A.G., Lian C.L., Shen Z.G. Cadmium accumulation and distribution in populations of *Phytolacca americana L.* and the role of transpiration. Chemosphere. 2010;78:1136-1141. DOI 10.1016/j.chemosphere.2009.12.030

33. Andres Schützendübel, & Polle, A. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. Journal of Experimental Botany.2002 53(372), 1351-1365. DOI 10.1093/jxb/53.372.1351

34. Guo Z., Yuan H., Yansong A.O. Effect of cadmium on photosynthesis and nutrient elements uptake of Solanum nigrum L. seedlings. Ecology & Environmental Sciences. 2009.

35. Foy C.D., R L Chaney a., White M.C. The Physiology of Metal Toxicity in Plants. Annual Review of Plant Physiology and Plant Molecular Biology. 1978;29:511-566. DOI 10.1146/annurev.pp.29.060178.002455

36. Shi-liang L., Xin-sheng S., Yuan-zhi P., Ji-jun D., Yang H., Li W. Effects of cadmium stress on growth,accumulation and distribution of biomass and nutrient in *Catharanthus roseus*. Acta Prataculturae Sinica. 2013; 22:154-161.

37. Sun Y.B., Zhou Q.X., Wang L., Liu W.T., Liu R. Characteristics of cadmium tolerance and bioaccumulation of Bidens pilosa L. seedlings. Environmental Science. 2009;30:3028. DOI http://dx.doi.org/

38. Zhouli L., Xingyuan H. Lonicera japonica Thunb. — a newly discovered Cd hyper-accumulator. Ecology\s&\senvironmental Sciences. 2013;22:666-670.

39. Xin N.Z., Na S.L., Sun T.H. Enrichment characteristics of Cd and Pb by four kinds of plant under hydroponic culture. Chinese Journal of Ecology. 2010;29:261-268. DOI 10.1016/S1872-5813(11)60001-7

40. Zhang Z., Wang H., Wang X., Bi Y. Nitric oxide enhances aluminum tolerance by affecting cell wall polysaccharides in rice roots. Plant Cell Reports. 2011;30:1701-1711. DOI 10.1007/s00299-011-1078-y

41. Krzesłowska M. The cell wall in plant cell response to trace metals: polysaccharide remodeling and its role in defense strategy. Acta Physiologiae Plantarum. 2011;33:35-51. DOI 10.1007/s11738-010-0581-z

42. M.C., Ruel K., † A.Y., Chabbert B., Jauneau A., Joseleau J.P., Boudet A.M. In situ analysis of lignins in transgenic tobacco reveals a differential impact of individual transformations on the spatial patterns of lignin deposition at the cellular and subcellular levels. Plant Journal. 2010;28:271-282. DOI 10.1046/j.1365-313X.2001.01159.x

43. Liu S.L., Yang R.J., Ming-Dong M.A., Jiang P., Zhao Y., University S.A. Effects of Soil Cadmium on Growth and Physiological Characteristics of Solanum nigrum L. Plants. Journal of Agro-Environment Science. 2015;34:240-247.

44. Cao X., Ma L.Q., Tu C.Antioxidative responses to arsenic in the arsenic-hyperaccumulator Chinese brake fern (*Pteris vittata L.*). Environmental Pollution.2004 128:317-325. DOI 10.1016/j.envpol.2003.09.018

45. Vázquez M.D., Poschenrieder C., Barceló J., Baker A.J.M., Hatton P., et al. Compartmentation of Zinc in Roots and Leaves of the Zinc Hyperaccumulator Thlaspi caerulescens J & C Presl. Plant Biology. 2015;107:243-250. DOI 10.1111/j.1438-8677.1994.tb00792.x

46. RodríguezSerrano M., RomeroPuertas M.C., Pazmiño D.M., Testillano P.S., Risueño M.C., Río L.A.d., Sandalio L.M. Cellular Response of Pea Plants to Cadmium Toxicity: Cross Talk between Reactive Oxygen Species, Nitric Oxide, and Calcium. Plant Physiology. 2009;150:229-243. DOI 10.1104/pp.108.131524

