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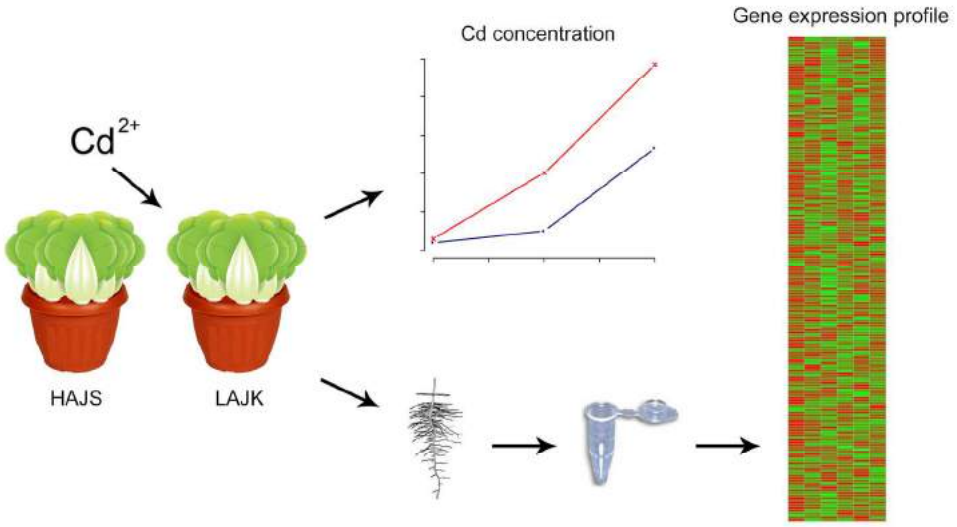

Table of contents and Abstract

Comparative transcriptome analysis between low- and high-cadmium-accumulating genotypes of pakchoi (*Brassica chinensis* L.) in response to cadmium stress

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ABSTRACT: To reduce cadmium (Cd) pollution of food chain, screening and breeding of low-Cd-accumulating cultivars are focused these decades. Two previously identified genotypes, a low-Cd-accumulating genotype (LAJK) and a high-Cd-accumulating genotype (HAJS) of pakchoi (*Brassica chinesis* L.), were stressed by Cd (12.5 μM) for 0 h (T0), 3 h (T3) and 24 h (T24). By comparative transcriptome analysis for root tissue, 3005 and 4343 differentially expressed genes (DEGs) were identified in LAJK at T3 (vs. T0) and T24 (vs. T3), respectively, while 8677 and 5081 DEGs were detected in HAJS. Gene expression pattern analysis suggested a delay of Cd responded transcriptional changes in LAJK comparing to HAJS. DEG functionalenrichments proposed genotype-specific biological processes coped with Cd stress. Cell wall biosynthesis and glutathione (GSH) metabolism were found to involve in Cd resistance in HAJS, while DNA repair and abscisic acid (ABA) signal transduction pathways played important roles in LAJK. Furthermore, the genes participating in Cd efflux such as *PDR8* were overexpressed in LAJK, while those responsible for Cd transport such as *YSL1* were more enhanced in HAJS, exhibiting different Cd transport processes between two genotypes. These

novel findings should be useful for molecular assisted screening and breeding of low-Cd-accumulating genotypes for pakchoi.

INTRODUCTION

Heavy metal contamination in soil presents a widespread serious environmental risk to plants and human health.[1] According to their metabolic roles in plant growth, heavy metals are grouped as two categories, one of which is essential but quite toxic with excessive concentrations such as copper (Cu), zinc (Zn) and iron (Fe), while the other is non-essential with recognized toxicity such as cadmium (Cd).[2] Cd is a typical toxic heavy metal that is hazardous to plant growth and development.[3] In recent years, Cd contamination in the arable soil has severely limited crop yield and threatened food safety.[4-6] Long-term exposure to Cd, even with low dose, would lead to chronic health problems, including liver and kidney damage, weakness and higher risk of illness.[7] Vegetables contributed 83% of the total Cd uptake in human bodies.[8] Comparing to root and fruit vegetables, leaf vegetables such as spinach (*Spinacia oleracea* L.) and coriander (*Coriandrum sativum* L.) have much higher capacities of heavy metal absorption and accumulation.[9-11] Thus, studies on strategies and technologies to lower the pollution risk of Cd in food chain, especially in leaf vegetables, are an urgent task and of great interest in the recent decade.

Previous studies have addressed the adverse impacts of Cd on the biochemical and physiological processes of plants, such as altering photosynthetic processes, reducing enzymes activities and nutrient uptake and breaking up homeostasis, which finally resulted in growth inhibition and diseases.[4, 5, 12] Under this scenario, plants have evolved a series of metabolic

strategies against the Cd stress, including immobilization, restriction of uptake and transport, efflux from cytoplasm to the outside of cell, chelation and sequestration in vacuoles through specific transporters.[13-16] Over the past decade, as the expansion of available transcriptional data, the genetic basis underlying these physiological processes have been identified and characterized, greatly improving our understanding on molecular mechanisms of Cd translocation and detoxification in some Cd hyperaccmulating plants such as *Arabidopsis halleri*,[5, 17] *Brassica juncea*,[18, 19] *Sedum alfredii*[20] and *Noccaea caerulescen*s,[21-23] as well as some cultivating plants such as pea (*Pisum sativum* L.),[24] barley (*Hordeum vulgare* L.),[25, 26] rice (*Oryza sativa* L.),[2, 27] tobacco (*Nicotiana tabacum* L.)[28] and ramie (*Boehmeria nivea* L.).[29] However, most studies mainly focused on the practical advantages in phytoremediation rather than food safety.[16, 30]

For food safety, screening and breeding the cultivars with low capacity of Cd accumulation or Cd pollution-safe cultivars (Cd-PSCs) is a low-cost strategy for restricting Cd transfer into the food chain.[10, 31, 32] The Cd-PSCs are kinds of crop cultivars containing a low enough level of Cd in edible part for safe consumption when growing in Cd contaminated soil.[31, 33] For plants, roots are thought to determine the Cd concentration in leaves. Several studies have addressed that the Cd content in above-ground tissue of plants is highly impacted by the capacities of Cd uptake from the soil to roots and the translocation from roots to shoot.[5, 18, 34, 35] Yamaguchi et al. implicated that down-regulation of one xylem-loading citrate transporter gene *ferric reductase defective 3* (*FRD3*), which inhabit Cd translocation from roots to shoot, played an important role in reducing Cd concentration in a low Cd-accumulating line of *Solanum torvum*.[36] However, our knowledge about the genome-wide molecular mechanism underlying the low capacity of Cd accumulation is

still quite limited.

Pakchoi (*Brassica chinensis* L.) is one of the most important worldwide leaf vegetables. In genus of *Brassica*, *B. juncea* and *B. napus* have been reported as Cd accumulative species.[18, 19, 37] Our previous study identified some genotypes of *B. chinensis* with significantly different capacities of Cd uptake and accumulation under Cd exposure.[38] In cell wall, chloroplast/trophoplast, organelle and soluble fractions of high Cd-accumulating genotypes, Cd concentrations were significantly higher than those low Cd-accumulating genotypes, which is a kind of Cd-PSCs. It provided us an ideal system to investigate the genome-wide differentiations underlying the differently physiological traits, which could shed light on the molecular assisted breeding methods of pakchoi.

In this study, we measured the Cd concentration in the edible parts of low- and high-Cd-accumulating genotypes of pakchoi at different time stages of Cd treatment to verify the Cd accumulating capacities. Comparative transcriptome analysis was then employed for the roots of the two genotypes to clarify two major issues: 1) what are the differences in the transcriptional responses to Cd stress associated to different treatment times and different genotypes; and 2) what is the genetic basis for the different capabilities of Cd accumulation between the two genotypes. Based on bioinformatics analysis, it is expected that results of this study could provide new insights into the molecular mechanisms brought about the low capacity of Cd accumulation in pakchoi, which would help to explore new ways for creating more efficient Cd-PSCs of pakchoi or even other leaf vegetables via molecular breeding methods.

MATERIALS AND METHODS

Plant Material and Cd Treatments. Based on previous study[38], two identified pakchoi genotypes, a low-Cd-accumulating cultivar (AJKSHY) and a high-Cd-accumulating cultivar (AJSZQ), were used in the present study. To make easy to distinguish the low- and high-Cd-accumulating genotypes, they were renamed as LAJK and HJAS in the present study. Seeds of the two genotypes were surface sterilized by soaking in 2% H_2O_2 for 10 min and fully rinsed with deionized water. After sterilizing, the seeds were soaked in deionized water at room temperature for 24 hours, and then germinated in sterilized moist sand substrate under constant temperature condition (25 ± 1°C) and photoperiod (14/10 h light/dark cycle). After two weeks, healthy seedlings with similar size of each genotype were selected and cultured in half-strength modified Hoagland nutrient solutions[38] in 500ml containers under the controlled temperature (25 ~30°C) and photoperiod (14/10 h light/dark cycle) in a greenhouse.

After 40 days of growth, three plants from three different containers of each genotype were used as biological replicates. For each container, the plant was treated with the fresh medium supplemented with $CdCl_2$ to final Cd concentrations of 12.5 μM, which is a mild stress condition that would not lead to any observable toxic symptoms for either of the two genotypes. Before the Cd treatment (denoted as T0) and in the 3rd and 24th hour after the Cd treatment (denoted as T3 and T24, respectively), shoots and roots from the plants of the two tested genotypes with similar size were harvested separately and washed three times with deionized water. Fresh root tissues were frozen in liquid nitrogen (N_2) and stored at -80°C for RNA extraction.

