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TRANSCRIPTOME ANALYSIS OF THE RESPONSES INVOLVED IN THE REGULATION OF CADMIUM STRESS BY EXOGENOUS HYDROGEN IN RICE (*ORYZA SATIVA*)

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Abstract

Cadmium is a major threat to agricultural production and human health. Exogenous hydrogen plays an important role in plant stress resistance through a mechanism that remains unknown. To elucidate this mechanism, this study investigated the effect of exogenous hydrogen on rice (*Oryza sativa*) gene expression under cadmium stress. Four libraries of the super hybrid rice LYP9 were sequenced through high-throughput sequencing technology. Approximately 13 GB of unique sequences were obtained. Comparative analysis identified 1329 differentially expressed genes in rice with normal growth exposed to exogenous hydrogen and 1217 differentially expressed genes in rice subjected to cadmium stress. The analyses of the functional enrichment and metabolic pathways of the differentially expressed genes revealed that exogenous hydrogen down-regulates the expression levels of stress-related genes under non-stress conditions and up-regulates the expression levels of stress-related genes under cadmium stress. Under non-stress conditions, exogenous hydrogen promotes the expression of genes related to photosynthesis and energy metabolism. Under cadmium stress, exogenous hydrogen down-regulates the expression of energy-metabolism-related genes while inhibiting rice growth by down-regulating the expression of cadmium-transporter-related genes. The latter effect prevents the absorption of cadmium by roots and increases the resistance of rice to cadmium toxicity. This study provides an experimental basis for the in-depth study of the mechanism underlying cadmium tolerance in rice.

Key words: Rice, Cadmium stress; Hydrogen; High-throughput sequencing; Transcriptome.

Introduction

Rapid industrialization and socialization have resulted in soil and water pollution by heavy metals. Heavy-metal pollution has consequently become a major threat against human health and sustainable social development (Emamverdian & Ding, 2018). Cadmium is one of the most toxic heavy metals and has a strong chemical activity in the soil. It can be absorbed easily by plants. Rice (*Oryza sativa*) and corn (*Zea mays*) are the primary cereal crops susceptible to cadmium absorption (Liu *et al.*, 2006; Yu *et al.*, 2006). Cadmium is enriched in the human body through the food chain and exerts adverse effects on human health (Imtiaz *et al.*, 2010).

Rice is one of the most important food crops worldwide and accounts for approximately 40% of China's total grain output. More than 65% of the country's population consume rice. However, rice is highly susceptible to cadmium contamination. Cadmium damage results in a series of symptoms that ultimately decrease rice quality (Wang, 1996; Huang *et al.*, 2007; Bai *et al.*, 2011). Thus, the mechanism of cadmium uptake and accumulation in rice must be elucidated to breed cadmium-resistant rice varieties and ensure food security.

Similar to other signal transduction molecules, such as NO, CO, and H_2S, hydrogen may possess a signal conditioning function and is thus known as "the fourth signaling gas molecule" (Atsunori *et al.*, 2009). The saturated solution of hydrogen in physiological saline can effectively replace the physiological role of hydrogen in living organisms (George & Agarwal, 2010). Hydrogen can protect cells by selectively reducing hydroxyl radicals and reactive oxygen species (Ohsawa *et al.*, 2007). It can also affect seed germination and crop growth (Giumarro & Siegel, 1964) and can enhance the paraquat-induced oxygen stress tolerance of *Medicago sativa* by regulating the heme oxygenase-1 signaling system (Jin *et al.*, 2013). Hydrogen enhances the salt tolerance of *Arabidopsis thaliana* by improving its antioxidant mechanism. In addition, salt stress can accelerate hydrogen production in *Arabidopsis* seedlings (Xie *et al.*, 2012). ABA can trigger rapid and sustained hydrogen release from *A. thaliana* by reducing the sizes of stomatal apertures, thus increasing drought resistance (Xie *et al.*, 2014). Hydrogen can enhance the plant tolerance to toxic substances, reduce heavy-metal accumulation in plants, diminish toxic effects on plants, alleviate the oxidative damage caused by heavy metals to cells, and maintain plant survival under the stress exerted by toxic substances (Chen *et al.*, 2014; Cui *et al.*, 2014). Expanding the knowledge on the molecular mechanisms that underlie the participation of hydrogen in plant resistance to metal stress can provide new insights into the molecular mechanisms of plant resistance to heavy metals. In addition, the application of exogenous hydrogen may increase the ability of plants to resist toxic substances and tolerate heavy metals.