47. Shao G., Hassan M.J., Zhang X., Zhang G. Effects of cadmium stress on plant growth and antioxidative enzyme system in different rice genotypes. Chinese Journal of Rice Science. 2004;18:239-

244. DOI 10.1007/s11769-003-0051-2

48. Sharma, & S. S. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. Journal of Experimental Botany. 2006;57(4), 711-726. DOI 10.1093/jxb/erj073

49. Kováčik J., Klejdus B., Hedbavny J., Bačkor M. Effect of copper and salicylic acid on phenolic metabolites and free amino acids in Scenedesmus quadricauda (*Chlorophyceae*). Plant Science. 2010;178:307-311. DOI 10.1016/j.plantsci.2010.01.009

50. Mishra S., Srivastava S., Tripathi R.D., Govindarajan R., Kuriakose S.V., Prasad M.N.V. Phytochelatin synthesis andresponse of antioxidants during cadmium stress in *Bacopamonnieri L*. Plant Physiology & Biochemistry. 2006;44:25-37. DOI 10.1016/j.plaphy.2006.01.007

51. Połeć-Pawlak K., Ruzik R., Abramski K., Ciurzyńska M., Gawrońska H. Cadmium speciation in *Arabidopsis thaliana* as a strategy to study metal accumulation system in plants. Analytica Chimica Acta. 2005;540:61-70. DOI 10.1016/j.aca.2004.10.048

52. Benavides M.P., Gallego S.M., Tomaro M.L. Cadmium toxicity in plants. Braz.j. plant Physiol .2005 17:21-34. DOI 10.1590/S1677-04202005000100003

53. Sun R.L., Zhou Q.X., Jin C.X. Cadmium accumulation in relation to organic acids in leaves of *Solanum nigrum L.* as a newly found cadmium hyperaccumulator. Plant & Soil. 2006;285:125-134. DOI 10.1007/s11104-006-0064-6

54. Sun R.L. Analysis on Ecological characteristics and pollution tolerance Mechanism of cadmium Superaccumulative plants. Shenyang Shenyang Institute of Applied Ecology, Chinese Academy of Sciences. 2006.

55. Lu H., Yan C., Liu J. Low-molecular-weight organic acids exuded by Mangrove (*Kandelia candel (L.) Druce*) roots and their effect on cadmium species change in the rhizosphere. Environmental & Experimental Botany. 2007;61:159-166. DOI 10.1016/j.envexpbot.2007.05.007

56. Lequeux H., Hermans C., Lutts S., *Verbruggen N.* Response to copper excess in *Arabidopsis thaliana*: impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile. plant physiol biochem. Plant Physiology and Biochemistry. 2010;48(8), 673-682. DOI 10.1016/j.plaphy.2010.05.005

57. Babula P., Klejdus B., Kovacik J., Hedbavny J., Hlavna M. Lanthanum rather than cadmium induces oxidative stress and metabolite changes in *Hypericum perforatum*. Journal of Hazardous Materials.2015 286:334-342. DOI 10.1016/j.jhazmat.2014.12.060

58. Tattini M, Galardi C, Pinelli P. Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. New Phytol. 2004;163 (3): 547–561.

DOI 10.1111/j.1469-8137.2004.01126.x

59. Ogundiwin E.A., Peace C.P., Nicolet C.M., Rashbrook V.K., Gradziel T.M., Bliss F.A., Parfitt D., Crisosto C.H. *Leucoanthocyanidin dioxygenase* gene (PpLDOX): a potential functional marker for cold storage browning in peach. Tree Genetics & Genomes. 2008;4:543-554. DOI 10.1007/s11295-007-0130-0

60. Kreuzaler F., Hahlbrock K. Enzymatic synthesis of aromatic compounds in higher plants: formation of naringenin (5,7,4'-trihydroxyflavanone) from p-coumaroyl coenzyme A and malonyl coenzyme A. Historical patterns of industrialization /. Longman. 1972. DOI 10.1016/0014-5793(72)80679-3