Determination of Shoot Cd Concentration. To detect shoot Cd concentration of the two

pakchoi genotypes, shoots from three plants as replicates for each of T0, T3 and T24 were dried at 70°C to a constant weight and then digested by HNO_3 and H_2O_2 in a microwave digester. Cd concentration was measured using FAAS (HITACHI Z-5300, Japan), following the manufacturer's instruction. A Certified Reference Material (CRM; GBW-07603, provided by the National Research Center for CRM, China) was applied to assess the precision of the analytical procedures for plant material. One-way Analysis Of Variance (ANOVA) and the least significant difference (LSD) tests were performed to identify the significant differences of the Cd concentration at each treatment stage between the two genotypes using the statistical package SPSS 13.0.

RNA Extraction, Sequencing and *De Novo* Assembly. Total RNA were extracted from the root tissues of the three plants as replicates for each of T0, T3 and T24 separately using an RN09-EASY spin plus Plant Kit (Aidlab Biotech, Beijing, China) following the manufacturer's instructions. The integrity of RNA was verified by RNase free agarose gel electrophoresis and the concentration was measured using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). High-quality RNA of the three plants from each treatment was mixed with equal quantity for the subsequent RNA sequencing.

cDNA library was constructed for each of the six mixed RNA samples and sequenced on the Illumina HiSeq™ 2000 platform (Illumina Inc., CA, USA). Before assembly, adapter sequences were removed from the raw reads. Then low quality reads with over 50% bases with quality scores of 5 or lower and/or over 10% bases unknown (N bases) were removed from each dataset to gain more reliable results. After that, the clean reads of high quality from all the six samples

were merged together and assembled using Trinity package[39] to construct unique consensus sequences as the reference sequences.

Normalization of Gene Expression Levels and Identification of Differentially Expressed Genes. Sequencing reads were remapped to the reference sequences by SOAPaligner/soap2.[40] For each gene, the expression level was measured by Reads Per Kilobase exon Model per Million mapped reads (RPKM) based on the number of uniquely mapped reads, to eliminate the influence of different gene lengths and sequencing discrepancies on the gene expression calculation. For genes with more than one alternative transcript, the longest transcript was selected to calculate the RPKM.

To infer the transcriptional changes over time in the two genotypes under Cd stress conditions, differentially expressed genes (DEGs) after 3 and 24 h of Cd treatment were identified by comparing the expression levels at T3 with those at T0 and the level at T24 with those at T3 in LAJK and HAJS, respectively. To correct for multiple testing, the false discovery rate (FDR) was calculated to adjust the threshold of p value.[41] Transcripts with a minimal 2-fold difference in expression ($|\log_2 \text{Ratio}| \geq 1$) and a FDR ≤ 0.001 were considered as differentially expressed between the two time points.[42] For convenience, DEGs with higher expression levels at T3 than those at T0, as well as those higher at T24 than those at T3, were donated as “up regulated”, while those in opposition were donated as “down regulated”.

To assess the gene expression patterns over time within each genotype, expression pattern analysis were performed, which assigned all the DEGs of LAJK and HAJS across the two Cd-treatment stages to eight expression profiles, using Short Time-series Expression Miner

(STEM) version 1.3.8.[43] DEGs belonging to the same cluster were proposed to have similar expression pattern with each other. For each genotype, the clustered profiles of DEGs with $p < 0.05$ were considered as significantly different from the reference set.

Gene Expression Validation. Eight genes with different expression patterns revealed by RNA sequencing were randomly selected for validation by quantitative real-time RT-PCR (qPCR). RNA extracted from the roots of the three independent biological replicates for each of T0, T3 and T24 were employed for qPCR validation. First-strand cDNA was synthesized using PrimeScript™ RT reagent Kit (TAKARA BIO Inc., Shiga, Japan). Gene copy specific primers for qPCR were designed based on the corresponding sequence on Primer3 website[44] and listed in Table S1 (Supporting information). Actin I was used as an internal control.[45] The qPCR was carried out using SYBR® Premix Ex Taq II (Tli RNaseH Plus; TAKARA BIO Inc., Shiga, Japan) and determined in LightCycler 480 (Roche, Basel, Switzerland) according to the manufacturer's instructions. Three technical replicates were performed for each gene. A regression analysis was performed between qPCR and RNA sequencing including all genes of the two genotypes at the three time points of Cd treatment using R package (version 3.1.3, http://cran.r-project.org/).

Functional Annotation and GO and KEGG Classification. All expressed genes were functional annotated against four databases, including NCBI non-redundant protein database (Nr), Clusters of Orthologous Groups of proteins database (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Swiss-Prot database, by BLASTX searches with an e-value cutoff of 1e-5 in Blast2GO.[46] For the gene matched to multiple protein sequences, the protein with the highest similarity score was considered as the optimal annotation.

For each treatment stage, Gene Ontology (GO) classification was performed for the up-regulated genes of LAJK and HAJS in WEGO,[47] respectively, and chi-square test was employed to figure out the GO terms of significant difference in gene proportion between the two genotypes, which were proposed to play different roles in response to Cd stress. For each KEGG pathway, the numbers of up- and down-regulated genes of each genotype were compared to the reference set by Fisher's exact test to find out the pathways enriched with up and down-regulated genes. GO and KEGG enrichment analysis were also carried out for all the eight gene expression profiles

RESULTS AND DISCUSSION

Difference of Shoot Cd Concentrations Between the Two Genotypes. Average shoot Cd concentrations at T0 were 0.28 and 0.42 mg/kg DW in LAJK and HAJS, respectively, where no significant difference was detected between the two genotypes (Figure 1). The Cd concentration in LAJK at T3 still remained at a relatively low level (0.4 mg/kg DW). In HAJS, by contrast, the Cd level at T3 increased to 1.15 mg/kg DW, which is approximately 2.9-fold higher than that in LAJK ($p < 0.01$). At T24, Cd concentration in LAJK and HAJS progressively reached 0.89 mg/kg DW and 2.12 mg/kg DW, respectively, with significant difference between the two genotypes ($p < 0.01$). These results verified the genotype dependent difference in shoot Cd accumulation of pakchoi as indicated in a previous study.[38] The genetic stability of shoot Cd accumulation at cultivar level in pakchoi as well as many other vegetable crops[48, 49] implies the difference in gene participation between different cultivars within the same species which has been partly clarified for limited crops especially for rice.[50, 51] Xue et al.[38] have suggested that the

lower capacity of Cd translocation from roots to shoot in LAJK comparing with HAJS is associated with subcellular distributions and chemical forms of Cd.

RNA Sequencing and *De Novo* Assembly of Root Transcriptome of the Two Genotypes. Approximately 24.63 - 36.59 million of 125 bp pair-end reads were generated for the six samples through RNA sequencing (Table 1). After sequence trimming, the retained high-quality reads of all the samples were merged together and *de novo* assembled into 59 271 unigenes as the reference transcripts of pakchoi, and 44 539 of them were functionally annotated with an e-value cutoff of 1e-5. The N50 of the assembled genes was 1294 bp and the average length was 804 bp with the maximum length of 14 696 bp, which were longer than those obtained in the experiments for *Cicer arietinum*,[52] *Elodea nuttallii*[53] and *Primrose* species (*P. poissonii* and *P. wilsonii*),[54] suggesting a good assembled quality of the transcriptome for pakchoi in the present study. By remapping to the reference transcripts, 46 753 - 49 391 expressed unigenes were identified for the two genotypes at the three time points. Using a cutoff of 2-fold difference in gene expression as methodological description, a total of 3005 and 8677 DEGs were detected in LAJK and HAJS at T3, respectively, as comparing with those at T0, while 4343 and 5801 genes were differentially expressed at T24, respectively, as comparing with those at T3 (Figure S1, Supporting information).

RNA Sequencing Validation by qPCR. To validate the expression data obtained from RNA sequencing, eight genes with different expression patterns were randomly selected to perform qPCR. The results showed a strong correlation between the data of RNA sequencing and qPCR ($r = 0.683$, $p < 0.001$, Figure 2). For each gene, the expression count values of

transcriptome data exhibited similar expression profile at all the three time stages comparing with the results of qPCR (Figure S2, Supporting information). It suggested a reliable expression results generated by RNA sequencing.

Gene Expression Pattern Analysis, and Clustering and Functional Enrichment of DEGs. DEGs of each LAJK and HAJS at different time stages were clustered in eight profiles based on gene expression pattern using STEM software. The profiles displayed a considerable difference in gene expression over time in response to Cd stress between the two genotypes (Figure 3A). In HAJS, the DEGs were significantly overrepresented in the profiles with apparent changes in expression level at T3 (Profile 1, 5 and 6, $p < 0.05$), while the major transcriptional changes in LAJK occurred at T24 concomitantly with the significant increase of Cd concentration (Profile 3, 4 and 7, $p < 0.05$). Consistent with the number of DEGs changes over time in both genotypes, these results also strongly suggested a delay in transcriptional responses to Cd stress in LAJK comparing with HAJS.