Materials and Methods

Plant growth and treatment: The super hybrid rice LYP9 was used as the test plant. Healthy and plump seeds were selected, cleaned for 5 min with 0.1% H_2O_2, soaked for 48 h in water at 30°C, and germinated for 24 h in a constant temperature incubator at 35°C. Seeds showing the same degree of germination were sown in porcelain plates filled with vermiculite. After 7 days, seedlings showing the same vigorous growth were transplanted into a nutrient pot containing Hoagland's culture solution (pH 4.5–5.5),

exposed to light for 12 h per day (photon flux=100μmol^{-2} s^{-1}), and cultured at 28℃/22℃ (day/night). The hydrogen-rich solution was prepared as follows: The H_2 source was a 99.99% (v/v) hydrogen gas generator (instrument model number) fed with 1000 mL of Hoagland treatment solution (pH 4.5–5.5, 25℃) at a rate of 150 mL.min^{-1} for 60min. The freshly prepared hydrogen-rich solution contained 0.22 mmol/L H_2 and was maintained at 25℃ for 12 h. Rice plants were subjected to the following treatments upon reaching the three-leaf stage: (1) 0 μmol/L $CdCl_2$, 0 mmol/L H_2; (2)0 μmol/L $CdCl_2$, 0.22 mmol/L H_2; (3)5.0 μmol/L $CdCl_2$, 0 mmol/L H_2; and (4) 5.0 μmol/L $CdCl_2$, 0.22 mmol/L H_2. The plants were maintained under the above lighting conditions for 7 days. The leaves of the same part of different seedlings were collected for RNA extraction and pooled as a mixed sample. The mixture was snap-frozen in liquid nitrogen and stored at −80°C until being used.

RNA library construction and sequencing: After total RNA extraction and DNA digestion with DNase, mRNA was enriched with magnetic beads containing oligo (dT). Interrupting reagents were added to break the mRNA into short fragments, and the interrupted mRNA was used as the template. One-stranded cDNA was synthesized using six-base random primers. Then, a two-stranded synthesis reaction system was used to synthesize double-stranded cDNA. Double-stranded cDNA was purified using a kit. The purified double-stranded cDNA was further subjected to terminal repair with the addition of an A-tail and a sequencing adapter. Afterward, fragments were selected on the basis of size, and PCR amplification was performed. The constructed library was qualified by using an Agilent 2100 Bio-analyzer and then sequenced by using an IlluminaHiSeqTM 2500 sequencer to yield 150 bp of double-ended data.

Sequencing data assembly: Raw data were filtered to remove linker sequences and low-quality reads and to obtain high-quality clean data. The sequence was assembled with Trinity (http://trinityrnaseq.sourceforge.net/). Bowtie2 was used to align the clean reads to the reference genome and reference genes in rice.

Unigene functional annotation: Functional annotation was performed on the basis of gene similarity. The BLAST comparison tool was used to compare sample gene sequences with sequences from Nr, Swiss-Prot, KEGG, COG, and GO databases.

Differential gene screening and functional prediction analysis: Gene expression was calculated through the Fragments Per kb Per Million Reads (FPKM) method (Trapnell *et al.*, 2010), i.e., the number of fragments per kilo base length from a gene per million fragments. FPKM considers the influence of sequencing depth and gene length on the fragment count. At present, it is the most commonly used method for the estimation of gene expression. When calculating differences in gene expression, htseq-count software was used to obtain the number of reads that fell under each sample. Data were normalized by using the Estimate Size Factors function of DESeq (2012) R package (Anders, 2012). P and fold-change values were calculated by using the nbinom Test Function for the comparison of differences in gene expression. Genes with differential expression at the significance level of $p<0.05$ were selected for GO and KEGG enrichment analysis. Transcripts with differential expression at the significance level of $p<0.05$ and fold-change>2 or fold-change <0.5 were selected. The GO and KEGG enrichment analysis of differential transcripts were performed to identify the biological function or pathway, mainly affected by differential gene expression.