61. Vogt, Thomas. Phenylpropanoid Biosynthesis. Molecular plant. 2010;3:2-20. DOI 10.1093/mp/ssp106

62. Christie P.J., Alfenito M.R., Walbot V. Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. Planta. 1994;194:541-549. DOI 10.1007/bf00714468

63. Izbiańska K., Arasimowiczjelonek M., Deckert J. Phenylpropanoid pathway metabolites promote tolerance response of lupine roots to lead stress. Ecotoxicology & Environmental Safety. 2014;110:61-67. DOI 10.1016/j.ecoenv.2014.08.014

64. Haag-Kerwer, & A. Cadmium exposure in brassica juncea causes a decline in transpiration rate and leaf expansion without effect on photosynthesis. Journal of Experimental Botany. 1999;50(341), 1827-1835. DOI 10.1093/jexbot/50.341.1827

65. Chen F., Wang F., Sun H., Cai Y., Mao W., Zhang G., Vincze E., Wu F. Genotype-Dependent Effect of Exogenous Nitric Oxide on Cd-induced Changes in Antioxidative Metabolism, Ultrastructure, and Photosynthetic Performance in Barley Seedlings (*Hordeum vulgare*). Journal of Plant Growth Regulation. 2010;29:394-408. DOI 10.1007/s00344-010-9151-2

66. W T., D M., E R., A H. Photosynthetic responses of the tropical spiny shrub Lycium nodosum (*Solanaceae*) to drought, soil salinity and saline spray. Annals of Botany. 2003;92:757-765. DOI 10.1093/aob/mcg199

67. Nield J., Orlova E.V., Morris E.P., Gowen B., Heel M.V., Barber J. 3D map of the plant photosystem II supercomplex obtained by cryoelectronmicroscopy and single particle analysis. Nature Structural Biology. 2000;7:44. DOI 10.1038/71242

68. Li HS. Principles and techniques of plant physiology and Biochemistry Experiments. In: Xiong QE editors. Plant Physiology Experiment Course. Sichuan Science and Technology Press p. 2003;68-92.

Tables

Table 1 Effect of Cd concentration on germination of *Verbena bonariensis*

Concentration (mg/L)	Germination rate (%)	7 d Germination index	Vigor index	14 d fresh weight per plant (mg)	Survival rate (%)
0	97.78±1.92^{ab}	25.33±0.29^{abc}	0.0532±0.0020^{abc}	2.10±0.10^{abc}	97.78±1.92^{ab}
5	97.78±1.92^{ab}	25.67±0.29^{ab}	0.0573±0.0056^{a}	2.23±0.23^{a}	97.78±1.92^{ab}
10	97.78±1.92^{ab}	25.67±0.58^{ab}	0.0574±0.0050^{a}	2.23±0.15^{a}	97.78±1.92^{ab}
20	100.00±0.00^{a}	26.50±0.50^{a}	0.0565±0.0025^{ab}	2.13±0.06^{ab}	98.89±1.92^{a}
50	97.78±1.92^{ab}	25.50±1.50^{ab}	0.0494±0.0043^{bcd}	1.93±0.06^{bcd}	46.67±6.67^{b}
100	96.67±5.77^{ab}	24.83±0.77^{bcd}	0.0471±0.0011^{cd}	1.90±0.10^{bcd}	0.00±0.00^{c}
150	95.56±1.93^{ab}	24.00±1.00^{cd}	0.0449±0.0069^{d}	1.87±0.21^{cd}	0.00±0.00^{c}
200	92.22±5.09^{b}	23.50±0.50^{d}	0.0423±0.0009^{d}	1.80±0.00^{d}	0.00±0.00^{c}