To determine the functional significance of the transcriptional changes in each genotype, GO and KEGG classifications were implemented for the genes belonging to the overrepresented profiles. In HAJS, genes involved in stress and stimulus resistance, starch and sucrose metabolism and pentose and glucuronate interconversions were enriched in Profile 5, where gene expressions were increased at T3 but decreased at T24 (Figure 3C; Table S2, Supporting Information), suggesting that these genes responded at the early stage of Cd stress. Similar pattern was also observed in *Arabidopsis thaliana* that the higher expressions of many genes responded to stress and stimulus were observed at 2 h of Cd exposure instead of one week.[17] In Profile 6, the

overrepresented GO and pathway included cell wall biosynthesis and organization, glucan and cellulose metabolism, transferase encoding, phenylpropanoid biosynthesis and glutamate metabolism. The expression level of these genes peaked at T3 and maintained at high level during the subsequent stage. Detailed gene functions would be discussed below. In LAJK, however, genes involved in response to oxygen-containing compounds were overrepresented in Profile 4, while genes responding to stimulus, regulatory region with DNA binding and the hormone signal transduction pathway were enriched in Profile 7 (Figure 3B; Table S2, Supporting Information). These results suggested an apparent genotype variation in genes and pathways responding to Cd stress. The differentiation in gene expression patterns between the two genotypes was corresponding to their distinct responses in Cd subcellular distribution as well as chemical forms after a long term of Cd treatment.[38]

Responses to Cd Stress were Faster in High-Cd-accumulating Genotype Than in Low One. Comparing to T0, only 1664 up- and 1341 down-regulated genes in LAJK were identified at T3, while they were 5138 and 3539 in HAJS (Figure S1, Supporting information). Concerning the up-regulated genes that may be responsible for Cd stress, GO enrichment analysis of up-regulated genes revealed a significant difference between the two genotypes (Figure S3A, Supporting Information). A total of 354 and 1123 genes were assigned into 138 and 171 GO terms at the third level in LAJK and HAJS, respectively. As a response to the mild damage caused by the slight increase in Cd concentration, only one category of genes encoding proteins with tetrapyrrole binding activity was significantly induced in LAJK at T3.

By contrast, more GO terms were overrepresented in HAJS in all the three GO categories, i.e.

246 biological process, cell component and molecular function, especially those related to stress tolerance.
247 In the category of biological process, the GO terms of response to stress, response to chemical
248 stimulus and response to abiotic stimulus, as well as those involved in metabolic process, were
249 exclusively enriched in HJAS ($p < 0.05$). Of them, seven genes were involved in activating and
250 encoding heat shock proteins (HSPs, Figure 4; Table S3, Supporting Information). All of these genes
251 were indicated to play an important role in protecting plant cells from the damage of metals exposure
252 by maintaining protein correct folding and stabilization.[27] It is noteworthy that, although Cd dose not
253 directly induce reactive oxygen species (ROS), glutathione **(**GSH)-derived phytochelatin (PC)-Cd
254 synthesis would deplete reduced GSH and alter oxidation state in the plant cell, as a by-product.[55]
255 Correspondingly, in HAJS, the genes category being responsible for oxidative stress resistance was
256 overrepresented at T3 (Figure 4; Table S3, Supporting Information), indicating a trade-off between
257 Cd chelation or compartmentalization and oxidative damage in HAJS to cope with the abrupt Cd
258 increase in cell. These results suggested that response changes in transcript level of HAJS to Cd stress
259 were more activated at the initial stage, which was consistent with the performance of Profile 5
260 (Figure 3A).

261 With regard to the subsequent treatment stage (T24), 1966 and 1782 genes were up regulated in
262 LAJK and HAJS, respectively, while 2377 and 4019 genes were down regulated in the two
263 genotypes (Figure S1, Supporting information). Different to the over-expression at T3, the genes
264 involved in response to stress and stimulus in HAJS were found to be down regulated at T24, as
265 inferred by the gene expression analysis (Figure S3B, Supporting Information). The decline of
266 expression level for the early-responsive genes also observed in *Arabidopsis thaliana* ,[17, 56] indicating

267 there are different stages of responses to Cd exposure in plants.. In LAJK, the Cd-responsive
268 transcriptional changes at T24 were more pronounced than in HAJS ($p < 0.05$, Figure S3B,
269 Supporting Information), which was especially observed in the genes involved in response to stress,
270 cellular response to stimulus and cell communication (Table S5, Supporting information). The slower
271 activation of the early stress-responsive genes in LAJK comparing to HAJS was considered to be
272 concomitant with the delay of Cd accumulation in LAJK.

273 **Enhanced Cell Wall Biosynthesis Resulted in High Cd Tolerance in High-**
274 **Cd-accumulating Genotype.** A total of ten GO terms involved in cell wall biosynthesis
275 exhibited a pattern that gene expression level increased at T3 and maintained a high level at T24
276 (Profile 6, Figure 3C), suggested that the cell wall relevant functions played important role in Cd
277 tolerance in HAJS after Cd exposure. Similarly, according to pathway enrichment analysis at T3,
278 the pentose and glucuronate interconversions pathway which involves in cell wall biosynthesis
279 consisted of higher percentages of up-regulated genes in HAJS than the reference set. Four of 27
280 overexpressed genes along this pathway encode the two key enzymes (pectinesterase and
281 polygalacturonase) involving in D-galacturonate biosynthesis (Figure 4), which is essential for
282 forming the backbone of pectic cell wall components and the borate-mediated cross-linking
283 within the cell wall.[57-59] This result implied that cell wall biosynthesis involved pathway might be
284 activated by Cd stress in HAJS at T3, which was consistent with the observations obtained from
285 the GO enrichment analysis of expression pattern (Profile 6). In another pathway of starch
286 metabolism, the two key enzymes (α-amylase and UGP2), catalyzing the biosynthesis of
287 UDP-glucose from α-D-glucose-1P, were up-regulated in HAJS at T3, but not in LAJK (Figure 4).

288 The enzyme GAUT, which is involved in the transmutation of UDP-glucose into pectin, was also
289 enhanced at this stage. Kieffer et al.[60] found that the activity of α-amylase was increased in
290 response to Cd stimulus in poplar. Therefore, the results from this study implied that the
291 overexpression of α-amylase may play an important role in Cd resistance via enhancing the cell
292 wall biosynthesis.

293 At T24, 23 up-regulated genes were assigned into the phenylpropanoid synthesis pathway,
294 participating in the biosynthesis of guaiacyl and syringyl lignin ($q < 0.001$, Table S6, Supporting
295 information; Figure 4). Guaiacyl and syringyl are crucial components presented in the cell wall of
296 angiosperm plants.[61] Similar increase in lignin synthesis in roots has been observed in Cd stressed
297 *A. thaliana*.[5] Therefore, the Cd induced transcriptional changes of genes or pathways that
298 participate in cell wall biosynthesis should be important molecular processes leading to the
299 genotype difference in Cd tolerance and accumulation in pakchoi.

300 **Glutathione (GSH) Metabolism and Phytochelatins (PCs) Responded More**
301 **Exclusively to Cd Stress in High-Cd-accumulating Genotype.** Plants employ an
302 important strategy in Cd detoxification through chelation and sequestration to restrict the
303 transport and circulation of free Cd ion in cytosol.[16] In this study, GSH-mediated Cd conjugation
304 was enhanced in HAJS under Cd stress condition. Key enzymes for cysteine biosynthesis including
305 3'-phosphoadenosine 5'-phosphosulfate synthase (PAPSS), sulfite reductase (Sir) and cysteine
306 synthase A (CysK) belonging to a sulfur assimilation pathway involved in GSH precursor
307 synthesis highly expressed in HAJS but down regulated in LAJK at both T3 and T24 stages
308 (Figure 4). Sulfur and cysteine have been reported to participate in Cd detoxification in *A.*

309 *thaliana*, *B. juncea* and *Populus italica*.[1, 5, 60, 62] Similarly, a pathway of the nitrogen metabolism
310 was also enriched in HAJS at T24, where three key enzymes, including assimilatory nitrate
311 reductase catalytic subunit (NasAB), glutamine synthetase (GLN) and glutamate dehydrogenase
312 (GLDH), were up regulated, and consistent with the observation in *A. thaliana*.[63, 64] Cysteine and
313 glutamate were both the important precursors of glutathione (GSH) metabolism. GSH plays an
314 important role in Cd detoxification via conjugating with Cd under the catalyzing of glutathione
315 S-transferases (GST)[60, 65]. Consistently, in this study, five and four GST encoding genes that are
316 highly expressed in HAJS at T3 and T24 were identified (Figure 4). It suggested that GSH-Cd
317 conjugation process is crucial for resistance to Cd in HAJS.

318 Furthermore, GSH could be also used to synthetize PCs, kinds of heavy metal complexing
319 peptides crucial for Cd detoxification in plants, catalyzed by phytochelatin synthase (PCS). The
320 expression levels of PCS in HAJS were higher than those in LAJK at both time stages (Figure 4).
321 Clemens et al.[66] proposed that the enhancement in PC generation resulted in increased Cd
322 accumulation. Moreover, proteins involved in PC-Cd complex transporting, such as multidrug
323 resistance-associated protein 2 and 3 (MRP2 and MPR3), were significantly overexpressed in HAJS at
324 T3 (Figure 4). MRP2 and MRP3 can increase Cd tolerance in *Arabidopsis* via mediating the transport
325 of PC-Cd into vacuole[67]. Therefore, the higher expression level of the PC formation and
326 immigration in HAJS might be associated with the higher Cd accumulation in HAJS than in
327 LAJK.

