

Comparative transcriptomic analysis of two *Vicia sativa* L. varieties with contrasting responses to cadmium stress reveals the important role of metal transporters in cadmium tolerance

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Abstract

Aims Cadmium (Cd) is one of the most toxic heavy metals. Cd tolerance ability differs among varieties in plants, but the underlying molecular mechanisms remain largely unknown. In this study, we identified genes that are involved in Cd stress responses and different Cd tolerances of two *V. sativa* varieties (Cd-tolerant variety (L3) and Cd-sensitive variety (ZM)).

Methods Transcriptomic analysis using Illumina pair-end sequencing was carried out on root tissues of L3 and ZM grown with 5 μM and 50 μM of Cd treatments. A de novo assembled *V. sativa* transcriptome was generated. Differentially expressed genes (DEGs) were assigned to Gene Ontology (GO) functions and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and enrichment analysis was performed. The expression of selected DEGs were confirmed by quantitative reverse transcription–polymerase chain reaction (qRT-PCR).

Results A total of 49,062 sequences were identified as unigenes. In the 5 μM Cd treatment, 69 and 28 differentially expressed unigenes were found as compared with the control in the L3 and ZM respectively, while in the 50 μM Cd treatment, 1036 and 335 differentially expressed unigenes were found in comparison with the control in L3 and ZM. Pathway enrichment analysis suggested that genes related to the cell wall, stress response, the glutathione pathway, metal transporters, and transcription factors are commonly up-regulated in response to Cd stress in both varieties. However, the expression of metal transporter genes and transcription factor genes showed significant differential responses to Cd stress.

Conclusions In addition to the regulation of transcription by transcription factors, metal transporters play a vital role in controlling the different Cd tolerances of *V. sativa* varieties L3 and ZM.

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Introduction

Cadmium is considered a non-essential metal element for plants and is one of the most toxic heavy metals. It causes a wide range of deleterious effects on plants, including oxidative stress, protein inactivation, nutrient uptake disturbance and even plant death (Mohamed et al. 2012). Plants have evolved several collective mechanisms to prevent excess Cd effects, such as cell

wall binding, extracellular exudates, redistribution by definite transporters, compartmentation in vacuole, chelation with phytochelatin, restoration of impaired proteins and antioxidant defense (Hall 2002; Mendoza-Cózatl et al. 2005; Weber et al. 2006; Ahsan et al. 2009; Lin and Aarts 2012; Gao et al. 2016; Singh et al. 2016). By limiting heavy metal transport activity, plants can diminish toxic metal inflow and enhance metal exclusion from the cytosol (Wysocki and Tamás 2010; Clemens et al. 2013). A number of heavy metal transporters involved in the acquisition, distribution, and homeostasis of Cd in plants have been characterized, comprising ATP-binding cassette transporters (ABC), heavy metal ATPases (HMA), metal transporter proteins (MTPs), low-affinity cation transporters (LCT), and natural resistance-associated macrophage proteins (Nramp) (Verrier et al. 2008; Thomine et al. 2000; Uruguchi et al. 2014; Slamet-Loedin et al. 2015; Yuan et al. 2012). OsNramp5, the uptake transporter for the essential element manganese (Mn), was found to enable rice to accumulate large amounts of Cd (Sasaki et al. 2012; Yoneyama et al. 2015). AtNramp3 and AtNramp4 play key roles in the export of vacuolar Cd (Thomine et al. 2003; Lanquar et al. 2010; Pottier et al. 2015), and AtNramp6 functions as an intracellular metal transporter, which can be modified to affect the distribution and availability of cadmium within the cell (Cailliatte et al. 2009). OsHMA2, an HMA family transporter, is involved in the translocation of Cd into shoots, while OsHMA3 helps sequester Cd in vacuoles (Ueno et al. 2010; Nocito et al. 2011; Satoh-Nagasawa et al. 2012; Takahashi et al. 2012). OsLCT1, which is localized at node I, plays an essential role in regulating Cd transport into rice grains (Uruguchi et al. 2011, 2014). The ABC transporter AtPDR8 is a Cd extrusion pump conferring heavy metal resistance in *Arabidopsis* (Kim et al. 2007), while the two closely related phytochelatin transporters, AtABCC1 and AtABCC2, mediate tolerance to Cd and mercury (Hg) (Park et al. 2012). Recently, another ABC-type transporter, AtABCC3, was recognized as a key factor for phytochelatin-mediated Cd tolerance in *Arabidopsis* (Brunetti et al. 2015).

Cd often induce the generation of toxic reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^\bullet), and superoxide anion ($O_2^{\bullet-}$), which are able to oxidize biological macromolecules such as proteins, lipids, and nucleic acids, and consequently promote enzyme inactivation, lipid peroxidation, and membrane impairment (Daud et al. 2016).

Plants have evolved antioxidant defense mechanisms, including enzymatic antioxidants such as peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and non-enzymatic antioxidants including reduced glutathione (GSH), ascorbic acid (AsA), and phenolics to circumvent the damaging effects of ROS (Ahsan et al. 2009; Mittler 2002). A few studies of heavy metal-induced antioxidant stress defense processes in legumes have been conducted using common vetch (*Vicia sativa* L.) (Romman 2016). Growth and antioxidative metabolism characteristics have been examined previously in *V. sativa* under Cd stress (Zhang et al. 2009). GSH is a biothiol tripeptide that plays a fundamental role as an antioxidant in mitigating the redox imbalance caused by heavy metal accumulation and as precursor of phytochelatin which play crucial role in Cd chelation. Several studies have indicated the importance of GSH metabolism in plant tolerance to heavy metals (Hernandez et al. 2015; Ahsan et al. 2008; Yang et al. 2007a). GSH also serves as a substrate for glutathione S-transferases (GSTs), which catalyze the conjugation of GSH with potentially dangerous xenobiotics and the reduction of toxic organic hydroperoxides (Edwards et al. 2000). Glutathione peroxidase (GPX) reduces H_2O_2 to H_2O using GSH as an electron donor, and glutaredoxins (GRXs) are small redox proteins that use GSH as a cofactor. The induction of GSTs, GRXs and GPX by heavy metals has been described previously in soybean, poplar and rice (Villiers et al. 2011; Song et al. 2013; Ma et al. 2012). Plant GSH synthesis depends on plant sulfur assimilation, beginning with the uptake and distribution of sulfate (SO_4^{2-}). Sulfate is activated by conversion to adenosine 5'-phosphosulfate (APS), which is reduced to sulfite (SO_3^{2-}) by adenylyl-sulfate reductase (APR). SO_3^{2-} is reduced to sulfide (S^{2-}), which is utilized to produce Cys and GSH (Hernandez et al. 2015).

Retention of Cd in cell wall is the first defense against Cd stress. Lignin is a branched phenylpropanoid polymer located in the cell wall, generally formed from three distinct phenylpropanoid alcohols including p -coumaryl, coniferyl, and sinapyl alcohol. More than 8 enzymes are involved in the biosynthesis of these precursors, including phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), hydroxycinnamoyl-CoA shikimate/quinic acid hydroxyl cinnamoyl transferase (HCT), p -coumarate 3-hydroxylase (C3H), cinnamoyl-

CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeoyl-CoA O-methyltransferase (CCoAOMT), caffeic acid O-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD) (Liu et al. 2015a, b). The polymerization of these precursors of lignin is catalyzed by POD in the presence of H₂O₂ and by laccases (LAC) in the presence of O₂. Increased lignin deposition occurs in a wide range of plant species in response to Cd (Kováčik and Klejdus 2008; Elobeid et al. 2012; Yang et al. 2007b). It has been reported that lignification of epidermis and exodermis cell walls limited the entry of cadmium into the xylem (Ederli et al. 2004), and that increased xylem lignification might prevent the translocation of Cd from the roots to the aerial parts (Ahsan et al. 2012).

However, until now plant Cd tolerance mechanisms have been poorly understood. Identifying the Cd stress response genes involved in Cd tolerance in *V. sativa* via modern biotechnological techniques such as transcriptomic analysis might be a useful method to explore the mechanisms of plant Cd tolerance and a useful step toward developing future transgenic lines resistant to Cd stress.

Quite a few studies have used transcriptomic analysis to investigate the transcriptional and post-transcriptional regulatory mechanisms involved in plant responses to Cd stress (He et al. 2015; Dal Corso et al. 2010; Yue et al. 2016; Xu et al. 2015; Dubey et al. 2014; Cao et al. 2014; Liu et al. 2015a, b). Genes related to the Cd response have been identified using various transcriptomic analysis methods, including chip analysis in *Arabidopsis* roots under Cd stress, a comparative transcriptomic study of Cd-treated roots of *A. thaliana* and *A. halleri* (a Cd-hypertolerant metallophyte), a time-course investigation of gene regulation under Cd toxicity in rice shoots and roots, and a study of early transcriptomic responses to Cd in rice roots (Lin et al. 2013; Herbette et al. 2006; Ogawa et al. 2009; Weber et al. 2006; Zhao et al. 2009). These transcriptomic analyses have given rise to a broad understanding of Cd-induced gene expression in model plants. However, few studies have been conducted on the response to Cd stress in non-model plants, and high-throughput sequencing approaches can lead to improved prospects for the identification of distinct tolerance mechanisms in non-model plants such as *V. sativa*.