Table 2 The top-15 significant enriched GO terms involving DEGs under Cd stress

Description	Term_type	Up-regulated DEGs number	Down-regulated DEGs number
structural constituent of cell wall	molecular_function	101	3
oxidation-reduction process	biological_process	1354	802
oxidoreductase activity	molecular_function	1337	773
plant-type cell wall organization	biological_process	111	5
plant-type cell wall organization or biogenesis	biological_process	111	5
catalytic activity	molecular_function	5244	3879
cell wall	cellular_component	160	35
external encapsulating structure	cellular_component	181	48
cell wall organization	biological_process	145	21
heme binding	molecular_function	333	160
tetrapyrrole binding	molecular_function	333	167
external encapsulating structure organization	biological_process	148	21
cell wall organization or biogenesis	biological_process	187	40
cell wall biogenesis	biological_process	141	20
single-organism metabolic process	biological_process	2820	1954

Table 3 The top-ten significant enriched KEGG pathways involving DEGs under Cd stress

Regulation	Pathway term	Rich factor	qvalue	Gene number
Up-regulated	Glutathione metabolism	0.223529	2.42E-10	133
	Citrate cycle (TCA cycle)	0.226221	3.80E-07	88
	Phenylpropanoid biosynthesis	0.179342	9.83E-07	158
	Proteasome	0.233974	1.13E-06	73
	Carbon fixation in photosynthetic organisms	0.180113	0.000261	96
	Glycolysis / Gluconeogenesis	0.157366	0.001086	141
	Flavone and flavonol biosynthesis	0.377778	0.00125	17
	Galactose metabolism	0.169091	0.001704	93
	Glyoxylate and dicarboxylate metabolism	0.172632	0.002082	82
	alpha-Linolenic acid metabolism	0.180723	0.004917	60
Down-regulated	Photosynthesis - antenna proteins	0.675676	2.18E-35	75
	Photosynthesis	0.345	1.82E-19	69
	Glycerophospholipid metabolism	0.16109	6.45E-12	130
	Glycerolipid metabolism	0.179704	6.71E-10	85
	Carotenoid biosynthesis	0.193133	3.94E-06	45
	Ether lipid metabolism	0.181818	3.94E-06	50
	Circadian rhythm - plant	0.178439	9.42E-06	48
	Starch and sucrose metabolism	0.112982	4.09E-05	161
	Vitamin B6 metabolism	0.301587	4.65E-05	19
	Plant hormone signal transduction	0.108998	0.000395	149

Figures

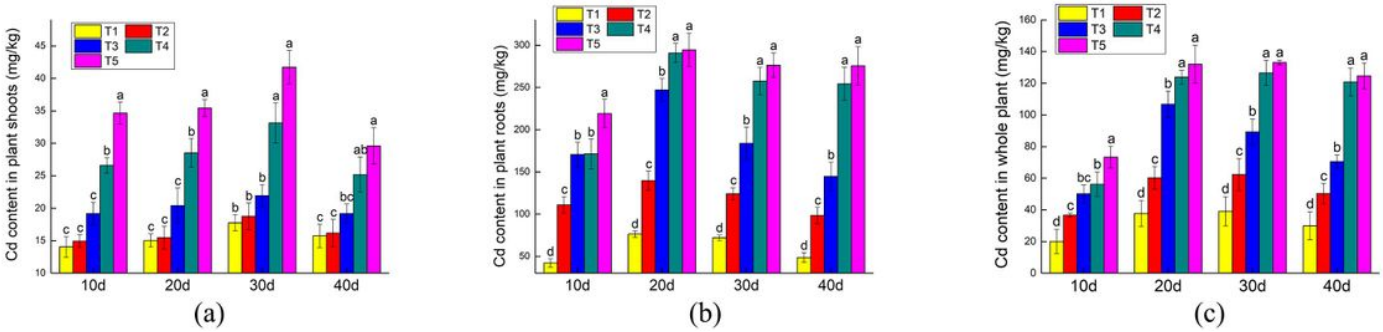

Figure 1

Enrichment of Cd in plants. (a) Cd content in plant shoots. (b) Cd content in plant roots. (c) Total Cd content in Verbena bonariensis. A total of 50 mg/kg (T1), 100 mg/kg (T2), 200 mg/kg (T3), 300 mg/kg (T4), and 400 mg/kg (T5) were set up for 5 Cd concentrations. Standard error of the mean for three repetitions is represented by the error bars. The different letters above the bars indicate the significant difference at $P < 0.05$ among the different treatments. The same below.