328 **Genes Involved in DNA Repair Acted as an Early Response to Cd Exposure in**
329 **Low-Cd-accumulating Genotype.** Besides the mutual early Cd responses, LAJK also

employed specific responsive mechanisms to cope with Cd stress. Among the up-regulated genes in LAJK, the pathways of ribosome formation and DNA replication was found to be overrepresented at T3 (Table S4, Supporting information), most of which are involved in forming helicase, the enzyme catalyzing the first step of DNA biosynthesis (Figure 4). Von Zglinichi et al. [68] implicated an enhanced DNA replication induced by low dose of Cd exposure, as a result of response amplifying actions. Moreover, helicase plays an indispensable role in DNA repair, which is required for coping with the oxidative damages under Cd stress. Because similar transcriptional changes did not observed in HAJS, the low Cd accumulating genotype should have more sensitive transcriptional responses to Cd stress than the high one at early stage of Cd stress.

Abscisic Acid **(ABA) Signal Transduction Pathway Responded Differently Between Low- and High-Cd-accumulating Genotype.** At T24 time point, plant hormone signal transduction was induced by Cd stress and played an exclusive role in response to Cd stress in LAJK (Table S6, Supporting information). The central signaling complex PYR/PYL-PP2Cs-SnRK2s (pyrabactin resistant - A-group proteins phosphatase 2C - sucrose non-fermentation kinase subfamily 2) of one abscisic acid (ABA) signaling pathway were importantly activated in LAJK. Among them, the genes encoding protein PYR/PYL and SnRK2s were up regulated, but the PP2Cs were down regulated (Figure 4). As reviewed by Guo et al. [69], PYR/PYL is an ABA receptor of the signaling complex. The overexpression of PYR/PYL could suppress PP2Cs, which release SnRK2s from the inhibition of PP2Cs and subsequently activate the downstream target ABRE-binding factor (ABF) transcription factor.[70-72] ABF could bind to and activate the promoter of another transcription factor DRE-binding protein 2A (DRE2A), which has been suggested a functional significance in the

response to osmotic stress.[73] However, this pathway, where PYR/PYL were highly depressed but PP2Cs were overexpressed, was suppressed in HAJS in either treatment stage, especially at T3. Therefore, the ABA-induced antioxidant pathway plays a genotype-specific role in countering the deleterious effects of Cd accumulation in LAJK.

Differential Expression of Genes Involved in Cd Transport Contributed to the Genotype Difference in Cd accumulating Capacities. To uncover the genes responsible for the different nature of Cd accumulation between LAJK and HAJS, pairwise comparisons between the two genotypes were performed and 9016, 8620 and 8253 DEGs at the three time stages of T0, T3 and T24 were identified, respectively. Since Cd is "opportunistic hitchhiker" with no specific transporter in plants, Cd usually enter plant cells using the transporters of the essential cations, such as Zn, Fe and Cu.[74] Between the two genotypes, 63 DEGs belonged to eight GO terms involved in the cation transport including the transporters of Cd, Zn, Cu, Fe, calcium (Ca), manganese (Mn) and nickel (Ni). Besides these genes, another 32 DEGs encoding transporters for metal and metal ligand, which have also been proposed to be related to heavy metal uptake and sequestration, were also identified (Table S7, Supporting information). The totally 95 DEGs showed apparently different expression pattern between the two genotypes (Figure S4, Supporting information).

Genes related to Cd efflux and transport were suggested to play the major role in the genotype difference of Cd accumulation.[66] The genes belonging to the pleiotropic drug resistance (PDR) subfamily of the ATP-binding cassette (ABC) transporter family showed markedly overexpressed in LAJK than HAJS at T24. The two genes encoding *PDR8* transporter were 3.1 and 3.4-fold

372 higher expressed in LAJK as compared to HAJS. In *A. thaliana*, *PDR8* has been confirmed as a
373 Cd efflux pump mainly presented in the plasma membrane of root hairs and epidermal cells.[75]
374 Moreover, *PDR8* has more functional significance in decreasing Cd concentration in shoots than
375 in roots.[75] The enhanced expressions of *PDR8* in LAJK may thus contribute to the low Cd uptake
376 in roots, leading to the low Cd accumulation in shoots.

377 In addition, genes involved in Cd absorption and translocation, such as the members from the
378 gene families of Yellow Stripe-like (YSL) and ZRT/IRT protein (ZIP), were overexpressed in
379 HAJS. YSL1 is responsible for iron-nicotianamine uptake by roots in response to iron shortage in
380 *Arabidopsis*.[76] It was found that three YSL1-encoding genes were strongly induced by Cd stress
381 with higher expression in HAJS than in LAJK at all the three stages (Figure 4), suggesting that
382 YSL1 might participate in Cd transport. Similar higher expression in HAJS was also observed in
383 the genes of IRT family, including *IRT1* and *IRT3*. IRT1 is mainly responsible for Fe uptake in
384 roots under Fe-deficient condition.[77] As Cd is absorbed concomitantly with iron uptake, the
385 overexpression of *IRT1* would also lead to Cd accumulation in plants.[78] Lin et al.[79] proposed that
386 the enhanced *IRT3* leaded to an increase in the concentration of Fe in roots and Zn in shoots of
387 *Arabidopsis*, suggesting an important role of IRT3 in Cd uptake across plasma membrane. The
388 overexpression of the genes responsible for Cd uptake was thought to result in the higher Cd
389 concentration in HAJS.

390 In conclusion, two new findings that 1) the high-Cd accumulating capacity of pakchoi should
391 be related to the fast transcriptomic response to Cd stress, and 2) ABA signaling pathways
392 seemed participated in the Cd detoxification in the low-Cd-accumulating genotype of pakchoi,

393 were explored in the present study. These novel findings greatly enriched our knowledge on
394 genetic basis of low Cd-accumulating pakchoi genotype. Furthermore, our works found the
395 linkage between the different capacities of Cd accumulation and the different metal transition
396 processes including Cd efflux, uptake and translocation. These results provided important clues
397 for molecular assisted screening and breeding of low Cd-accumulating cultivars for pakchoi.
398

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406 **Notes**

407 The authors declare no competing financial interest.
408

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414

ASSOCAIATED CONTENT

Supporting Information Available

Figure S1: Pairwise comparison of differentially expressed genes (DEGs) among the three time points of Cd treatment in LAJK and HAJS.

Figure S2: Expression of the selected eight genes inferred by RNA sequencing and qPCR. In each panel, red bars represented the RPKM values of each gene in the two genotypes at the three time points of Cd treatment inferred by RNA sequencing, while blue bars represented the average expression levels of the gene at the corresponding time points verified by qPCR.

Figure S3: Gene Ontology (GO) distribution for the DEGs of the two pakchoi genotypes at T3 and T24. A. GO distribution for the DEGs of LAJK (blue) and HAJS (red) at T3 of Cd treatment. B. GO distribution for the DEGs of LAJK (blue) and HAJS (red) at T24 of Cd treatment. For both frame, annotation results were mapped to categories in the third level of GO terms. GO terms that contain less than 1% of total genes in both genotypes were excluded from the graphs. *, $p < 0.05$; **, $p < 0.01$.

Figure S4: A heatmap of Cd transport-related DEGs. Expression values of six samples are presented after being normalized and log-transformed. DEGs of down- (blue) and up-regulation (red) are distinguished from different genotypes and stages. L represented LAJK, while H represented HAJS.Table S1: Gene IDs, descriptions and primer sequences for the eight genes used for qPCR verification. Table S2: KEGG pathway significantly overrepresented in the six enriched profiles of gene expression versus the reference set in HAJS and LAJK. Table S3: List of DEGs belonging to the GO terms significantly overrepresented in LAJK and HAJS at T3. Table S4: List of the KEGG pathway significantly overrepresented with up- and down-regulated genes in LAJK and HAJS at T3versus the reference set. Table S5: List of DEGs belonging

436 to the GO terms significantly overrepresented in LAJK and HAJS at T24 Table S6: List of the KEGG
437 pathway significantly overrepresented with up- and down-regulated genes in LAJK and HAJS at T24
438 versus the reference set. Table S7: Gene IDs and RPKM values of DEGs correlating to the transport of
439 cations such as Cd, Cu, Fe, Ca, Mn, Ni and Zn, and Cd sequestration in the two genotypes at the three time
440 points of Cd treatment.