Results and Analysis

Sequencing results and assembly: A total of 15 Gb of clean data were obtained for the four libraries. Approximately 13 Gb of sequences were mapped to the genome. Approximately 13 Gb sequences had a unique alignment position on the reference sequence. Each sample had clean data of 3.8 Gb and Q30 base percentage of over 94.65%. The uniquely mapped sequence of the control and hydrogen-treated groups reached more than 84%, whereas those of the cadmium-treated group and the composite-treated group were below 70%. These results suggest that gene expression in rice may decrease after exposure to cadmium stress (Table 1).

Analysis of differentially expressed genes: Compared with the hydrogen treatment (HL) group, the control (CL) group had 1329 differentially expressed genes, of which 730 were up-regulated, and 599 were down-regulated. Compared with the HR group, the CR group had 1217 differentially expressed genes, of which 604 were up-regulated, and 613 were down-regulated (Fig. 1).

Calculations for several high-level differentially expressed genes in the CL group vs. HL group and CR group vs. HR groups are shown in Tables 2 and 3. For example, LOC_Os07g05360 encodes for a predicted 10kDa PSII polypeptide chain that functions to assist protein transport across the thylakoid membrane(Webber *et al.*, 1989). The up-regulated expression of this gene in the HL group indicates that hydrogen may affect photosynthesis in rice. LOC_Os10g37340 encodes for a cystathionine gamma-synthase that has an important role in the regulation of cellular methionine metabolism (Hacham *et al.*, 2002).The down-regulated expression of this gene suggests that exogenous hydrogen may inhibit the cellular metabolism of specific amino acids. LOC_Os01g73200, LOC_Os07g48020, and LOC_Os07g48010 encode for peroxidases. The down-regulation of these three genes in the HL group indicates that exogenous hydrogen treatment may induce oxidative stress in rice roots.

Table 1. The mapping information of the sequencing reads.

Sample[a]	CL	CR	HL	HR
Total reads	39066186	38174470	38050832	42144592
Total mapped	34430451(88.13%)	27158747(71.14%)	33610838(88.33%)	28932441(68.65%)
Multiple mapped	1428897(3.66%)	1083029(2.84%)	1361362(3.58%)	1098689(2.61%)
Uniquely mapped	33001554(84.48%)	26075718(68.31%)	32249476(84.75%)	27833752(66.04%)

[a]CL, control group; CR, Cd treatment group; HL, H2 treatment group; HR, Cd and H2 treatments group