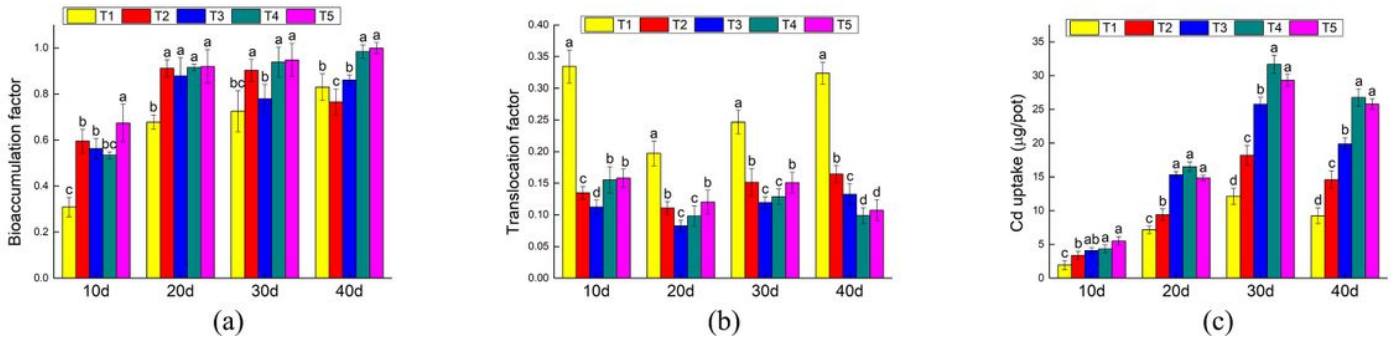

Figure 2

The impacts of Cd in soil on the bioaccumulation factor, translocation factor and Cd uptake of Verbena bonariensis. (a) bioaccumulation factor of Cd in roots. (b) translocation factor of Cd in Verbena bonariensis. (c) Cd uptake by Verbena bonariensis.