441 This information is available free of charge via the Internet at http://pubs.acs.org.

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443 **REFERNCES**

444 (1) Villiers, F.; Ducruix, C.; Hugouvieux, V.; Jarno, N.; Ezan, E.; Garin, J.; Junot, C.; Bourguignon, J.
445 Investigating the plant response to cadmium exposure by proteomic and metabolomic approaches.
446 *Proteomics* **2011,** *11* (9), 1650-1663.
447 (2) Oono, Y.; Yazawa, T.; Kawahara, Y.; Kanamori, H.; Kobayashi, F.; Sasaki, H.; Mori, S.; Wu, J.; Handa,
448 H.; Itoh, T. Genome-wide transcriptome analysis reveals that cadmium stress signaling controls the
449 expression of genes in drought stress signal pathways in rice. *PLOS one* **2014,** *9* (5), e96946.
450 (3) Wu, F.; Zhang, G. Genotypic differences in effect of Cd on growth and mineral concentrations in
451 barley seedlings. *Bulletin of Environmental Contamination and Toxicology* **2002,** *69* (2), 219-227.
452 (4) Di Toppi, L. S.; Gabbrielli, R. Response to cadmium in higher plants. *Environmental and*
453 *Experimental Botany* **1999,** *41* (2), 105-130.
454 (5) Herbette, S.; Taconnat, L.; Hugouvieux, V.; Piette, L.; Magniette, M.-L.; Cuine, S.; Auroy, P.; Richaud,
455 P.; Forestier, C.; Bourguignon, J. Genome-wide transcriptome profiling of the early cadmium response of
456 *Arabidopsis* roots and shoots. *Biochimie* **2006,** *88* (11), 1751-1765.
457 (6) Rodríguez, L.; Ruiz, E.; Alonso-Azcárate, J.; Rincón, J. Heavy metal distribution and chemical
458 speciation in tailings and soils around a Pb–Zn mine in Spain. *Journal of Environmental Management* **2009,**
459 *90* (2), 1106-1116.
460 (7) Moulis, J.-M.; Thévenod, F. New perspectives in cadmium toxicity: an introduction. *Biometals* **2010,**
461 *23* (5), 763-768.
462 (8) Oskarsson, A.; Widell, A.; Olsson, M.; Grawé, K. P. Cadmium in food chain and health effects in
463 sensitive population groups. *Biometals* **2004,** *17* (5), 531-534.
464 (9) McLaughlin, M. J.; Parker, D.; Clarke, J. Metals and micronutrients–food safety issues. *Field crops*
465 *research* **1999,** *60* (1), 143-163.
466 (10) Grant, C.; Clarke, J.; Duguid, S.; Chaney, R. Selection and breeding of plant cultivars to minimize
467 cadmium accumulation. *Science of the Total Environment* **2008,** *390* (2), 301-310.
468 (11) Arora, M.; Kiran, B.; Rani, S.; Rani, A.; Kaur, B.; Mittal, N. Heavy metal accumulation in vegetables

irrigated with water from different sources. *Food Chemistry* **2008,** *111* (4), 811-815.
(12) Siedlecka, A.; Krupa, Z. Interaction between cadmium and iron and its effects on photosynthetic capacity of primary leaves of *Phaseolus vulgaris*. *Plant Physiology and Biochemistry* **1996,** *34* (6), 833-841.
(13) Zenk, M. H. Heavy metal detoxification in higher plants-a review. *Gene* **1996,** *179* (1), 21-30.
(14) Cobbett, C. S. Phytochelatins and their roles in heavy metal detoxification. *Plant physiology* **2000,** *123* (3), 825-832.
(15) Hall, J. Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of experimental botany* **2002,** *53* (366), 1-11.
(16) Hossain, M. A.; Piyatida, P.; da Silva, J. A. T.; Fujita, M. Molecular mechanism of heavy metal toxicity and tolerance in plants: central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. *Journal of Botany* **2012,** *2012* (2012), Article ID 872875. doi:10.1155/2012/872875.
(17) Weber, M.; Trampczynska, A.; Clemens, S. Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd^{2+}-hypertolerant facultative metallophyte *Arabidopsis halleri*. *Plant, Cell & Environment* **2006,** *29* (5), 950-963.
(18) Farinati, S.; DalCorso, G.; Varotto, S.; Furini, A. The *Brassica juncea* BjCdR15, an ortholog of *Arabidopsis* TGA3, is a regulator of cadmium uptake, transport and accumulation in shoots and confers cadmium tolerance in transgenic plants. *New Phytologist* **2010,** *185* (4), 964-978.
(19) Fusco, N.; Micheletto, L.; Dal Corso, G.; Borgato, L.; Furini, A. Identification of cadmium-regulated genes by cDNA-AFLP in the heavy metal accumulator *Brassica juncea* L. *Journal of Experimental Botany* **2005,** *56* (421), 3017-3027.
(20) Gao, J.; Sun, L.; Yang, X.; Liu, J.-X. Transcriptomic analysis of cadmium stress response in the heavy metal hyperaccumulator *Sedum alfredii* Hance. *PloS one* **2013,** *8* (6), e64643.
(21) Halimaa, P.; Lin, Y.-F.; Ahonen, V. H.; Blande, D.; Clemens, S.; Gyenesei, A.; Häikiö, E.; Kärenlampi, S. O.; Laiho, A.; Aarts, M. G. Gene expression differences between *Noccaea caerulescens* ecotypes help to identify candidate genes for metal phytoremediation. *Environmental science & technology* **2014,** *48* (6), 3344-3353.
(22) Milner, M. J.; Mitani-Ueno, N.; Yamaji, N.; Yokosho, K.; Craft, E.; Fei, Z.; Ebbs, S.; Clemencia Zambrano, M.; Ma, J. F.; Kochian, L. V. Root and shoot transcriptome analysis of two ecotypes of *Noccaea caerulescens* uncovers the role of NcNramp1 in Cd hyperaccumulation. *The Plant Journal* **2014,** *78* (3), 398-410.
(23) Lin, Y.-F.; Severing, E. I.; te Lintel Hekkert, B.; Schijlen, E.; Aarts, M. G. A comprehensive set of transcript sequences of the heavy metal hyperaccumulator *Noccaea caerulescens*. *Frontiers in plant science* **2014,** *5* (261).
(24) Romero-Puertas, M. C.; Corpas, F. J.; Rodríguez-Serrano, M.; Gómez, M.; Luis, A.; Sandalio, L. M. Differential expression and regulation of antioxidative enzymes by cadmium in pea plants. *Journal of plant physiology* **2007,** *164* (10), 1346-1357.
(25) Tamás, L.; Dudíková, J.; Ďurčeková, K.; Halušková, L. u.; Huttová, J.; Mistrík, I.; Ollé, M. Alterations of the gene expression, lipid peroxidation, proline and thiol content along the barley root exposed to cadmium. *Journal of plant physiology* **2008,** *165* (11), 1193-1203.

(26) Cao, F.; Chen, F.; Sun, H.; Zhang, G.; Chen, Z. H.; Wu, F. Genome-wide transcriptome and functional analysis of two contrasting genotypes reveals key genes for cadmium tolerance in barley. *BMC Genomics* **2014,** *15* (1), 1.

(27) Zhang, M.; Liu, X.; Yuan, L.; Wu, K.; Duan, J.; Wang, X.; Yang, L. Transcriptional profiling in cadmium-treated rice seedling roots using suppressive subtractive hybridization. *Plant physiology and biochemistry* **2012,** *50*, 79-86.

(28) Martin, F.; Bovet, L.; Cordier, A.; Stanke, M.; Gunduz, I.; Peitsch, M. C.; Ivanov, N. V. Design of a tobacco exon array with application to investigate the differential cadmium accumulation property in two tobacco varieties. *BMC genomics* **2012,** *13* (1), 674.

(29) Liu, T.; Zhu, S.; Tang, Q.; Tang, S. Genome-wide transcriptomic profiling of ramie (*Boehmeria nivea* L. Gaud) in response to cadmium stress. *Gene* **2015,** *558* (1), 131-137.

(30) Verbruggen, N.; Hermans, C.; Schat, H. Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist* **2009,** *181* (4), 759-776.

(31) Yu, H.; Wang, J.; Fang, W.; Yuan, J.; Yang, Z. Cadmium accumulation in different rice cultivars and screening for pollution-safe cultivars of rice. *Science of the total environment* **2006,** *370* (2), 302-309.

(32) Zhu, Y.; Yu, H.; Wang, J.; Fang, W.; Yuan, J.; Yang, Z. Heavy metal accumulations of 24 asparagus bean cultivars grown in soil contaminated with Cd alone and with multiple metals (Cd, Pb, and Zn). *Journal of Agricultural and Food Chemistry* **2007,** *55* (3), 1045-1052.

(33) Reeves, P. G.; Chaney, R. L. Bioavailability as an issue in risk assessment and management of food cadmium: A review. *Science of the Total Environment* **2008,** *398* (1), 13-19.

(34) Salt, D. E.; Blaylock, M.; Kumar, N. P.; Dushenkov, V.; Ensley, B. D.; Chet, I.; Raskin, I. Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. *Nature biotechnology* **1995,** *13* (5), 468-474.

(35) Mori, S.; Uraguchi, S.; Ishikawa, S.; Arao, T. Xylem loading process is a critical factor in determining Cd accumulation in the shoots of *Solanum melongena* and *Solanum torvum*. *Environmental and Experimental Botany* **2009,** *67* (1), 127-132.

(36) Yamaguchi, H.; Fukuoka, H.; Arao, T.; Ohyama, A.; Nunome, T.; Miyatake, K.; Negoro, S. Gene expression analysis in cadmium-stressed roots of a low cadmium-accumulating solanaceous plant, *Solanum torvum*. *Journal of experimental botany* **2010,** *61* (2), 423-437.

(37) Salt, D. E.; Prince, R. C.; Pickering, I. J.; Raskin, I. Mechanisms of cadmium mobility and accumulation in Indian mustard. *Plant Physiology* **1995,** *109* (4), 1427-1433.

(38) Xue, M.; Zhou, Y.; Yang, Z.; Lin, B.; Yuan, J.; Wu, S. Comparisons in subcellular and biochemical behaviors of cadmium between low-Cd and high-Cd accumulation cultivars of pakchoi (*Brassica chinensis* L.). *Frontiers of Environmental Science & Engineering* **2014,** *8* (2), 226-238.

(39) Grabherr, M. G.; Haas, B. J.; Yassour, M.; Levin, J. Z.; Thompson, D. A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature biotechnology* **2011,** *29* (7), 644-652.

(40) Li, R.; Yu, C.; Li, Y.; Lam, T.-W.; Yiu, S.-M.; Kristiansen, K.; Wang, J. SOAP2: an improved ultrafast tool for short read alignment. *Bioinformatics* **2009,** *25* (15), 1966-1967.