The leguminous plant *Vicia sativa* has been cultivated largely in areas at high altitude with extremely cold

climates because of its high nutritional value and tolerance to cold and drought. It can be used as food for humans as well as fodder for livestock. Kim et al. (2015) conducted transcriptomic analysis of two *V. sativa* subspecies, mining molecular markers to enhance genomic resources for vetch improvement. Recently, we identified two varieties of *V. sativa* with distinct tolerances to Cd (Rui et al. 2016). In that study, we found elevated levels of ROS and a greater extent of lipid peroxidation in the roots of Cd-sensitive variety (ZM) than in the Cd-tolerant variety (L3), and observed that hydrogen peroxide (H₂O₂) and superoxide anions (O₂^{•-}) mainly accumulated in the cell walls and extracellular spaces in response to Cd treatment. In addition, we elucidated the physiological relationships of Cd tolerance with apoplastic H₂O₂ accumulation, guaiacol peroxidase (GPOD) and LAC activities and lignin deposition in roots. In the present study, we aimed to identify the molecular mechanisms and defensive genes responsible for avoiding Cd toxicity in the same two varieties of *V. sativa*.

Materials and methods

Plant materials, growth conditions, and treatments

According to our previous study (Rui et al. 2016), seeds of two *Vicia sativa* varieties (L3, ZM) were surface-sterilized with 3% H₂O₂ and germinated in plastic dishes at 20–25 °C filled with vermiculite. After 3 days, seedlings were shifted to vessels comprising Hoagland nutrient solution. The growth parameters were set as follows: 25/20 °C day/night temperatures, 60–80% relative humidity, and 12/12-h day/night photoperiod. 7-day-old seedlings were treated with 0, 5 and 50 μM Cd (added as CdCl₂·2.5H₂O) for 24 h. The experiment was arranged in a completely randomized design with three replicate vessels having 10 seedlings correspondingly. The pH of the nutrient solution was adjusted to 5.5 with NaOH or HCl and nutrient solution was resupplied every 3 days. Subsequent to 24-h Cd treatments, roots, stems and leaves samples were harvested and immediately frozen in liquid nitrogen and stored at –80 °C for RNA extraction and subsequent de-novo library construction, RNA sequencing (RNA-Seq) and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis. All the experiments were performed at least twice, each with three biological replicates.

RNA isolation, RNA-Seq library preparation, and sequencing

Total RNA for RNA-Seq was extracted from roots, stems, and leaves of samples as described above using a plant RNA kit (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's protocol. Purified RNA was quantified using an ND-8000 spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE, USA), a 2100-Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), or agarose gel electrophoresis. RNA samples with no smearing observed on agarose gels, a 260/280 ratio above 2.0, and an RNA integrity number greater than 8.0 were selected for use in subsequent processes. For *de novo* transcriptome analysis, equal amounts of RNA from roots, stems, and leaves were mixed to construct the cDNA library. For comparative RNA-Seq analysis, we mixed three replicate samples of roots into one total RNA sample for each treatment, which was then sent to Majorbio Bio-pharm Technology Corporation (<http://www.majorbio.com/>) for sequencing. The cDNA libraries were constructed using the TruSeq™ RNA Sample Preparation Kit (Illumina, Inc.) according to the manufacturer's instructions. Briefly, the protocol consists of the following steps: Poly(A)-containing mRNA was purified from 5 µg of total RNA using oligo(dT) magnetic beads and fragmented into ~200-bp pieces using divalent cations at 94 °C for 5 min. The cleaved RNA fragments were copied into first-strand cDNA using SuperScript II reverse transcriptase (Life Technologies, Inc. Carlsbad, CA, USA) and random primers. After second-strand cDNA synthesis, fragments were end-repaired, A-tailed, and indexed adapters were ligated. The products were purified and amplified via PCR to create the final cDNA library. Target bands were harvested by 2% agarose gel electrophoresis and quantified using a TBS380 mini-fluorometer (Picogreen). The tagged cDNA libraries were pooled at equal ratios and used for 101-bp paired-end sequencing on the Illumina HiSeq2000 platform, according to the manufacturer's instructions.

Data processing, assembly, and annotation

The original image data were processed by base calling, generating sequenced reads, namely, raw reads in the FASTQ format. Next, the data were subjected to four-

step quality control: (1) base mistake ratio counting: <1%; (2) A/T/G/C percentage and distribution analysis, removal of AT/GC separated sequences, and transforming raw reads into clean reads; (3) removal of reads with more than 10% N (uncertain base type); and (4) removal of the adaptor and reads less than 20 bp in final length. Trinity software (v.2013-02-25) (Grabherr et al. 2011) was used for assembly, during which clean reads of different isoforms derived from one gene were assembled into distinct transcripts of the same subcomponent, which can be regarded as a gene. The longest transcript of each subcomponent was defined as a "unigene" for functional annotation. All assembled unigenes from the three tissues were searched against the Nr (NCBI non-redundant protein sequences) database to identify putative mRNA functions using BLASTX (Version 2.2.25) with an E-value cut-off of 10^{-5} . Additionally, Gene Ontology (GO) terms were extracted from the best hits obtained from BLASTX against Nr using the Blast2GO program (Conesa and Gotz 2008). The BLAST algorithm was also used to align unique sequences by applying String software (Szklarczyk et al. 2011), the Pfam database (Punta et al. 2012), and the KEGG Ortholog (KO) database (Kanehisa et al. 2008) (with an E-value cut-off of 10^{-5}) to predict possible functional classifications and molecular pathways. BLAST analysis were performed in a local protein sequence database downloading all protein sequences (proteomes) of various organisms used in NR, Swissprot, STRING, Pfam, and KEGG database.

Gene expression analysis

After mapping the results from all samples to the reference genome, RSEM (RNA-Seq by Expectation-Maximization) (<http://deweylab.biostat.wisc.edu/rsem/>) was used to calculate the reads per kilobase of transcript per million mapped reads (RPKM) value of each gene or transcript, which represents the relative expression level of that gene or transcript in the samples. EdgeR (<http://www.bioconductor.org/packages/2.12/bioc/html/edgeR>) was adopted to identify differentially expressed genes among groups of samples using the following criteria: FDR (false discovery rate) <0.05 and \log_2 (fold change) ≥ 1 . A Venn diagram of differentially expressed genes was generated using the GeneVenn application (Pirooznia et al. 2007).

Validation of gene expression

To validate the RNA-Seq results, the expression of selected up- or down-regulated genes were confirmed by quantitative reverse transcription–polymerase chain reaction (qRT-PCR). After 7-days of growth, *V. sativa* seedlings were treated with or without 5 and 50 μM CdCl_2 solutions for 24 h, and the roots samples were harvested for RNA extraction. After RNA extraction, genomic DNA was removed with the RNase free DNase I Set (Omega, USA) following the manufacturer's guidelines. RNA was reverse-transcribed using a Super Reverse transcription kit (BioTeke, China). Gene-specific PCR products were amplified with SYBR pre-mix EX Taq (TaKaRa Bio, Japan), and the quantitative real-time PCR was performed on Mastercycler ep realplex (Eppendorf) with the primer sets shown in Supplemental Table 1. The PCR protocol was as follows: initial denaturation at 95 °C for 30 s, followed by 95 °C for 5 s, and 60 °C for 34 s in a 40-cycle reaction, then followed by dissociation stage. $2^{-\Delta\Delta\text{Ct}}$ method calculation was used for data analysis and *ACTIN2* was used as an internal standard.

Results

De novo assembly of a *V. sativa* reference transcriptome

To compare the transcriptomes of *V. sativa* varieties (L3 and ZM) treated with different CdCl_2 concentrations, a de novo assembled *V. sativa* transcriptome was generated. In total, 61,335,826 raw reads were generated from the mixed samples (Table 1). After filtration, 58,789,686 clean reads were obtained. More than 95.21% of bases in raw reads had a Q value ≥ 20 (an error probability of 0.0312%) and more than 97.15% of bases in clean reads had a Q value ≥ 20 (an error probability of 0.0275%). The GC contents were 43.33% and 43.25% for raw reads and clean reads, respectively. Clean reads data

were used for de novo assembly. Trinity software generated 63,100 total transcripts (Table 2) with an average length of 921.55 bp and a N50 of 1532 bp, and a total of 49,062 sequences were identified as unigenes. Among these unigenes, 9398 (32.9%) were more than 500 bp in length, while 3854 (13.5%) of unigenes were longer than 1 kb (Supplemental Fig. 1).