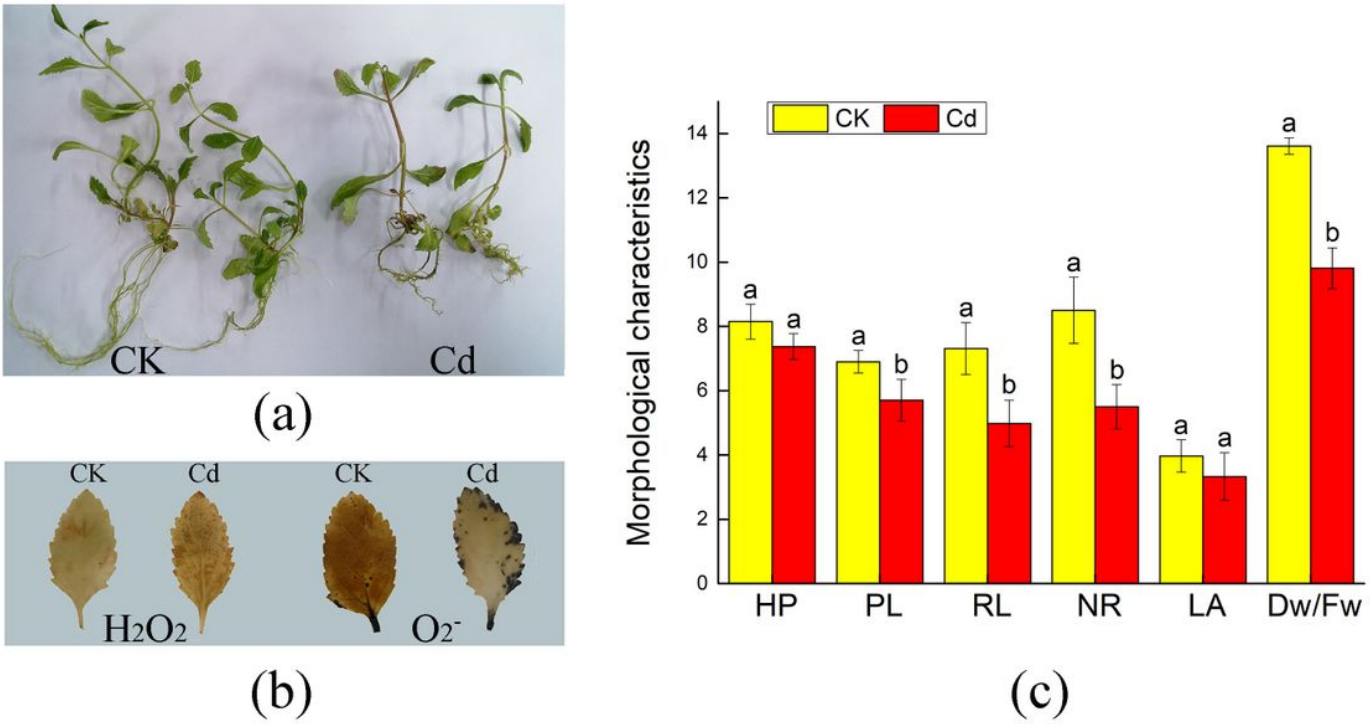

Figure 3

Effects of Cd stress on external morphology and active oxygen metabolism in Verbena bonariensis. (a) The comparison of vitro morphology of plants from CK and Cd treated. (b) The comparison of ROS staining of leaves from control and Cd-treated Verbena bonariensis plants. (c) The indexes of morphological characteristics. Plants were grown with 100 mg/kg Cd for 20 d.

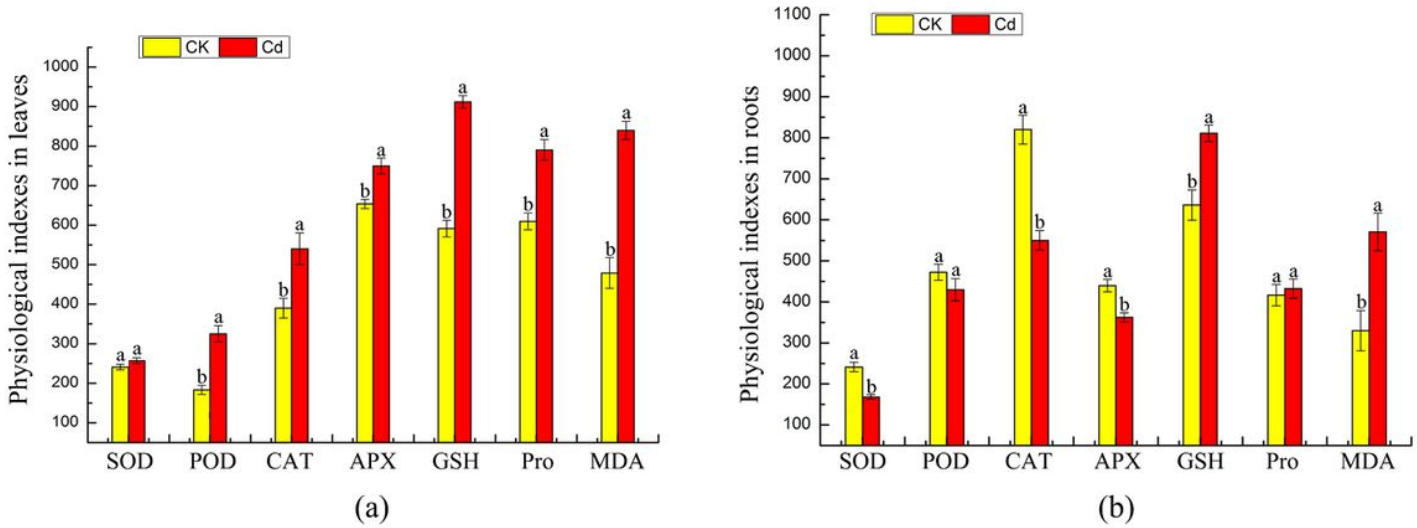

Figure 4

Effects of Cd on physiological indexes of Verbena bonariensis. (a) The changes of leaves under Cd stress; (b) The changes of roots under Cd stress. SOD and APX activity as u·g-1□POD activity was expressed as u·g-1·min-1, CAT as 10-1·u·g-1·min-1, GSH as 10-2·u·g-1 FW, proline as ng·ml-1 and MDA as 10-1·nmol·L-1.