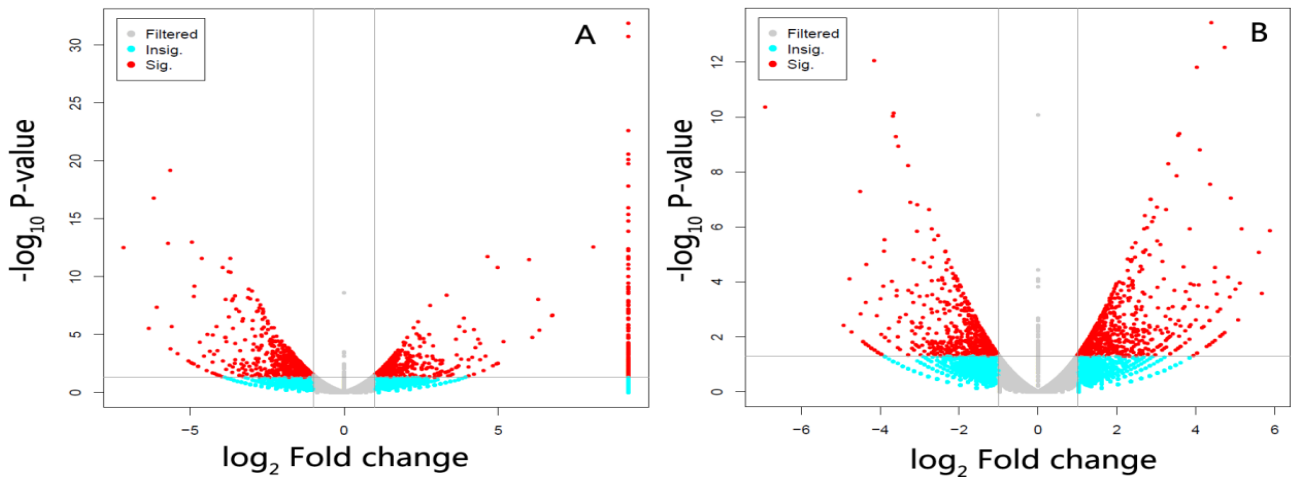

Fig. 1. The volcano plot of differentially expressed genes.
A, comparision of CL and HL; B, comparision of CR and HR

Table 2. List of some differentially expressed genes and their annotations in CL vs. HL group.

ID	CL	HL	log2FC[a]	pval	Annotation
LOC_Os04g38600	245.91	514.01	1.06	0.037	glyceraldehyde-3-phosphate dehydrogenase
LOC_Os10g18340	200.24	499.68	1.32	0.003	uncharacterized protein
LOC_Os12g33130	178.09	434.99	1.29	0.004	uncharacterized protein
LOC_Os02g47510	169.49	402.19	1.25	0.008	9-cis-epoxycarotenoid dioxygenase 1
LOC_Os12g19470	129.06	343.61	1.41	0.005	ribulosebisphosphate carboxylase small chain
LOC_Os01g08700	110.70	236.82	1.10	0.034	GIGANTEA
LOC_Os07g05360	88.72	250.39	1.50	0.001	photosystem II 10 kDa polypeptide
LOC_Os08g39430	85.17	215.95	1.34	0.001	thylakoid lumenal 19 kDa protein
LOC_Os01g59080	70.25	162.75	1.21	0.006	uncharacterized protein
LOC_Os01g22980	70.12	146.46	1.06	0.015	OsSCP3
LOC_Os01g03310	1289.03	421.15	-1.53	0.004	BBTI1
LOC_Os12g38170	844.31	218.13	-1.86	0.000	osmotin
LOC_Os01g21250	800.27	384.37	-1.02	0.033	late embryogenesis abundant protein
LOC_Os01g03340	741.71	100.40	-2.79	0.000	BBTI4
LOC_Os01g03320	732.50	125.85	-2.48	0.000	BBTI2
LOC_Os05g15770	638.79	233.27	-1.37	0.008	glycosyl hydrolase
LOC_Os01g73200	618.64	137.35	-2.06	0.000	peroxidase
LOC_Os07g48020	523.07	111.27	-2.14	0.000	peroxidase
LOC_Os10g37340	513.37	55.74	-3.11	0.000	cystathionine gamma-synthase
LOC_Os07g48010	506.47	157.82	-1.58	0.002	peroxidase

a, FC, fold change

In the CR group vs. HR group, LOC_Os01g71830, LOC_Os11g47560, and LOC_Os05g15880 encode for glycosyl hydrolase. The up-regulated expression of these genes suggest that the addition of exogenous hydrogen promotes the hydrolysis of polysaccharides under cadmium stress. LOC_Os02g38920 encodes for a glyceraldehyde-3-phosphate dehydrogenase, which is an important enzyme that is involved in glycolysis and in numerous subcellular activities, such as protein phosphorylation, membrane fusion, and transport. Moreover, this enzyme promotes apoptosis, regulates protein expression, participates in DNA damage repair, and acts as a transferrin receptor (Fu & Huang, 2013). The down-regulated expression of this gene indicates that the energy metabolism of rice cells may be affected by exposure to exogenous hydrogen under cadmium stress.