(41) Rajkumar, A. P.; Qvist, P.; Lazarus, R.; Lescai, F.; Ju, J.; Nyegaard, M.; Mors, O.; Børglum, A. D.; Li, Q.; Christensen, J. H. Experimental validation of methods for differential gene expression analysis and

sample pooling in RNA-seq. *BMC genomics* **2015,** *16* (1), 1.
(42) Audic, S.; Claverie, J.-M. The significance of digital gene expression profiles. *Genome research* **1997,** *7* (10), 986-995.
(43) Ernst, J.; Bar-Joseph, Z. STEM: a tool for the analysis of short time series gene expression data. *BMC bioinformatics* **2006,** *7* (1), 191.
(44) Untergrasser, A.; Koressaar, T.; Ye, J.; Faircloth, B.; Remm, M.; Rozen, S. 582 2012. Primer3-new capabilities and interfaces. *Nucleic Acids Research 40*, e115.
(45) Xing, J.; Jiang, R.; Ueno, D.; Ma, J.; Schat, H.; McGrath, S.; Zhao, F. Variation in root-to-shoot translocation of cadmium and zinc among different accessions of the hyperaccumulators *Thlaspi caerulescens* and *Thlaspi praecox*. *New Phytologist* **2008,** *178* (2), 315-325.
(46) Conesa, A.; Götz, S.; García-Gómez, J. M.; Terol, J.; Talón, M.; Robles, M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **2005,** *21* (18), 3674-3676.
(47) Ye, J.; Fang, L.; Zheng, H.; Zhang, Y.; Chen, J.; Zhang, Z.; Wang, J.; Li, S.; Li, R.; Bolund, L. WEGO: a web tool for plotting GO annotations. *Nucleic acids research* **2006,** *34* (suppl 2), W293-W297.
(48) Wang, J.; Yuan, J.; Yang, Z.; Huang, B.; Zhou, Y.; Xin, J.; Gong, Y.; Yu, H. Variation in cadmium accumulation among 30 cultivars and cadmium subcellular distribution in 2 selected cultivars of water spinach (*Ipomoea aquatica* Forsk.). *Journal of agricultural and food chemistry* **2009,** *57* (19), 8942-8949.
(49) Qiu, Q.; Wang, Y.; Yang, Z.; Xin, J.; Yuan, J.; Wang, J.; Xin, G. Responses of Different Chinese Flowering Cabbage (*Brassica parachinensis* L.) Cultivars to Cadmium and Lead Exposure: Screening for Cd+ Pb Pollution-Safe Cultivars. *CLEAN–Soil, Air, Water* **2011,** *39* (11), 925-932.
(50) Li, J. C.; Guo, J. B.; Xu, W. Z.; Ma, M. RNA Interference-mediated Silencing of Phytochelatin Synthase Gene Reduce Cadmium Accumulation in Rice Seeds. *Journal of Integrative Plant Biology* **2007,** *49* (7), 1032-1037.
(51) Ishimaru, Y.; Takahashi, R.; Bashir, K.; Shimo, H.; Senoura, T.; Sugimoto, K.; Ono, K.; Yano, M.; Ishikawa, S.; Arao, T. Characterizing the role of rice NRAMP5 in manganese, iron and cadmium transport. *Scientific reports* **2012,** *2*, Article number: 286 (2012) doi:10.1038/srep00286.
(52) Garg, N.; Chandel, S. Effect of mycorrhizal inoculation on growth, nitrogen fixation, and nutrient uptake in *Cicer arietinum* (L.) under salt stress. *Turkish Journal of Agriculture and Forestry* **2011,** *35* (2), 205-214.
(53) Regier, N.; Baerlocher, L.; Münsterkötter, M.; Farinelli, L.; Cosio, C. Analysis of the Elodea nuttallii transcriptome in response to mercury and cadmium pollution: development of sensitive tools for rapid ecotoxicological testing. *Environmental science & technology* **2013,** *47* (15), 8825-8834.
(54) Zhang, L.; Yan, H.-F.; Wu, W.; Yu, H.; Ge, X.-J. Comparative transcriptome analysis and marker development of two closely related Primrose species (*Primula poissonii* and *Primula wilsonii*). *BMC genomics* **2013,** *14* (1), 1.
(55) De Vos, C. R.; Vonk, M. J.; Vooijs, R.; Schat, H. Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. *Plant Physiology* **1992,** *98* (3), 853-858.
(56) VAN DE MORTEL, J. E.; Schat, H.; Moerland, P. D.; VAN THEMAAT, E. V. L.; VAN DER ENT, S.; Blankestijn, H.; Ghandilyan, A.; Tsiatsiani, S.; AARTS, M. G. Expression differences for genes involved in

lignin, glutathione and sulphate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd-hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell & Environment* **2008,** *31* (3), 301-324.
(57) Carpita, N. C.; Gibeaut, D. M. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal* **1993,** *3* (1), 1-30.
(58) Mohnen, D. Biosynthesis of pectins and galactomannans. *Comprehensive natural products chemistry* **1999,** *3*, 497-527.
(59) Ridley, B. L.; O'Neill, M. A.; Mohnen, D. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* **2001,** *57* (6), 929-967.
(60) Kieffer, P.; Planchon, S.; Oufir, M.; Ziebel, J.; Dommes, J.; Hoffmann, L.; Hausman, J.-F.; Renaut, J. Combining proteomics and metabolite analyses to unravel cadmium stress-response in poplar leaves. *Journal of proteome research* **2008,** *8* (1), 400-417.
(61) Obst, J. R. Guaiacyl and syringyl lignin composition in hardwood cell components. *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood* **1982,** *36* (3), 143-152.
(62) Alvarez, S.; Berla, B. M.; Sheffield, J.; Cahoon, R. E.; Jez, J. M.; Hicks, L. M. Comprehensive analysis of the *Brassica juncea* root proteome in response to cadmium exposure by complementary proteomic approaches. *Proteomics* **2009,** *9* (9), 2419-2431.
(63) Sarry, J. E.; Kuhn, L.; Ducruix, C.; Lafaye, A.; Junot, C.; Hugouvieux, V.; Jourdain, A.; Bastien, O.; Fievet, J. B.; Vailhen, D. The early responses of *Arabidopsis thaliana* cells to cadmium exposure explored by protein and metabolite profiling analyses. *Proteomics* **2006,** *6* (7), 2180-2198.
(64) Semane, B.; Dupae, J.; Cuypers, A.; Noben, J.-P.; Tuomainen, M.; Tervahauta, A.; Kärenlampi, S.; Van Belleghem, F.; Smeets, K.; Vangronsveld, J. Leaf proteome responses of *Arabidopsis thaliana* exposed to mild cadmium stress. *Journal of plant physiology* **2010,** *167* (4), 247-254.
(65) Kieffer, P.; Schröder, P.; Dommes, J.; Hoffmann, L.; Renaut, J.; Hausman, J.-F. Proteomic and enzymatic response of poplar to cadmium stress. *Journal of proteomics* **2009,** *72* (3), 379-396.
(66) Clemens, S.; Kim, E. J.; Neumann, D.; Schroeder, J. I. Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *The EMBO Journal* **1999,** *18* (12), 3325-3333.
(67) Brunetti, P.; Zanella, L.; De Paolis, A.; Di Litta, D.; Cecchetti, V.; Falasca, G.; Barbieri, M.; Altamura, M. M.; Costantino, P.; Cardarelli, M. Cadmium-inducible expression of the ABC-type transporter AtABCC3 increases phytochelatin-mediated cadmium tolerance in *Arabidopsis*. *Journal of experimental botany* **2015,** *66* (13), 3815-3829.
(68) Von Zglinicki, T.; Edwall, C.; Ostlund, E.; Lind, B.; Nordberg, M.; Ringertz, N.; Wroblewski, J. Very low cadmium concentrations stimulate DNA synthesis and cell growth. *Journal of Cell Science* **1992,** *103* (4), 1073-1081.
(69) Guo, J.; Yang, X.; Weston, D. J.; Chen, J. G. Abscisic Acid Receptors: Past, Present and Future. *Journal of integrative plant biology* **2011,** *53* (6), 469-479.
(70) Umezawa, T.; Sugiyama, N.; Anderson, J. C.; Takahashi, F.; Ishihama, Y.; Peck, S. C.; Shinozaki, K., Protein Phosphorylation Network in Abscisic Acid Signaling. In *Plant and Microbe Adaptations to Cold in a Changing World*, Springer: 2013; pp 155-164.
(71) Vlad, F.; Rubio, S.; Rodrigues, A.; Sirichandra, C.; Belin, C.; Robert, N.; Leung, J.; Rodriguez, P. L.;