Functional annotation

All assembled transcripts and unigenes were searched against the Nr, Swissprot, String, Pfam and KEGG databases using BLAST (Version 2.2.25, E-value $< 1\text{E}^{-5}$). Among unigenes, 57.30% showed homology with *Medicago truncatula*, 27.85% showed homology with *Cicer arietinum*, 4.32% showed homology with *Glycine max*, and 1.40% showed homology with *Pisum sativum* in the Nr database (Fig. 1). The numbers of transcripts with significant similarity to sequences in the Pfam, Swissprot, KEGG, String, and Nr databases were 24,093, 25,930, 13,247, 19,411, and 40,402, respectively. The numbers of unigenes with significant similarity to sequences in the Pfam, Swissprot, KEGG, String, and Nr databases were 17,227, 18,662, 9543, 13,854, and 29,354, respectively.

During GO classification, all unigenes were classified based on three functional categories: molecular function, biological process, and cellular component. In biological process analysis, unigenes were clustered into 22 classifications, with the most abundant being metabolic processes, cellular processes, and single-organism processes, containing 11,943, 10,345, and 8575 unigenes, respectively. In the cellular component category, unigenes were clustered into 17 classifications, with the largest being the cell, cell parts, and organelle classes, which contained 7823, 7823, and 5582 unigenes, respectively. In terms of molecular function, unigenes were clustered into 17 classifications, the largest of which were binding and catalytic activity, containing 9509 and 9636 unigenes.

Table 1 Summary of RNA-seq of *V. sativa* transcriptome

Data	Reads	bp	Gb	Error%	Q20%	Q30%	GC%
Raw data	61,335,826	7,728,314,076	7.7	0.0312	95.21	90.87	43.33
Clean data	58,789,686	7,199,419,842	7.1	0.0275	97.15	93.72	43.25

Table 2 Summary of de novo assembly of *V. sativa* transcriptome

	Unigenes	Transcripts
Total sequence number	49,062	63,100
Total sequence base	40,960,032	58,150,008
Percent GC	39.27	39.34
Largest transcript	17,933	17,933
Smallest transcript	201	201
Average length	834.86	921.55
N50	1442	1532

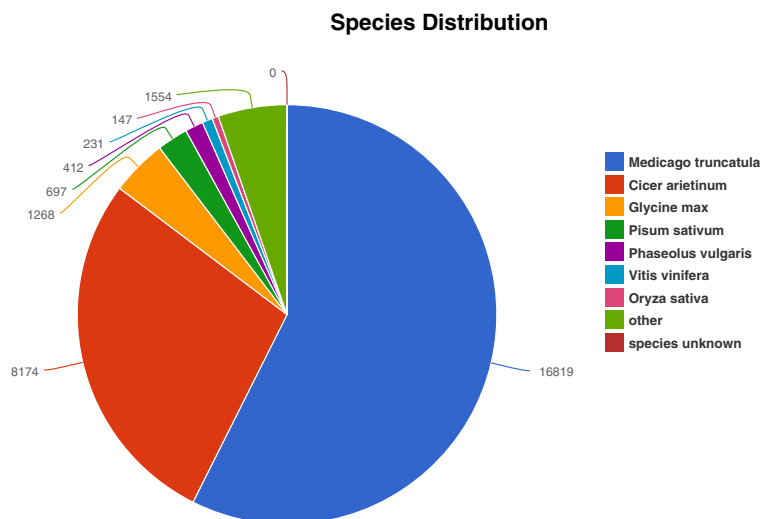
A total of 10,218 unigenes were clustered into 25 Eukaryotic Orthologous Group (KOG) categories (Fig. 2). Among these categories, the largest was general function prediction only, accounting for 1829 unigenes; the second largest category was signal transduction mechanisms, including 1141 unigenes; and the third was post-translational modification/protein turnover/chaperones, comprising 1070 unigenes.

A total of 13,247 unigenes had BLAST hits in KO database with E-values $<1E^{-5}$, and 7237 were assigned to 330 metabolism pathways. Among these pathways, the six with the most genes were ribosome, plant hormone signal transduction, starch and sucrose metabolism, protein processing in the endoplasmic reticulum, plant-pathogen interaction, and phenylpropanoid biosynthesis.

Differential gene expression between L3 and ZM under Cd stress

To identify genes in *V. sativa* that are involved in Cd stress responses and those that contribute to the differing Cd tolerances between the two varieties, L3 and ZM, 7-day-old seedlings of both varieties were treated with 5 or 50 μM solutions of Cd^{2+} or solution without Cd (control) for 24 h, after which root samples were harvested from each treatment and labeled as L3(0); without Cd, L3(5); with 5 μM Cd and L3(50); with 50 μM Cd) and ZM(0), ZM(5), and ZM(50) accordingly. Libraries were constructed from the six RNA samples separately and Illumina Hiseq sequencing was performed. More than 27 million raw reads were obtained, and more than 24 million clean reads remained for each sample after processing (Supplemental Table 2). Using the de novo assembled sequence as a reference, clean reads were mapped, with results between 23 and 34 million reads (Supplemental Table 3).

Based on the results above, gene expression was calculated using the RPKM method, which takes into account the influences of both sequencing depth and gene length on read count. On the basis of the criteria applied [$\text{FDR} < 0.05$ and $\log_2(\text{fold change}) \geq 1$], the two cultivars grown under low- and high-Cd conditions were compared with the control. Under low-Cd treatment, few genes had different expression levels from the control in both varieties (i.e. 69 in L3 and 28 in ZM). Under high-Cd treatment, 1036 genes showed differential expression in L3,

Fig. 1 Species distribution of matched proteins in NR database in *V. sativa* transcriptome

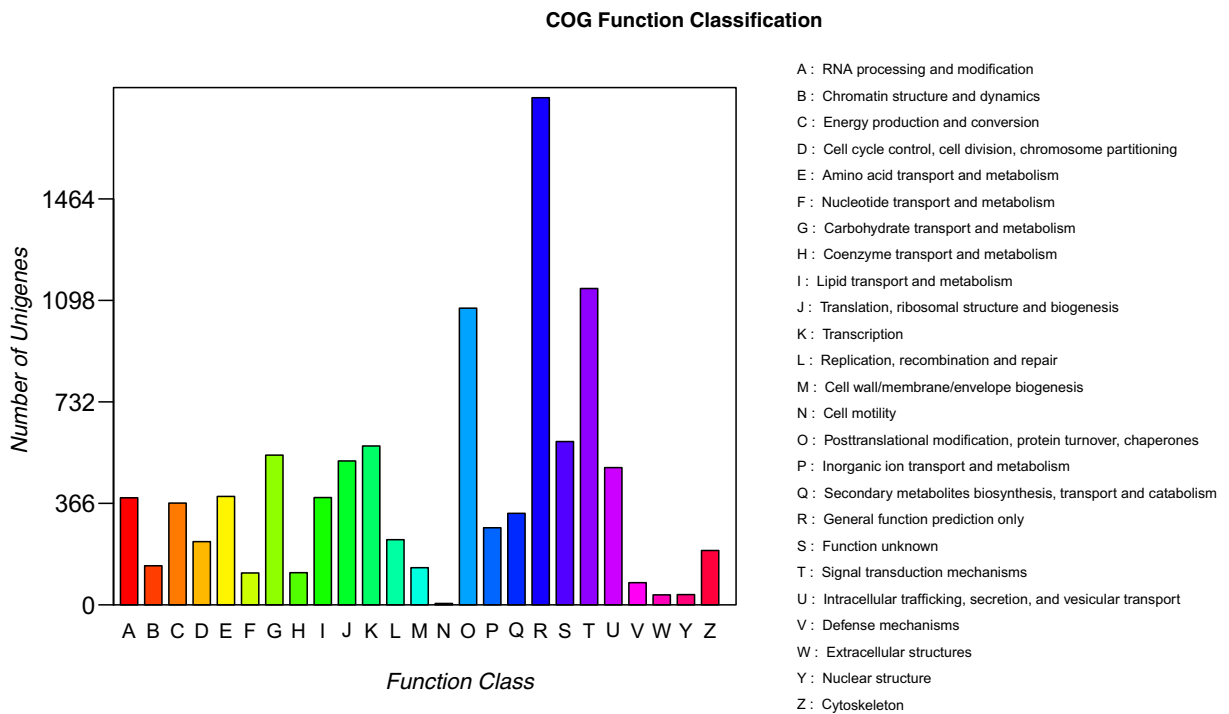
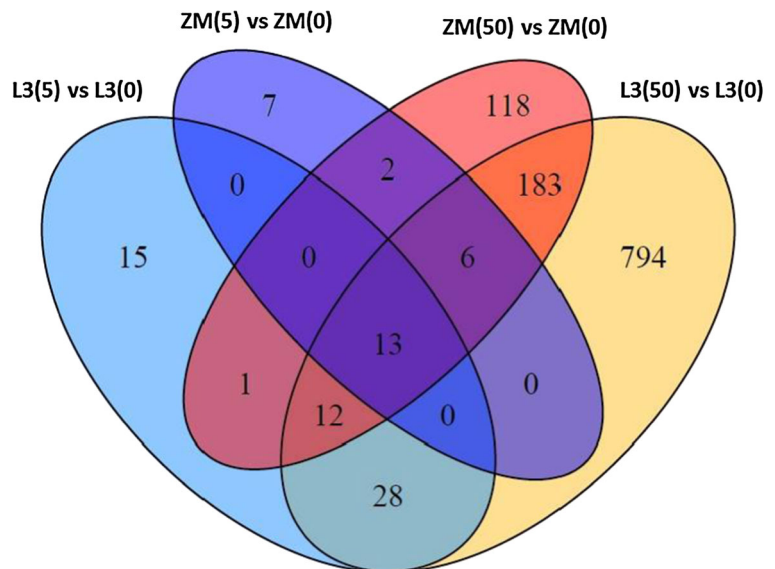


Fig. 2 Histogram presentation of KOG classification of unigenes in *V. sativa* transcriptome

whereas in ZM, only 335 genes had altered expression levels. As shown in the Venn diagram (Fig. 3), in L3, 53 genes were differentially expressed under both low- and high-Cd stress, 16 genes were solely expressed under low-Cd stress, and 983 genes specifically changed expression under high-Cd stress. In ZM, 21 genes were differentially

expressed under both low- and high-Cd stress, 7 genes were specifically expressed under low-Cd stress, and 314 genes changed expression solely under high-Cd stress. As commonalities between the L3 and ZM varieties, there were 13 and 214 genes differentially expressed under low- and high-Cd treatments, respectively.