GO annotation of differentially expressed genes: GO functional annotation analysis was performed to identify the function of differentially expressed genes in the four libraries. Figure 2 shows that in the CL group vs. HL group, the genes related to the metabolic process, the response to biotic/abiotic stimulus, and the response to stress in the biological process subset are enriched. Further analysis revealed that these significantly enriched genes are mainly down-regulated. In the cellular component subset, most genes are enriched in plastids and thylakoids and are mainly up-regulated. In the subset of molecular function, the genes related to catalytic activity and oxygen binding are enriched. In the CR group vs. HR group, the genes related to the response to stress in the biological process subset are enriched and are mainly up-regulated. In the cellular component subset, most genes are enriched in plastids and thylakoids and are mainly down-regulated. In the subset of molecular function, genes related to catalytic activity and oxygen binding are enriched and are mostly down-regulated.

Table 3. List of some differentially expressed genes and their annotations in CR vs. HR group.

ID	CR	HR	log2FC[a]	pval	Annotation
LOC_Os01g42860	888.77	3043.95	1.78	0.011	inhibitor I family protein
LOC_Os03g52390	374.00	707.77	1.06	0.049	PIII1
LOC_Os04g50970	339.31	667.55	1.22	0.037	seed specific protein Bn15D1B
LOC_Os01g71830	270.70	533.49	1.30	0.047	glycosyl hydrolases family 17
LOC_Os11g47560	222.26	518.71	1.58	0.009	glycosyl hydrolase
LOC_Os05g15880	206.31	627.38	1.92	0.004	glycosyl hydrolase
LOC_Os01g03360	204.98	708.97	2.07	0.002	BBTI5
LOC_Os03g46060	192.75	405.98	1.40	0.010	thaumatin family domain containing protein
LOC_Os08g10500	168.89	385.99	1.39	0.017	uncharacterized protein
LOC_Os01g04620	168.74	379.24	1.46	0.027	transposon protein
LOC_Os02g37190	1852.68	335.35	-2.47	0.003	uncharacterized protein
LOC_Os11g24140	883.20	81.68	-3.43	0.000	plastocyanin-like domain containing protein
LOC_Os07g18750	633.56	106.78	-2.57	0.000	LTPL42
LOC_Os07g01410	629.52	201.74	-1.64	0.036	peroxidase precursor
LOC_Os02g55890	585.18	145.83	-2.00	0.044	inorganic H+ pyrophosphatase
LOC_Os02g38920	542.39	120.48	-2.17	0.010	glyceraldehyde-3-phosphate dehydrogenase
LOC_Os01g68740	465.69	124.09	-1.91	0.003	keratin, type I cytoskeletal 9
LOC_Os03g01700	441.99	129.40	-1.77	0.024	uncharacterized protein
LOC_Os03g12290	389.44	127.29	-1.61	0.040	glutamine synthetase
LOC_Os04g55600	346.36	148.68	-1.22	0.048	uncharacterized protein

a, FC, fold change

KEGG annotation of differentially expressed genes: The KEGG database was used to analyze the pathways of the differentially expressed genes to understand their regulatory information. Differentially expressed genes in the CL group vs. HL group are ascribed to160 metabolic pathways, including ribosome, phenylpropanoid biosynthesis, carbon metabolism, and plant hormone signal. The genes of the four metabolic pathways are significantly enriched. Differentially expressed genes in the CR group vs. HR group are attributed to 149 metabolic pathways, including glycolysis/ gluconeogenesis, carbon metabolism, phenylpropanoid biosynthesis, and cysteine and methionine metabolism. The genes of these metabolic pathways are significantly enriched (Fig. 3).