633 Laurière, C.; Merlot, S. Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by
634 abscisic acid in *Arabidopsis*. *The Plant Cell* **2009,** *21* (10), 3170-3184.
635 (72) Fujii, H.; Zhu, J.-K. *Arabidopsis* mutant deficient in 3 abscisic acid-activated protein kinases reveals
636 critical roles in growth, reproduction, and stress. *Proceedings of the National Academy of Sciences* **2009,**
637 *106* (20), 8380-8385.
638 (73) Kim, J.-S.; Mizoi, J.; Yoshida, T.; Fujita, Y.; Nakajima, J.; Ohori, T.; Todaka, D.; Nakashima, K.;
639 Hirayama, T.; Shinozaki, K. An ABRE promoter sequence is involved in osmotic stress-responsive
640 expression of the DREB2A gene, which encodes a transcription factor regulating drought-inducible genes
641 in *Arabidopsis*. *Plant and Cell Physiology* **2011,** *52* (12), 2136-2146.
642 (74) Clemens, S. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants.
643 *Biochimie* **2006,** *88* (11), 1707-1719.
644 (75) Kim, D. Y.; Bovet, L.; Maeshima, M.; Martinoia, E.; Lee, Y. The ABC transporter AtPDR8 is a
645 cadmium extrusion pump conferring heavy metal resistance. *The Plant Journal* **2007,** *50* (2), 207-218.
646 (76) Jean, M. L.; Schikora, A.; Mari, S.; Briat, J. F.; Curie, C. A loss-of-function mutation in AtYSL1
647 reveals its role in iron and nicotianamine seed loading. *The Plant Journal* **2005,** *44* (5), 769-782.
648 (77) Guerinot, M. L. The ZIP family of metal transporters. *Biochimica et Biophysica Acta*
649 *(BBA)-Biomembranes* **2000,** *1465* (1), 190-198.
650 (78) Connolly, E. L.; Fett, J. P.; Guerinot, M. L. Expression of the IRT1 metal transporter is controlled by
651 metals at the levels of transcript and protein accumulation. *The Plant Cell* **2002,** *14* (6), 1347-1357.
652 (79) Lin, Y. F.; Liang, H. M.; Yang, S. Y.; Boch, A.; Clemens, S.; Chen, C. C.; Wu, J. F.; Huang, J. L.; Yeh,
653 K. C. *Arabidopsis* IRT3 is a zinc-regulated and plasma membrane localized zinc/iron transporter. *New*
654 *Phytologist* **2009,** *182* (2), 392-404.
655

656 Table 1. Sequencing and assembly statistics for the six transcriptome data of two pakchoi
657 genotypes at three time stages of Cd treatment.

Sample ID		No. of reads ($\times 10^6$)	No. of basepairs ($\times 10^9$)	No. of mapped reads ($\times 10^6$)	Mapped percentage (%)
HAJS	T0	26.92	3.36	14.05	52.21
	T3	33.16	4.15	13.95	42.06
	T24	36.59	4.57	15.68	42.85
LAJK	T0	30.19	3.77	15.31	50.71

T3	24.64	3.08	12.43	50.45
T24	25.93	3.24	12.85	49.55

No. is short for number.

Figure Captions:

Figure 1. Shoot Cd concentrations in LAJK (blue bars) and HASJ (red bars) at T0, T3 and T24. Different small letters indicate significant differences at $p < 0.05$ level of LSD test between two genotypes at the same time point. Different capital letters indicate significant differences at $p < 0.05$ level among different time points in the same genotype.

Figure 2. Correlation between qPCR and RNA sequencing for the eight selected genes. Each point represents a value of fold change of expression level at T3 or T24 comparing with that at T0 or T3. Fold-change values were $\log_{10}$ transformed.

Figure 3. Patterns of gene expressions and GO enrichment across three time points in LAJK and HAJS. A. Patterns of gene expressions across three time points in LAJK and HAJS inferred by STEM analysis. In each frame, the light grey lines represented the expression pattern of each gene, while the black line represented the expression tendency of all the genes. The number of genes belonging to each pattern was labeled above the frame. B. Gene Ontology (GO) enrichment analysis of three significant clusters in LAJK.

676 C. Gene Ontology (GO) enrichment analysis of three significant clusters in HAJS. The
677 significance of the most represented GO-slims in each main cluster is indicated by
678 *p*-value. The red areas represented the significant *p*-values, while the dark grey
679 represented the non-significant values.

680

681 **Figure 4.** Transcriptional changes of genes responsible for Cd tolerance in roots of the
682 two pakchoi genotypes. The metabolites, transporter proteins and transcriptional factors
683 in response to Cd are represented in orange boxes, while the other metabolitesarein gray
684 boxes. For enzymes reactions, the arrows between two metabolites represented the
685 directions of catalytic reactions. The name (s) and expression pattern over the three time
686 points in both two genotypes of the genes encoding corresponding enzyme (s) are given
687 above or under the arrow. Hash arrows represented multiple enzyme reactions, which
688 were no concerned in this study. For transporters, the arrows cross the orange boxes
689 represented the directions of Cd transport. For transcriptional factors, the arrows pointed
690 to the products of transcriptions.