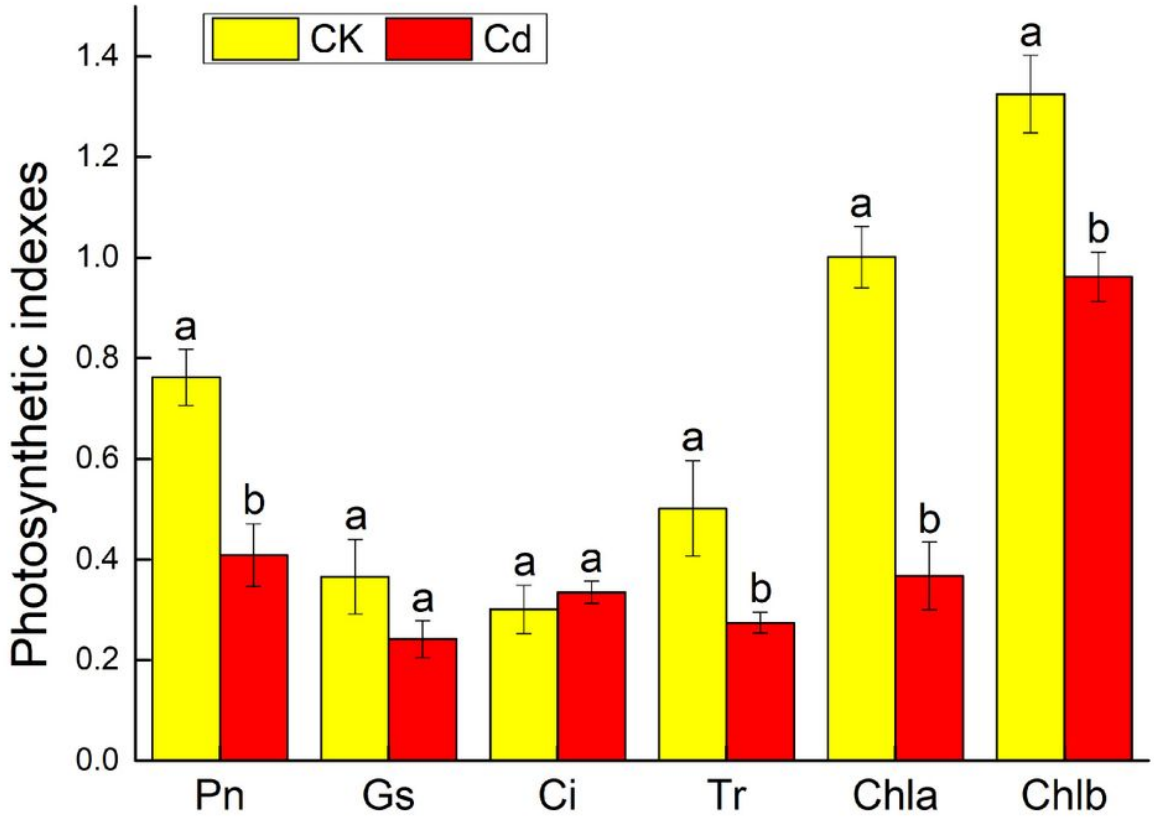

Figure 5

Effects of photosynthesis under Cd stress in Verbena bonariensis leaves. Pn and Tr were expressed as umol·m-2·s-1, Gs was expressed as 10-1·mol·m-2·s-1, Ci as ml·L-1, Chla and Chlb as mg·g-1.