Fig. 3 The Venn diagram of the number of differentially expressed unigenes in each comparison group



To identify possible biological processes or pathways that were altered under the two levels of Cd stress, in relation to the two varieties of *V. sativa*, Gene Ontology (GO) and KEGG pathway enrichments were performed using an FDR adjusted p -value ≤ 0.05 as the criterion. Our analysis revealed that the common differentially expressed genes were highly enriched in cell wall organization or biogenesis, phenylpropanoid biosynthesis, drug metabolism-cytochrome P450, metabolism of xenobiotics by cytochrome P450, cutin, suberine, and wax biosynthesis, protein processing in the endoplasmic reticulum, glutathione metabolism, starch and sucrose metabolism, the tricarboxylic acid cycle, and glycerophospholipid metabolism (Supplemental File 1).

Cell wall-related genes

Cutin, suberine, and wax biosynthesis genes were considerably overrepresented in L3(50) vs. L3(0) as compared to ZM(50) vs. ZM(0). Five genes were significantly up-regulated in L3(50) vs. L3(0); in contrast, only one gene (FAR) showed increased expression in ZM(50) vs. ZM(0) (Supplemental Table 4). Lignin biosynthesis pathway genes were overrepresented in both varieties [L3(50) vs. L3(0) and ZM(50) vs. ZM(0)]; one *COMT*, two *CCR*, and three *CAD* genes showed similar up-regulation patterns. However, one *4CL* gene displayed decreased expression in the comparison of L3(50) vs. L3(0) and one *CCoAOMT* gene down-regulated in the comparison of ZM(50) vs. ZM(0). Among *POD* genes, 13 were up-regulated and 1 gene was down-regulated in L3(50) vs. L3(0), whereas 10 and 3 *POD* genes were up- and down-regulated, respectively, in ZM(50) vs. ZM(0). Among these *POD* genes, 9 were up-regulated in both varieties (Supplemental Table 5).

Common stress response genes

Under Cd stress, both varieties differentially expressed many heat shock protein (HSP) genes and other chaperone/co-chaperone protein-encoding genes (Supplemental Table 6). In total, 19 and 16 *HSP* genes were up-regulated in L3(50) vs. L3(0) and ZM(50) vs. ZM(0), respectively, and 15 genes were shared among them. Eleven of these (c21159_g1; c24577_g1; c45652_g1; c28022_g1; c39617_g1; c8867_g1; c32996_g1; c3732_g1; c25243_g1; c27249_g1; c9550_g1) are

among the 13 genes that are differentially expressed in the presence of Cd regardless of dose or variety. For chaperone/co-chaperone genes, 5 were up-regulated and 2 down-regulated in L3(50) vs. L3(0), while 3 were significantly up-regulated in ZM(50) vs. ZM(0). We also identified that two germin-like protein-encoding genes were commonly up-regulated in both groups. In contrast, one pathogenesis-related protein (PR) gene was up-regulated in L3(50) vs. L3(0), but not ZM(50) vs. ZM(0).

GSH pathway genes

We identified 11 *GST* genes that were significantly up-regulated in the assessment of L3(50) vs. L3(0), while only one *GST* gene showed increased background expression in ZM. Two *GPX* and 6 *GRX* genes were significantly down-regulated in the background of L3, while no *GPX* and 4 *GRX* genes were down-regulated in the ZM background. For the *APR* gene, 4 and 3 genes were significantly up-regulated in the background of L3 and ZM, respectively (Supplemental Table 7).

Metal transporter genes

We found significant differences in metal transporter gene expression regulation between L3(50) vs. L3(0) and ZM(50) vs. ZM(0) (Supplemental Tables 8, 9 and 10). More than 10 transporter genes were differentially regulated under Cd exposure, and most of these gene expression changes were variety-specific. There were 9 ABC transporter family genes which up-regulated. Among them, one PDR family ABC transporter gene (c28232_g2) was up-regulated in ZM but not in L3, whereas 8 ABC family genes (c20094_g1, c21832_g1, c2540_g1, c28624_g2, c27925_g1, c13627_g1, c28427_g1, c25102_g1) were up-regulated in L3 background, but not in ZM. Moreover, four MATE (Multi-drug and Toxic Compound Extrusion) efflux family protein genes, three auxin transporter genes and five phosphate transporter genes were up- or down-regulated in L3 but not in ZM. As for sugar transporter genes, ZIP (ZRT/IRT-like protein) family transporter genes and lipid-transfer protein genes, the numbers of changed gene were much greater in L3 than in ZM. However, three sulfate transporter genes and two Nrmap3 genes were only up-regulated in ZM but not in L3. In general, the change in expression of transporter family genes was much higher in the L3 than in ZM under Cd stress.

Transcription factors

Transcription factors (TFs) play a major role in the network of Cd-responsive genes. We identified several Cd-inducible TFs belonging to different families. Significant differences in TF gene expression regulation between L3(50) vs. L3(0) and ZM(50) vs. ZM(0) were observed (Supplemental Tables 11 and 12). Two GRAS family TF genes were down-regulated in L3(50) vs. L3(0) and one was down-regulated in ZM(50) vs. ZM(0). Six bHLH TF genes were down-regulated in L3(50) vs. L3(0), whereas only one down-regulated bHLH TF gene was identified in ZM(50) vs. ZM(0). Four and two myeloblastosis protein (MYB) family TF genes were up-regulated in L3(50) vs. L3(0) and ZM(50) vs. ZM(0), respectively, while four MYB TFs were repressed in L3(50) vs. L3(0). Besides, four up-regulated ERF (ethylene-responsive) genes, two up-regulated zinc finger protein (ZFP) TF genes, one up-regulated NAC TF-like gene, one down-regulated RF2a-like TF gene, two basic leucine zipper (bZIP) TF genes, and two up-regulated and one down-regulated WKRY TF genes were identified in L3(50) vs. L3(0), while none was found in ZM(50) vs. ZM(0).

qPCR validation

To test the reliability of RNA-Seq results, we selected several transporter gene family members for real-time quantitative polymerase chain reaction (RT-PCR) validation. As shown in Fig. 4, the differential expression changes were largely enhanced in the L3 background, especially in the case of two ABC transporter genes, ABCC3 and ABCC9, under 5 μM Cd stress. Under 50 μM Cd, the differential expression was significant and much higher (Fig. 4).

Discussion

Plants have evolved different strategies for improving their heavy metal tolerance (Gallego et al. 2012). In our previous work (Rui et al. 2016), we observed that the *V. sativa* variety L3 had a higher Cd tolerance than the variety ZM. In this study, using high-throughput sequencing technologies, we aimed to provide a deeper exploration of the molecular basis of responses to Cd stress, revealing Cd tolerance genes in two varieties with contrasting Cd tolerance traits at the global

transcriptome level. On one hand, the different expression regulation patterns under different levels of Cd stress suggested that the genes related to cell wall organization or biogenesis, phenylpropanoid biosynthesis, GSH metabolism and common stress response play essential roles in response to Cd in *V. sativa* plants, while the difference between Cd tolerances in the L3 and ZM varieties is correlated with metal transporter genes and transcription factors.