Discussion

The level of gene expression in organisms changes under stress. High-throughput transcriptomic sequencing is an effective approach for understanding these gene changes. In this study, we investigated whether exogenous hydrogen can regulate the response of rice to cadmium stress. We constructed four cDNA libraries for high-throughput transcriptomic sequencing. We obtained more than 15 Gb of raw data, of which 12 Gb comprised unique sequences. This result indicates that sequencing coverage is good. Compared with that under the control treatment, 1329 genes are differentially expressed after exogenous hydrogen treatment, among which 730 are up-regulated. Compared with that under cadmium stress, 1217 genes are differentially expressed (including 604 with up-regulated expression) under exogenous hydrogen treatment. GO functional analysis revealed that stress-related genes are significantly enriched after exogenous hydrogen treatment. Interestingly, most of these genes are down-regulated. In general, the expression of stress-related genes in plants will be up-regulated to respond to stress (Shinozaki & Yamaguchi-Shinozaki, 1997; Xiong *et al.*, 2002; Chinnusamy *et al.*, 2007). In rice seedlings, the expression levels of stress-related genes are down-regulated after exogenous hydrogen treatment. This finding suggests that in rice under non-stress conditions, hydrogen does not activate the stress signal transduction pathway but rather induces the low-level expression of stress-related genes. Further analysis revealed that genes associated with photosynthesis, precursor metabolite generation, and energy were up-regulated in rice grown under normal conditions (data not shown). These genes are mostly distributed in organelles, such as plastids and thylakoids. This result indicates that in rice grown under normal conditions, exogenous hydrogen decreases the expression level of stress-related genes while promoting growth by up-regulating the expression of genes related to photosynthesis and energy metabolism. By contrast, in the CR group, stress-related genes are significantly enriched after exogenous hydrogen treatment. Most of these genes are up-regulated, suggesting that hydrogen further increases the expression level of stress-related genes in response to cadmium stress. Similarly, under cadmium stress, the genes associated with metabolic process and cell cycle are down-regulated after hydrogen treatment. This expression pattern suggests that hydrogen treatment can reduce the metabolism and growth of rice cells under cadmium stress. These effects improve the resistance of rice to cadmium stress.