691

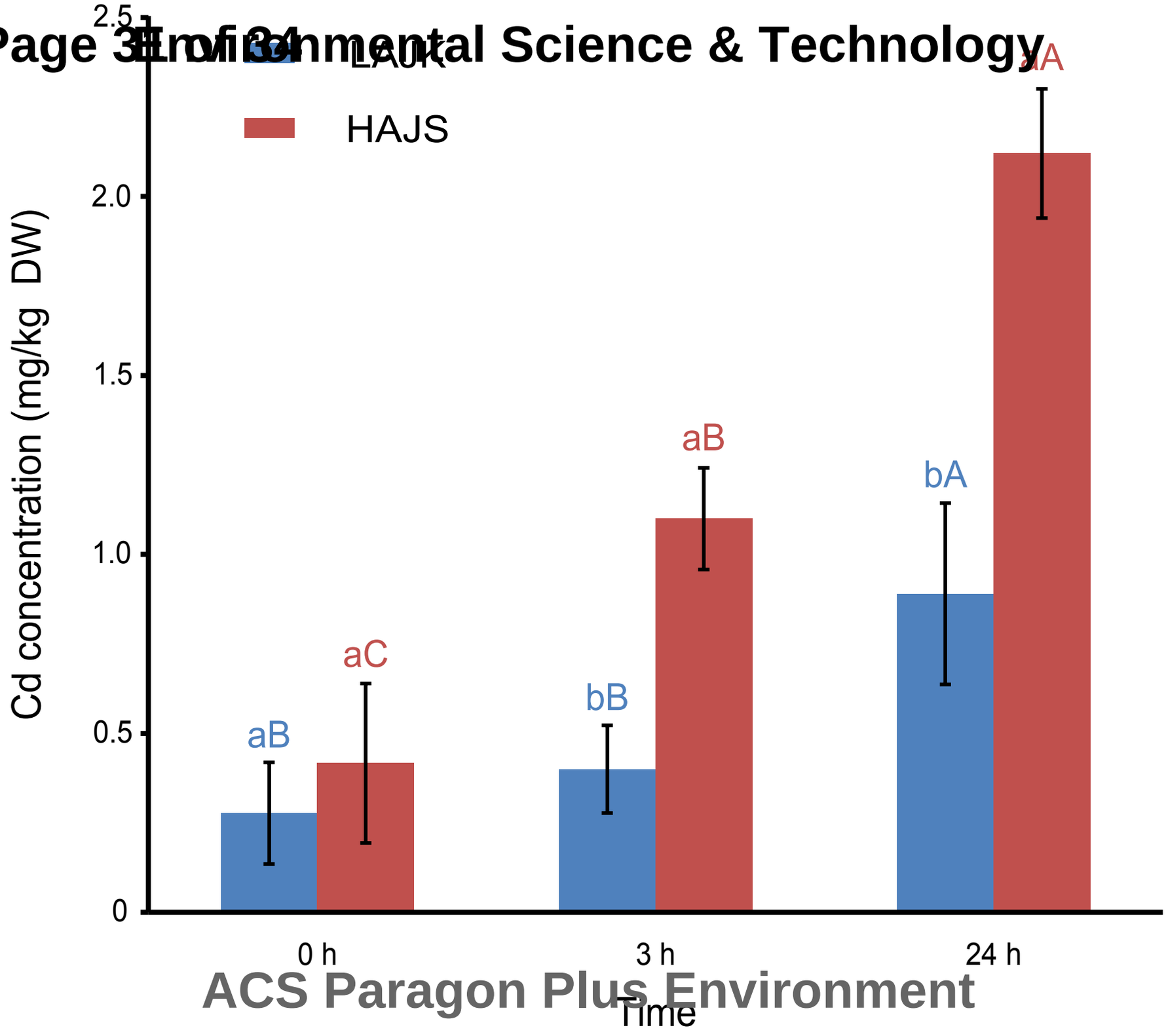


Figure 1

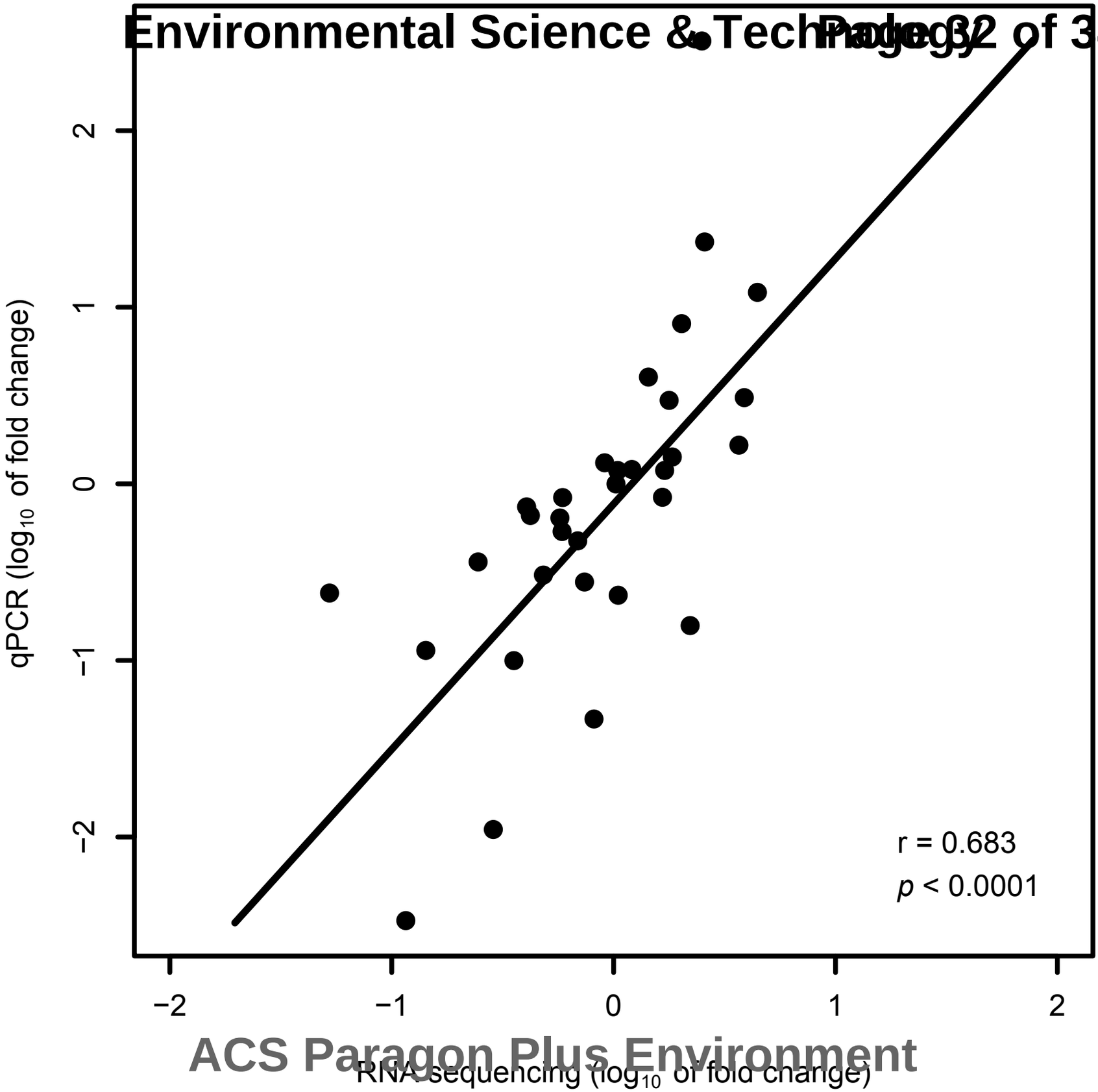


Figure 2

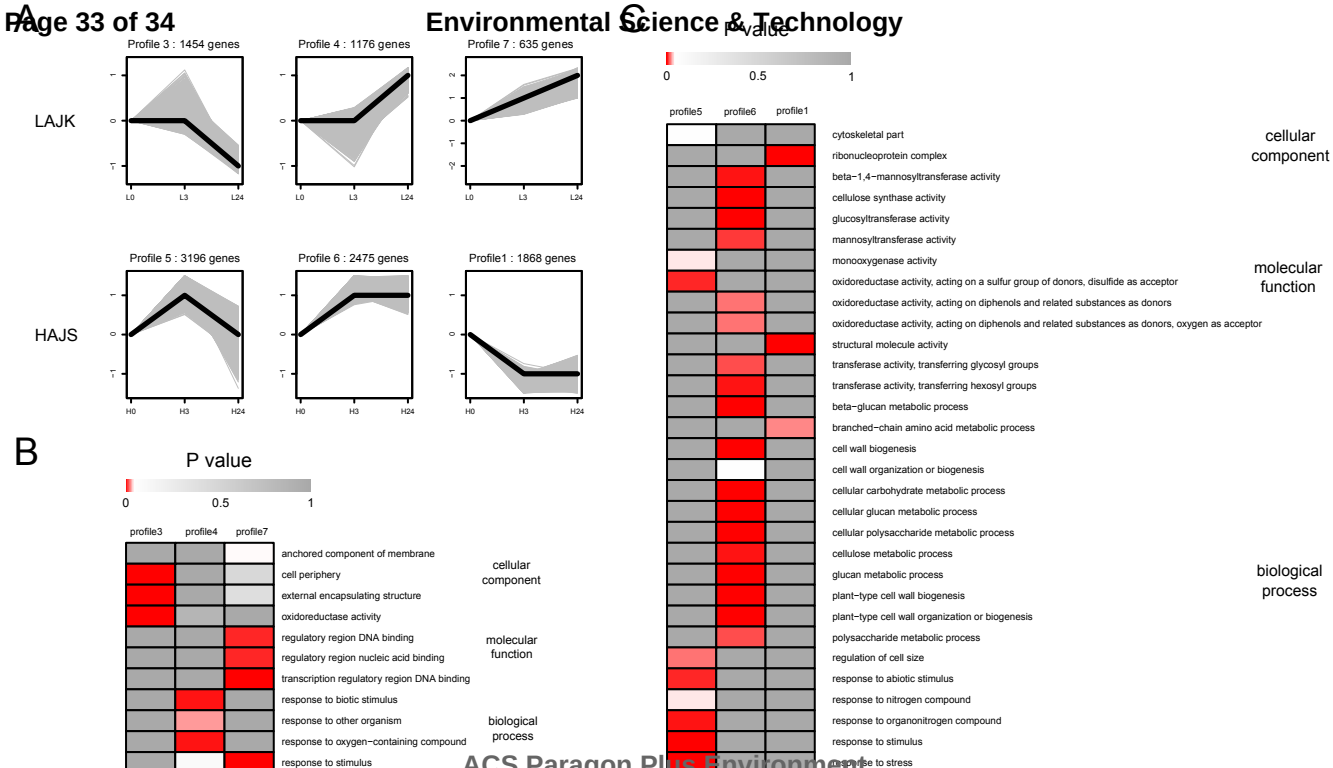
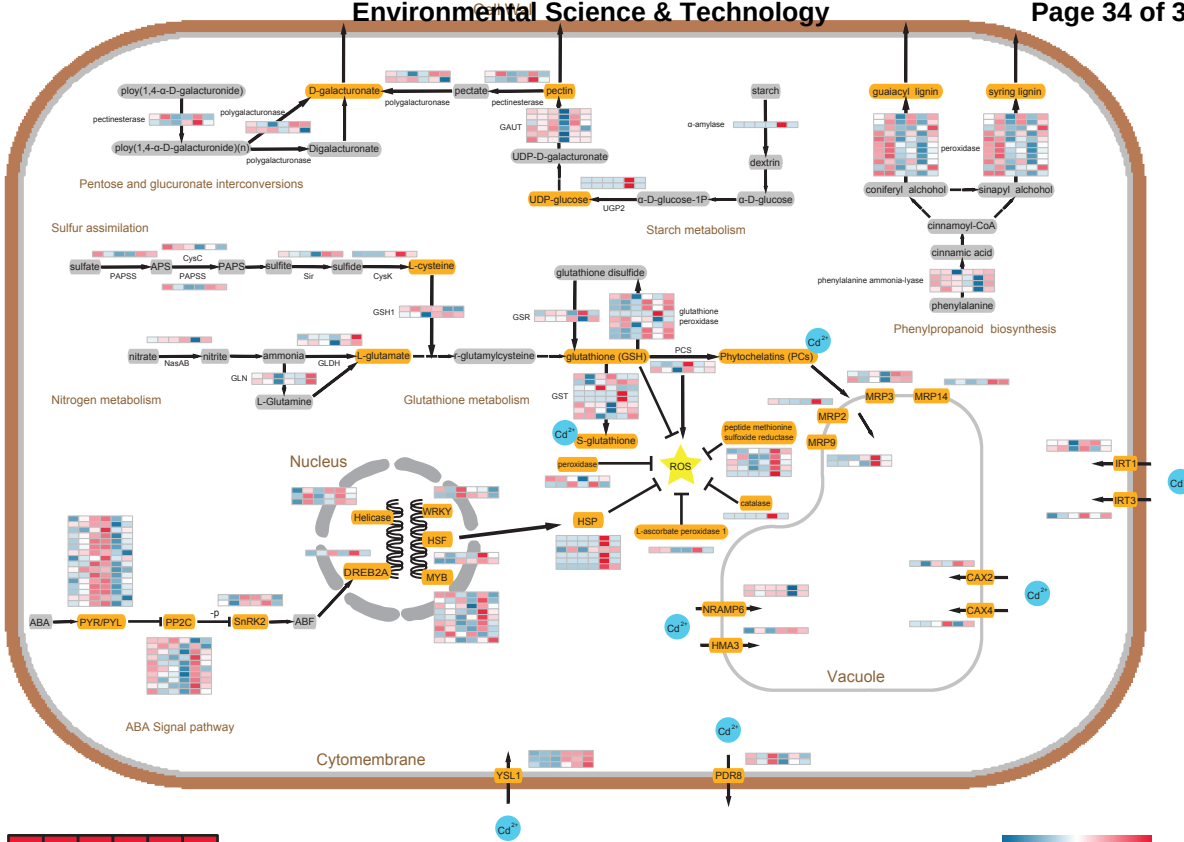
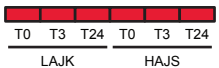


Figure 3





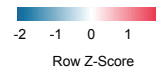


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Figure 4