In our previous study (Rui et al. 2016), we noted that lignin accumulation in the roots was enhanced under Cd stress, and this accumulation was more significant in the sensitive variety, ZM. In RNA-Seq data, the lignin biosynthesis pathway was found to be significantly over-represented in KEGG pathway analysis. One *COMT*, two *CCR*, and three *CAD* genes were significantly up-regulated in both varieties. However, aside from one *4CL* gene and one *LAC* gene that displayed decreased expression only in L3(50) vs. L3(0) and one *CCoAOMT* gene that displayed decreased expression only in ZM(50) vs. ZM(0), there were no differences in lignin biosynthesis pathway gene expression between the two varieties. As a number of lignin biosynthesis pathway genes have previously been reported as inducible by Cd stress in *Arabidopsis* and copper (Cu) toxicity in rice (Liu et al. 2015a), we suggest that this pathway is a conserved adaptation mechanism that plants have gained through evolution. In addition, GPOD (both ionically bound cell wall GPOD and soluble GPOD) and LAC activity in the roots of two *V. sativa* varieties increased rapidly under 10 μM Cd stress (Rui et al. 2016), which is analogous with our study on Cu in rice roots (Liu et al. 2015a). Phenols can participate in lignin synthesis, acting as the electron donor for POD (Sgherri et al. 2004), and LAC can also oxidize phenolic substrates, a process which requires O_2 as a secondary substrate. So we suggest that lignin accumulation is a common response by plants to heavy metal stress. Moreover, one KEGG pathway (cutin, suberine, and wax biosynthesis) showed significant enrichment only in L3(50) vs. L3(0). Metabolites including cutin, suberine, and wax on the cuticle can decrease water loss during dehydration stress, and thus enrichment of the cutin, suberine, and wax pathway may play a role in Cd-tolerance differences found between the two varieties.

There are a greater number of GSH metabolism genes up- or down-regulated in L3(50) vs. L3(0) than in ZM(50) vs. ZM(0), in particular, there are 11 *GST* genes up-regulated in L3(50) vs. L3(0) but only one

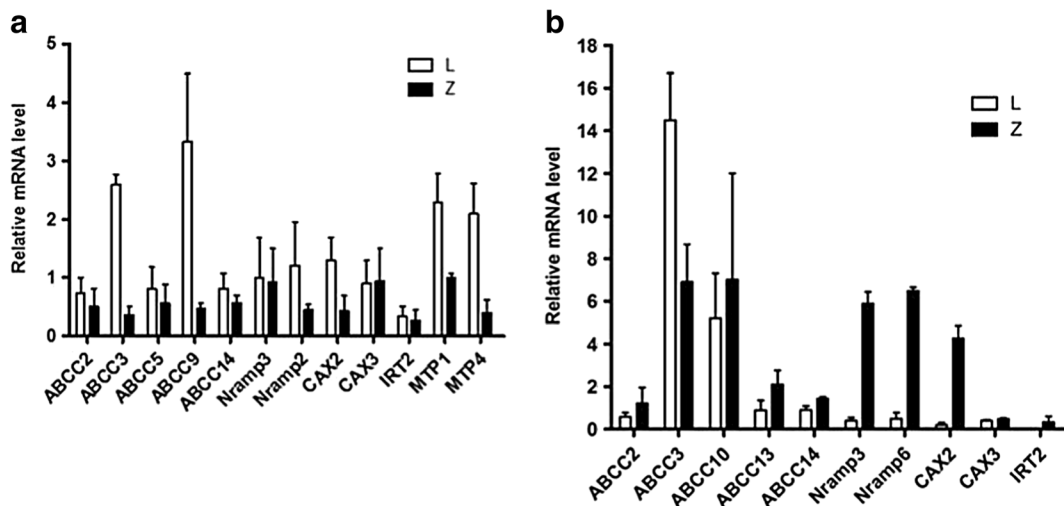


Fig. 4 Validation of RNA-Seq data by real-time qRT-PCR under Cd-treated condition in two *V. sativa* varieties L3 (Cd-tolerant) and ZM (Cd-sensitive). The relative mRNA levels of several genes under 5 µM (a) and 50 µM (b) CdCl₂ stress for 24 h. Steady-state mRNA levels in roots were normalized against *actin2* gene and

were expressed relative to those of control plants, which were given a value of 1 (not shown). Bar values are means ± SD of three biological replicates, each corresponding to different RNA extractions from roots of three plants grown independently

GST gene up-regulated in ZM(50) vs. ZM(0), which suggested that the GSH metabolism had an important role in the difference of Cd tolerance in two *V. sativa* varieties.

Molecular chaperones (e.g. HSPs) are protein-repairing bodies, and are active in cells under both normal and adverse conditions. HSPs, including HSP70s, HSP90s, HSP60s, and small heat-shock proteins (sHSPs), are stress-responsive proteins, protecting plants from damage under stress (Xu et al. 2015). In the present experiment, up-regulation of a large number of HSPs during Cd stress indicates that HSPs in particular play important roles in protecting plants against stress by re-establishing normal protein conformation and thus cellular homeostasis (Dubey et al. 2014). There are more stress-responsive genes in L3(50) vs. L3(0) than in ZM(50) vs. ZM(0), which may contribute to the different levels of cadmium tolerance of these two varieties.

Heavy metal accumulation can be mediated by a group of metal transporters in plants. Plant metal transporters can be classified into different families based on their sequence specificity, subcellular location, and the metals they carry (Hall 2002). Several metal transporter family members are implicated in heavy metal detoxification in plants (Yoneyama et al. 2015), including Nramp, MATE, HMA, ZIP, and ABC. Cd is biologically non-essential and often accumulate in plants through the same transporters used for essential divalent

metals, such as Ca, Fe, Mn and Zn due to their structural similarity (Verbruggen et al. 2009). The repression of these transporters under Cd toxicity could reduce plant Cd uptake and translocation and increase Cd tolerance, however, in the other hand might cause secondary effects on essential metal uptake and distribution in *planta* which will also affect plant Cd tolerance. *OsNramp5* is a major transporter of Mn and Cd in rice root. The *OsNramp5* knockout line lost the ability to take up Mn and Cd and resulted in a significant reduction in rice growth and grain yield (Sasaki et al. 2012). It has been reported that Nramp3 and Nramp4 are responsible for transporting Cd²⁺ out of the vacuole in *Arabidopsis*. The *nramp3nramp4* mutant impaired in release of vacuolar metal stores showed hypersensitive to cadmium stress (Molins et al. 2013). In *V. sativa*, up-regulation of two *Nramp3* under Cd stress in ZM but not in L3 might play some important roles in different Cd tolerance of these two varieties. As a large family of membrane proteins, ABC transporters are involved in the transport of a broad range of substances and in plant responses to various environmental stresses. Both *OsPDR9* and *AtPDR8* encode PDR-type ABC transporters, which are induced by Cd in rice and *Arabidopsis* as an efflux pump of Cd²⁺ or Cd conjugates (Kim et al. 2007; Moons 2003). In *V. sativa*, one PDR family ABC transporter gene was up-regulated in ZM but not in L3. In contrast, there were 8 ABC family genes that showed a response

to Cd in L3 background, but not in ZM. This is also confirmed by the qRT-PCR results (Fig. 4). The sensitivity expression response of ABC transporter genes in the L3 background is an adaptation mechanism to detoxify Cd from cytosol. We speculate these difference in the ABC transporters in response to Cd stress is at least one main contribution in the difference in Cd-tolerance of L3 and ZM. MATE proteins bind to a variety of potentially toxic compounds and function as proton dependent efflux transporters to remove toxic compounds from the cell (Dubey et al. 2014). A MATE-related efflux protein from *Arabidopsis* (AtDTX1) was reported to serve as an efflux transporter for plant-derived alkaloids, antibiotics, and other toxic compounds involving in detoxification of heavy metal Cd (Li et al. 2002). Four MATE efflux protein genes were down-regulated in Cd-tolerant L3 but not in Cd-sensitive ZM, if these differences were responsible for their different Cd tolerances, it needs further research. The uptake of Cd may occur via ZIP family transporters. One root iron transporter protein gene was down-regulated in L3 but not in ZM and one ZIP family protein was up-regulated in both varieties, which indicated that *V. sativa* might regulate root Cd or iron/zinc uptake through ZIP transporter expression regulation, which might influence plant Cd tolerance by control of Cd absorption and essential metal utilization. Besides, there are three auxin transporter genes up- or down-regulated and five phosphate transporter genes up-regulated in L3 but not in ZM, which indicated that auxin regulation and phosphate transport might play important role in response to Cd stress in L3. Cd affected sulfur assimilation pathway in both of the two *V. sativa* varieties. Three sulphate transporter genes were up-regulated in ZM but not in L3, which might mean that ZM absorbed more sulphate than L3 in response to Cd stress. Corresponding to this, three APR genes were up-regulated in L3 and four APR genes were up-regulated in ZM (Supplemental Table 7). Sulfur assimilation leads to the activation of pathway responsible for GSH biosynthesis (Gill and Tuteja 2011). GSH acts as an important antioxidant in mitigating Cd-induced oxidative stress and plays an important role in phytochelatin synthesis (Hernandez et al. 2015). These results indicated that sulphur assimilation pathway and synthesis of GSH were crucial steps for alleviating the phytotoxicity of Cd in two *V. sativa* varieties. In our study, we observed differential expression of various transporter genes between L3 and ZM under Cd stress.

These results indicated that transporters might play an essential role in the differential tolerance to Cd stress among the two *V. sativa* varieties.