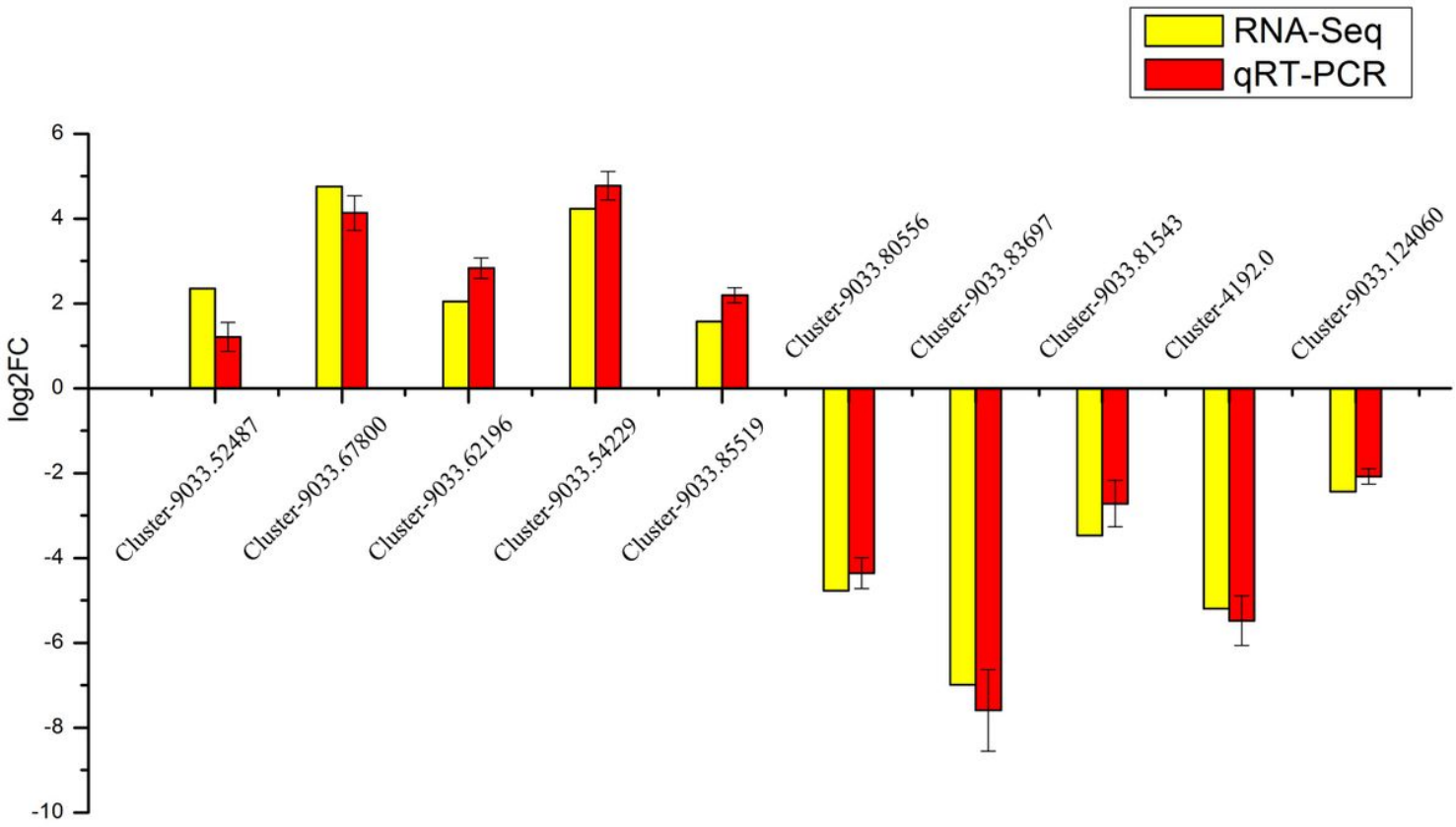

Figure 6

Validation of RNA-Seq results using qRT-PCR. The gene primers used for RT-qPCR analysis are shown in Table S2. Standard error of the mean for three repetitions is represented by the error bars.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.docx
- supplement2.docx
- supplement3.docx
- supplement3.docx
- supplement5.docx
- supplement6.docx
- supplement7.docx

- supplement8.docx