Transcription factors are involved in many processes related to plant stress signaling. TFs, such as the bZIP, WRKY, NAC, ERF, and MYB play a vital part in directing the expression of specific stress-related genes in response to Cd stress (Dal Corso et al. 2010). Genes including *MYB*, *WRKY*, *NAC*, and *AP2* were up-regulated in rice roots treated with 10 μM Cd for 3 h. It has also been shown in *Arabidopsis thaliana* roots that some genes encoding C2H2 ZFP, AP2 domain-containing protein, HSF, and MYB TFs were specifically induced by Cd^{2+} but not by Cu^{2+} (Weber et al. 2006). Wheat ZAT7 and ZAT12 in the C2H2 ZFP family were shown to improve ROS tolerance in *Arabidopsis* (Ali-Benali et al. 2013). In *Arabidopsis*, RAP2.6, an AP2/ERF transcription factor, contributes to abscisic acid (ABA), osmotic, and salt toxicity responses (Zhu et al. 2010). *SNAC2*, isolated from *Oryza sativa*, is a stress-inducible NAC gene that can elevate stress tolerance in rice (Hu et al. 2008). In addition, wheat overexpressing the *TaNAC6* gene was shown to exhibit greater resistance to stress (Xue et al. 2011). *MYB* genes can also respond to one or more stress treatments, such as elevated salinity, exogenous ABA, and polyethylene glycol (PEG) (Zhang et al. 2012). A number of *WRKY* genes are also stimulated by lead (Pb) heavy metal stress in *Arabidopsis* (Liu et al. 2009). For example, *WRKY38* from barley has been implicated in responses to drought and cold stress (Marè et al. 2004). *TcWRKY53* is also induced markedly by NaCl, drought, cold, and salicylic acid stresses in Cd-treated *Thlaspi caerulescens* (Wei et al. 2009). Four up-regulated *WRKY* genes comprising *OsWRKY71*, *OsWRKY28*, *OsWRKY22*, and *OsWRKY42* were also shown to be involved in plant stress resistance responses (Liu et al. 2007; Abbruscato et al. 2012; Chujo et al. 2013; Han et al. 2014). In rice, *OsWRKY42* also inhibits *OsMT1d* and induces ROS production and senescence of leaves. One up-regulated gene, *OsWRKY72*, was found to be involved in the ABA signal and auxin transport pathway (Song et al. 2010). In our experiment, we observed differential expression of various TF genes (*WRKY*, *GRAS*, *MYB*, *bHLH*, *ZFP*, *ERF*, and *NAC* transcription factors) in two *V. sativa* varieties under Cd stress. These results suggested that TFs play significant role in Cd stress response of *V. sativa*, especially in Cd-tolerant variety L3 which had greater number of up- or down-

regulated TF genes in response to Cd stress. Further analysis of these TF genes may lead to decipher the network related to the whole pathway that may differ in Cd stresses. Currently, we are testing their potential role in heavy metal tolerance.

In conclusion, we demonstrated that Cd caused lignification in the roots of *V. sativa* at the transcriptome level, which is consistent with our previous physiological data (Rui et al. 2016). More interestingly, our gene expression analysis results suggest that the differential expression of transcription factors and transporter genes plays a major role in controlling the contrasting Cd stress sensitivities of the L3 and ZM varieties of *V. sativa*.

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References

- Abbruscato P, Nepusz T, Mizzi L, Del Corvo M, Morandini P, Fumasoni I, Michel C, Paccanaro A, Guiderdoni E, Schaffrath U, Morel JB, Piffanelli P, Faivre-rampant O (2012) OsWRKY22, a monocot WRKY gene, plays a role in the resistance response to blast. *Mol Plant Pathol* 13:828–841. <https://doi.org/10.1111/J.1364-3703.2012.00795.X>
- Ahsan N, Lee DG, Alam I, Kim PJ, Lee JJ, Ahn YO, Kwak SS, Lee IJ, Bahk JD, Kang KY, Renaut J, Komatsu S, Lee BH (2008) Comparative proteomic study of arsenic-induced differentially expressed proteins in rice roots reveals glutathione plays a central role during As stress. *Proteomics* 8:3561–3576. <https://doi.org/10.1002/pmic.200701189>
- Ahsan N, Renaut J, Komatsu S (2009) Recent developments in the application of proteomics to the analysis of plant responses to heavy metals. *Proteomics* 9: 2602–2621. <https://doi.org/10.1002/pmic.20080093>
- Ahsan N, Nakamura T, Komatsu S (2012) Differential responses of microsomal proteins and metabolites in two contrasting cadmium (Cd)-accumulating soybean cultivars under Cd stress. *Amino Acids* 42:317–327. <https://doi.org/10.1007/s00726-010-0809-7>
- Ali-Benali MA, Badawi M, Houde Y, Houde M (2013) Identification of oxidative stress-responsive C2H2 zinc fingers associated with Al tolerance in near-isogenic wheat lines. *Plant Soil* 366:199–212. <https://doi.org/10.1007/s11104-012-1417-y>
- Brunetti P, Zanella L, De Paolis A, Di Litta D, Cecchetti V, Falasca G, Barbieri M, Altamura MM, Costantino P, Cardarelli M (2015) Cadmium-inducible expression of the ABC-type transporter AtABCC3 increases phytochelatin-mediated cadmium tolerance in Arabidopsis. *J Exp Bot* 66:3815–3829. <https://doi.org/10.1093/jxb/erv185>
- Caillaite R, Lapeyre B, Briat JF, Mari S, Curie C (2009) The NRAMP6 metal transporter contributes to cadmium toxicity. *Biochem J* 422:217–228. <https://doi.org/10.1042/bj20090655>
- Cao F, Chen F, Sun H, Zhang G, Chen ZH, Wu F (2014) Genome-wide transcriptome and functional analysis of two contrasting genotypes reveals key genes for cadmium tolerance in barley. *BMC Genomics* 15:611–624. <https://doi.org/10.1186/1471-2164-15-611>
- Chujo T, Miyamoto K, Shimogawa T, Shimizu T, Otake Y, Yokotani N, Nishizawa Y, Shibuya N, Nojiri H, Yamane H, Minami E, Okada K (2013) OsWRKY28, a PAMP-responsive transcription factor, negatively regulates innate immune responses in rice against rice blast fungus. *Plant Mol Biol* 82:23–37. <https://doi.org/10.1007/s11103-013-0032-5>
- Clemens S, Aarts MG, Thomine S, Verbruggen N (2013) Plant science: the key to preventing slow cadmium poisoning. *Trends Plant Sci* 18:92–99. <https://doi.org/10.1016/j.tplants.2012.08.003>
- Conesa A, Gotz S (2008) Blast2GO: a comprehensive suite for functional analysis in plant genomics. *Int J Plant Genomics* 2008:619832. <https://doi.org/10.1155/2008/619832>
- Dal Corso G, Farinati S, Furini A (2010) Regulatory networks of cadmium stress in plants. *Plant Signal Behav* 5:663–667. <https://doi.org/10.4161/psb.5.6.1142>
- Daud MK, Mei L, Azizullah A, Dawood M, Ali I, Mahmood Q (2016) Leaf-based physiological, metabolic, and ultrastructural changes in cultivated cotton cultivars under cadmium stress mediated by glutathione. *Environ Sci Pollut Res Int* 23: 15551–15564. <https://doi.org/10.1007/s11356-016-6739-5>
- Dubey S, Shri M, Misra P, Lakhwani D, Bag SK, Asif MH (2014) Heavy metals induce oxidative stress and genome-wide modulation in transcriptome of rice root. *Funct Integr Genomics* 14:401–417. <https://doi.org/10.1007/s10142-014-0361-8>
- Ederli L, Reale L, Ferranti F, Pasqualini S (2004) Responses induced by high concentration of cadmium in *Phragmites australis* roots. *Physiol Plantarum* 121:66–74. <https://doi.org/10.1111/j.0031-9317.2004.00295.x>
- Edwards R, Dixon DP, Walbot V (2000) Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. *Trends Plant Sci* 5:193–198. [https://doi.org/10.1016/s1360-1385\(00\)01601-0](https://doi.org/10.1016/s1360-1385(00)01601-0)
- Eloheid M, Göebel C, Feussner I, Polle A (2012) Cadmium interferes with auxin physiology and lignification in poplar. *J Exp Bot* 63:1413–1421. <https://doi.org/10.1093/jxb/err384>
- Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, Iannone MP, Rosales EP, Zawoznik MS, Groppa MD, Benavides MP (2012) Unravelling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. *Environ Exp Bot* 83:33–46. <https://doi.org/10.1016/j.envexpbot.2012.04.006>
- Gao L, Chang J, Chen R, Li H, Lu H, Tao L, Xiong J (2016) Comparison on cellular mechanisms of iron and cadmium accumulation in rice: prospects for cultivating Fe-rich but Cd-free rice. *Rice* 9:39–51. <https://doi.org/10.1186/s12284-016-0112-7>

- Gill SS, Tuteja N (2011) Cadmium stress tolerance in crop plants: probing the role of sulfur. *Plant Signal Behav* 6(2):215–222. <https://doi.org/10.4161/psb.6.2.14880>
- Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnol* 29:644–652. <https://doi.org/10.1038/nbt.1883>
- Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. *J Exp Bot* 53:1–11. <https://doi.org/10.1093/jxb/53.366.1>
- Han M, Kim CY, Lee J, Lee SK, Jeon JS (2014) OsWRKY42 represses OsMT1d and induces reactive oxygen species and leaf senescence in rice. *Mol Cells* 37:532–539. <https://doi.org/10.14348/molcells.2014.0128>
- He F, Liu Q, Zheng L, Cui Y, Shen Z, Zheng L (2015) RNA-Seq analysis of rice roots reveals the involvement of post-transcriptional regulation in response to cadmium stress. *Front Plant Sci* 6:1136. <https://doi.org/10.3389/fpls.2015.01136>
- Herbette S, Taconnat L, Hugouvieux V, Piette L, Magniette ML, Cuine S, Auroy P, Richaud P, Forestier C, Bourguignon J, Renou JP, Vavasseur A, Leonhardt N (2006) Genome-wide transcriptome profiling of the early cadmium response of *Arabidopsis* roots and shoots. *Biochimie* 88:1751–1765. <https://doi.org/10.1016/j.biochi.2006.04.018>
- Hernandez LE, Sobrino-Plata J, Montero-Palmero MB, Carrasco-Gil S, Flores-Caceres ML, Ortega-Villasante C, Escobar C (2015) Contribution of glutathione to the control of cellular redox homeostasis under toxic metal and metalloids stress. *J Exp Bot* 66:2901–2911. <https://doi.org/10.1093/jxb/erv063>
- Hu H, You J, Fang Y, Zhu X, Qi Z, Xiong L (2008) Characterization of transcription factor gene SNAC2 conferring cold and salt tolerance in rice. *Plant Mol Biol* 67:169–181. <https://doi.org/10.1007/s11103-008-9309-5>
- Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T, Yamanishi Y (2008) KEGG for linking genomes to life and the environment. *Nucleic Acids Res* 36:480–484. <https://doi.org/10.1093/nar/gkm882>
- Kim DY, Bovet L, Maeshima M, Martinoia E, Lee Y (2007) The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. *Plant J* 50:207–218. <https://doi.org/10.1111/j.1365-313X.2007.03044.x>
- Kim TS, Raveendar S, Suresh S, Lee GA, Lee JR, Cho JH, Lee SY, Ma KH, Cho GT, Chung JW (2015) Transcriptome analysis of two *Vicia sativa* subspecies: mining molecular markers to enhance genomic resources for vetch improvement. *Genes* 6:1164–1182. <https://doi.org/10.3390/genes6041164>
- Kováčik J, Klejdus B (2008) Dynamics of phenolic acids and lignin accumulation in metal-treated *Matricaria chamomilla* roots. *Plant Cell Rep* 27:605–615. <https://doi.org/10.1007/s00299-007-0490-9>
- Lanquar V, Ramos MS, Lelièvre F, Barbier-Brygoo H, Krieger-Liszak A, Kraemer U, Thomine S (2010) Export of vacuolar manganese by AtNRAMP3 and AtNRAMP4 is required for optimal photosynthesis and growth under manganese deficiency. *Plant Physiol* 152:1986–1999. <https://doi.org/10.1104/pp.109.150946>
- Li L, He Z, Pandey GK, Tsuchiya T, Luan S (2002) Functional cloning and characterization of a plant efflux carrier for multidrug and heavy metal detoxification. *J Biol Chem* 277:5360–5368. <https://doi.org/10.1074/jbc.M108777200>
- Lin YF, Aarts MG (2012) The molecular mechanism of zinc and cadmium stress response in plants. *Cell Mol Life Sci* 69:3187–3206. <https://doi.org/10.1007/s00018-012-1089-z>
- Lin CY, Trinh NN, Fu S, Hsiung YC, Chia LC, Lin CW, Huang HJ (2013) Comparison of early transcriptome responses to copper and cadmium in rice roots. *Plant Mol Biol* 81:507–522. <https://doi.org/10.1007/s11103-013-0020-9>
- Liu X, Bai X, Wang X, Chu C (2007) OsWRKY71, a rice transcription factor, is involved in rice defense response. *J Plant Physiol* 164:969–979. <https://doi.org/10.1016/j.jplph.2006.07.006>
- Liu T, Liu S, Guan H, Ma L, Chen Z, Gu H, Qu L (2009) Transcriptional profiling of *Arabidopsis* seedlings in response to heavy metal lead (Pb). *Environ Exp Bot* 67:377–386. <https://doi.org/10.1016/j.envexpbot.2009.03.016>
- Liu Q, Zheng L, He F, Zhao FJ, Shen Z, Zheng L (2015a) Transcriptional and physiological analyses identify a regulatory role for hydrogen peroxide in the lignin biosynthesis of copper-stressed rice roots. *Plant Soil* 387:323–336. <https://doi.org/10.1007/s11104-014-2290-7>
- Liu T, Zhu S, Tang Q, Tang S (2015b) Genome-wide transcriptomic profiling of ramie (*Boehmeria nivea* L. gaud) in response to cadmium stress. *Gene* 558:131–137. <https://doi.org/10.1016/j.gene.2014.12.05>
- Ma BH, Gao L, Zhang HX, Cui J, Shen ZG (2012) Aluminum-induced oxidative stress and changes in antioxidant defenses in the roots of rice varieties differing in Al tolerance. *Plant Cell Rep* 31:687–696. <https://doi.org/10.1007/s00299-011-1187-7>
- Marè C, Mazzucotelli E, Crosatti C, Francia E, Cattivelli L (2004) HvWRKY38: a new transcription factor involved in cold- and drought-response in barley. *Plant Mol Biol* 55:399–416. <https://doi.org/10.1007/s11103-004-0906-7>
- Mendoza-Cózatl D, Loza-Tavera H, Hernández-Navarro A, Moreno-Sánchez R (2005) Sulfur assimilation and glutathione metabolism under cadmium stress in yeast, protists and plants. *FEMS Microbiol Rev* 29:653–671. <https://doi.org/10.1016/j.femsre.2004.09.004>
- Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* 7:405–410. [https://doi.org/10.1016/s1360-1385\(02\)02312-9](https://doi.org/10.1016/s1360-1385(02)02312-9)
- Mohamed AA, Castagna A, Ranieri LS, Sanità di Toppi L (2012) Cadmium tolerance in *Brassica juncea* roots and shoots is affected by antioxidant status and phytochelatin biosynthesis. *Plant Physiol Biochem* 57:15–22. <https://doi.org/10.1016/j.plaphy.2012.05.002>
- Molins H, Michelet L, Lanquar V, Agorio A, Giraudat J, Roach T, Krieger-Liszak A, Thomine S (2013) Mutants impaired in vacuolar metal mobilization identify chloroplasts as a target for cadmium hypersensitivity in *Arabidopsis thaliana*. *Plant Cell Environ* 36(4):804–817. <https://doi.org/10.1111/pce.12016>
- Moons A (2003) OsPDR9, which encodes a PDR-type ABC transporter, is induced by heavy metals, hypoxic stress and redox perturbations in rice roots. *FEBS Lett* 553:370–376. [https://doi.org/10.1016/s0014-5793\(03\)01060-3](https://doi.org/10.1016/s0014-5793(03)01060-3)

- Nocito FF, Lancilli C, Dendena B, Lucchini G, Sacchi GA (2011) Cadmium retention in rice roots is influenced by cadmium availability, chelation and translocation. *Plant Cell Environ* 34: 994–1008. <https://doi.org/10.1111/j.1365-3040.2011.02299.x>
- Ogawa I, Nakanishi H, Mori S, Nishizawa NK (2009) Time course analysis of gene regulation under cadmium stress in rice. *Plant Soil* 325:97–108. <https://doi.org/10.1007/s11104-009-0116-9>
- Park J, Song WY, Ko D, Eom Y, Hansen TH, Schiller M, Lee TG, Martinoia E, Lee Y (2012) The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. *Plant J* 69:278–288. <https://doi.org/10.1111/j.1365-313X.2011.04789.x>
- Pirooznia M, Nagarajan V, Deng Y (2007) GeneVenn-A web application for comparing gene lists using Venn diagrams. *Bioinformatics* 1:420–422. <https://doi.org/10.6026/97320630001420>
- Pottier M, Oomen R, Picco C, Giraudat J, Scholz-Starke J, Richaud P, Carpaneto A, Thomine S (2015) Identification of mutations allowing natural resistance associated macrophage proteins (NRAMP) to discriminate against cadmium. *Plant J* 83:625–637. <https://doi.org/10.1111/tpj.12914>
- Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J (2012) The Pfam protein families database. *Nucleic Acids Res* 40:290–301. <https://doi.org/10.1093/nar/gkm960>
- Romman SA (2016) Molecular characterization of a catalase gene (*VsCat*) from *Vicia sativa*. *Int J Biol* 8:66–76. <https://doi.org/10.5539/ijb.v8n3p66>
- Rui H, Chen C, Zhang X, Shen Z, Zhang F (2016) Cd-induced oxidative stress and lignification in the roots of two *Vicia sativa* L. varieties with different Cd tolerances. *J Hazard Mater* 301: 304–313. <https://doi.org/10.1016/j.jhazmat.2015.08.052>
- Sasaki A, Yamaji N, Yokosho K, Ma JF (2012) Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice. *Plant Cell* 24:2155–2167. <https://doi.org/10.1105/tpc.112.096925>
- Satoh-Nagasawa N, Mori M, Nakazawa N, Kawamoto T, Nagato Y, Sakurai K, Takahashi H, Watanabe A, Akagi H (2012) Mutations in rice (*Oryza sativa*) heavy metal ATPase 2 (*OsHMA2*) restrict the translocation of zinc and cadmium. *Plant Cell Physiol* 53:213–224. <https://doi.org/10.1093/pcp/pcr166>
- Sgherri C, Stevanovic B, Navari-Izzo F (2004) Role of phenolics in the antioxidative status of the resurrection plant *Ramonda serbica* during dehydration and rehydration. *Physiol Plant* 122:478–485. <https://doi.org/10.1111/j.1399-3054.2004.00428.x>
- Singh S, Singh A, Bashri G, Prasad SM (2016) Impact of Cd stress on cellular functioning and its amelioration by phytohormones: an overview on regulatory network. *Plant Growth Regul* 80:253–263. <https://doi.org/10.1007/s10725-016-0170-2>
- Slamet-Loedin IH, Johnson-Beebout SE, Impa S, Tsakirpaloglou N (2015) Enriching rice with Zn and Fe while minimizing Cd risk. *Front Plant Sci* 6:121. <https://doi.org/10.3389/fpls.2015.00121>
- Song Y, Chen L, Zhang L, Yu D (2010) Overexpression of *OsWRKY72* gene interferes in the abscisic acid signal and auxin transport pathway of *Arabidopsis*. *J Biosci* 35:459–471. <https://doi.org/10.1007/s12038-010-0051-1>
- Song YF, Cui J, Zhang HX, Wang GP, Zhao FJ, Shen ZG (2013) Proteomic analysis of copper stress responses in the roots of two rice (*Oryza sativa* L.) varieties differing in Cu tolerance. *Plant Soil* 366:647–658. <https://doi.org/10.1007/s11104-012-1458-2>
- Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P, Doerks T, Stark M, Muller J, Bork P, Jensen LJ, von Mering C (2011) The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res* 39:561–568. <https://doi.org/10.1093/nar/gkq973>
- Takahashi R, Ishimaru Y, Shimo H, Ogo Y, Senoura T, Nishizawa NK, Nakanishi H (2012) The OsHMA2 transporter is involved in root-to-shoot translocation of Zn and Cd in rice. *Plant Cell Environ* 35:1948–1957. <https://doi.org/10.1111/j.1365-3040.2012.02527.x>
- Thomine S, Wang R, Ward JM, Crawford NM, Schroeder JI (2000) Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to Nramp genes. *Proc Natl Acad Sci U S A* 97:4991–4996. <https://doi.org/10.1073/pnas.97.9.4991>
- Thomine S, Lelievre F, Debarbieux E, Schroeder JI, Barbier-Brygoo H (2003) AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. *Plant J* 34:685–695. <https://doi.org/10.1046/j.1365-313X.2003.01760.x>
- Ueno D, Yamaji N, Kono I, Huang CF, Ando T, Yano M, Ma JF (2010) Gene limiting cadmium accumulation in rice. *Proc Natl Acad Sci U S A* 107:16500–16505. <https://doi.org/10.1073/pnas.1005396107>
- Uraguchi S, Kamiya T, Sakamoto T, Kasai K, Sato Y, Nagamura Y, Yoshida A, Kyoizuka J, Ishikawa S, Fujiwara T (2011) Low-affinity cation transporter (OsLCT1) regulates cadmium transport into rice grains. *Proc Natl Acad Sci U S A* 108: 20959–20964. <https://doi.org/10.1073/pnas.1116531109>
- Uraguchi S, Kamiya T, Clemens S, Fujiwara T (2014) Characterization of OsLCT1, a cadmium transporter from indica rice (*Oryza sativa*). *Physiol Plant* 151:339–347. <https://doi.org/10.1111/ppl.12189>
- Verbruggen N, Hermans C, Schat H (2009) Mechanisms to cope with arsenic or cadmium excess in plants. *Curr Opin Plant Bio* 12(3):364–372. <https://doi.org/10.1016/j.pbi.2009.05.001>
- Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu U, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EJ, Yazaki K, Theodoulou FL (2008) Plant ABC proteins—a unified nomenclature and updated inventory. *Trends Plant Sci* 13:151–159. <https://doi.org/10.1016/j.tplants.2008.02.001>
- Villiers F, Ducruix C, Hugouvieux V, Jarno N, Ezan E, Garin J, Junot C, Bourguignon J (2011) Investigating the plant response to cadmium exposure by proteomic and metabolomic approaches. *Proteomics* 11:1650–1663. <https://doi.org/10.1002/pmic.201000645>
- Weber M, Trampeczynska A, Clemens S (2006) Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd²⁺ hypertolerant facultative metallophyte *Arabidopsis halleri*. *Plant Cell Environ* 29: 950–963. <https://doi.org/10.1111/j.1365-3040.2005.01479.x>
- Wei W, Chai T, Zhang Y, Han L, Xu J, Guan Z (2009) The *Thlaspi caerulescens* NRAMP homologue TcNRAMP3 is capable of divalent cation transport. *Mol Biotechnol* 41:15–21. <https://doi.org/10.1007/s12033-008-9088-x>

- Wysocki R, Tamás MJ (2010) How *Saccharomyces cerevisiae* copes with toxic metals and metalloids. *FEMS Microbiol Rev* 34: 925–951. <https://doi.org/10.1111/j.1574-6976.2010.00217.x>
- Xu L, Wang Y, Liu W, Wang J, Zhu X, Zhang K (2015) De novo sequencing of root transcriptome reveals complex cadmium-responsive regulatory networks in radish (*Raphanus sativus* L.) *Plant Sci* 236:313–323. <https://doi.org/10.1016/j.plantsci.2015.04.015>
- Xue GP, Way HM, Richardson T, Drenth J, Joyce PA, McIntyre CL (2011) Overexpression of TaNAC69 leads to enhanced transcript levels of stress up-regulated genes and dehydration tolerance in bread wheat. *Mol Plant* 4:697–712. <https://doi.org/10.1093/mp/ssr013>
- Yang Q, Wang Y, Zhang J, Shi W, Qian C, Peng X (2007a) Identification of aluminum-responsive proteins in rice roots by a proteomic approach: cysteine synthase as a key player in al response. *Proteomics* 7:737–749. <https://doi.org/10.1002/pmic.200600703>
- Yang YJ, Cheng LM, Liu ZH (2007b) Rapid effect of cadmium on lignin biosynthesis in soybean roots. *Plant Sci* 172:632–639. <https://doi.org/10.1016/j.plantsci.2006.11.018>
- Yoneyama T, Ishikawa S, Fujimaki S (2015) Route and regulation of zinc, cadmium, and iron transport in rice plants (*Oryza sativa* L.) during vegetative growth and grain filling: metal transporters, metal speciation, grain Cd reduction and Zn and Fe biofortification. *Int J Mol Sci* 16:19111–19129. <https://doi.org/10.3390/ijms160819111>
- Yuan L, Yang S, Liu B, Zhang M, Wu K (2012) Molecular characterization of a rice metal tolerance protein, OsMTP1. *Plant Cell Rep* 31:67–79. <https://doi.org/10.1007/s00299-011-1140-9>
- Yue R, Lu C, Qi J, Han X, Yan S, Guo S, Liu L, Fu X, Chen N, Yin H, Chi H, Tie S (2016) Transcriptome analysis of cadmium-treated roots in maize (*Zea Mays* L.). *Front Plant Sci* 7:1298. <https://doi.org/10.3389/fpls.2016.01298>
- Zhang FQ, Zhang HX, Wang GP, LL X, Shen ZG (2009) Cadmium-induced accumulation of hydrogen peroxide in the leaf apoplast of *Phaseolus aureus* and *Vicia sativa* and the roles of different antioxidant enzymes. *J Hazard Mater* 168:76–84. <https://doi.org/10.1016/j.jhazmat.2009.02.002>
- Zhang L, Zhao G, Jia J, Liu X, Kong X (2012) Molecular characterization of 60 isolated wheat MYB genes and analysis of their expression during abiotic stress. *J Exp Bot* 63:203–214. <https://doi.org/10.1093/jxb/err264>
- Zhao FY, Liu W, Zhang SY (2009) Different responses of plant growth and antioxidant system to the combination of cadmium and heat stress in transgenic and non-transgenic rice. *J Integr Plant Biol* 51:942–950. <https://doi.org/10.1111/j.1744-7909.2009.00865.x>
- Zhu Q, Zhang J, Gao X, Tong J, Xiao L, Li W, Zhang H (2010) The *Arabidopsis* AP2/ERF transcription factor RAP2.6 participates in ABA, salt and osmotic stress responses. *Gene* 457:1–12. <https://doi.org/10.1016/j.gene.2010.02.011>