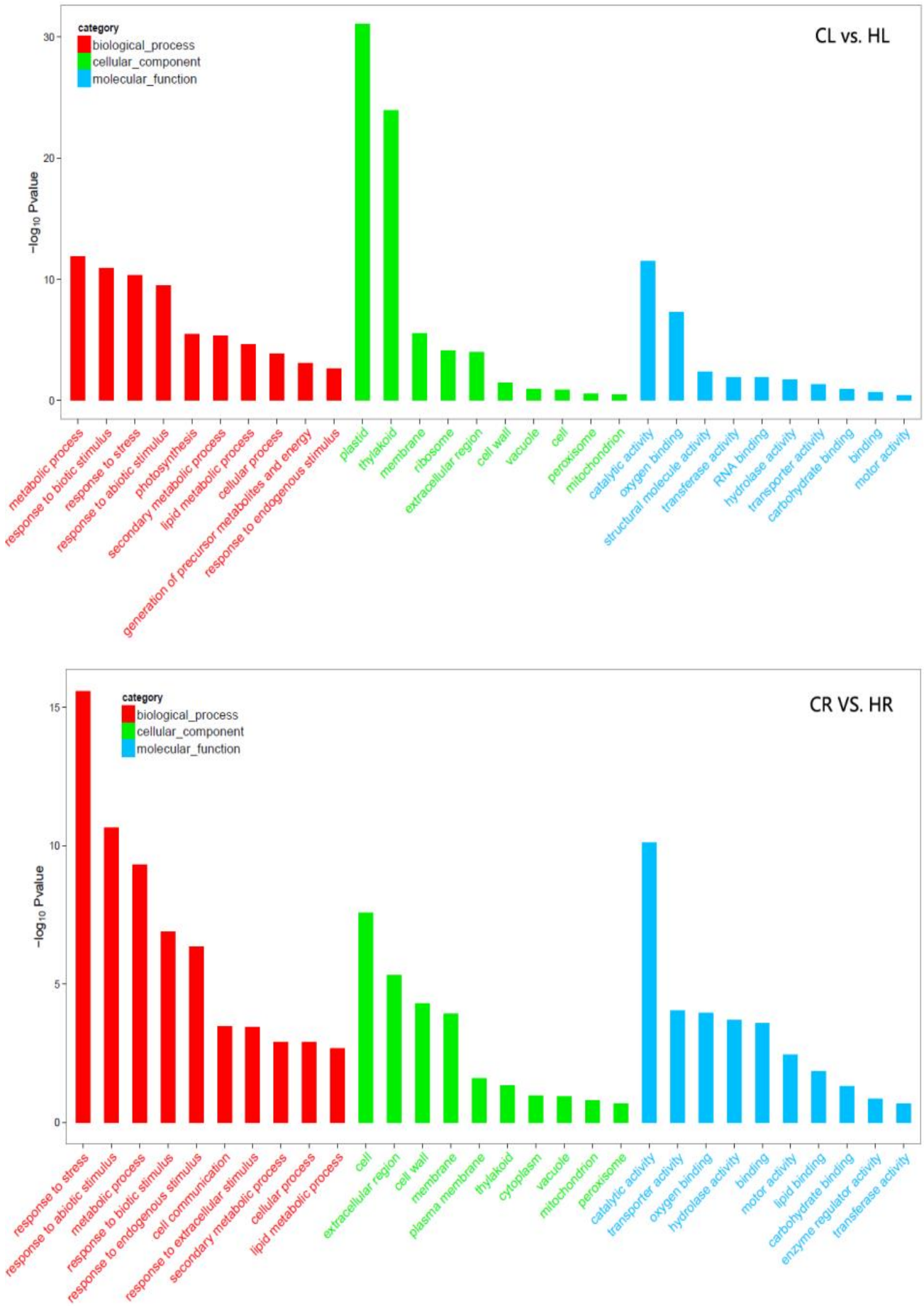

Fig. 2. GO functional annotations of differentially expressed genes.

Plant glycosyl hydrolase (GH) likely is likely important role in plant biological activities by involvement in plant growth and development and in various signal transduction processes in response to environmental stimuli (Barleben *et al.*, 2005). We identified several genes that encoded for GH. Moreover, we found that in rice grown under normal conditions, the expression level of GH decreased after exposure to exogenous hydrogen. By contrast, in rice under cadmium stress, the expression level of GH increases after exposure to exogenous hydrogen. We speculated that under adverse stress, exogenous hydrogen may promote the hydrolysis of sugar chains by increasing the expression of GH genes. This results in the release of signal susceptibility factors and the transduction of stress signals. That is, hydrogen mediates the transduction pathway of cadmium stress signals. Plant natural resistance-associated macrophage protein (Nramp) family proteins act as channels in metal-ion transport (Nevo & Nelson, 2006). The over-expression of Nramp6 can enhance the sensitivity of *A. thaliana* to cadmium toxicity without increasing cadmium uptake (Cailliatte *et al.*, 2010). This effect may be attributed to the mechanism that regulates cadmium distribution in cells. We found that LOC_Os07g15460 encodes for a Nramp6 gene. Moreover, we found that its expression decreased when exogenous hydrogen gas passed through rice plants grown under cadmium stress. Therefore, we speculated that exogenous hydrogen could regulate the spatial distribution of cadmium in rice cells by decreasing the expression level of Nramp6. This effect, in turn, increases the cadmium resistance of rice.

Ribosome protein has numerous other functions aside from being a component of ribosomes and a participant in protein biosynthesis (Subramanian, 1983; Arturas & Dinman, 2008; Jang *et al.*, 2012). We found that in the HL group, genes related to ribosome metabolism are significantly enriched. These genes include 24 up-regulated genes related to ribosomal proteins, such as L3, L4, L5, L6, L29, S1, and S20. This finding implies that exogenous hydrogen up-regulates the expression of ribosomal protein genes at the transcriptional level by acting as a signaling molecule. In the HR group, genes associated with glycolysis/ gluconeogenesis metabolic pathways are significantly enriched and are mainly down-regulated. These genes include those that encode for phosphofructo kinase, triosephosphateisomerase, pyruvate decarboxylase, and glyceraldehyde 3-phosphate dehydrogenase. The decrement in the expression level of these genes implies that the energy metabolism and energy consumption of cells have decreased. This phenomenon reduces the metabolic activity of cells and the absorption of cadmium, thereby increasing the resistance of rice to cadmium stress.

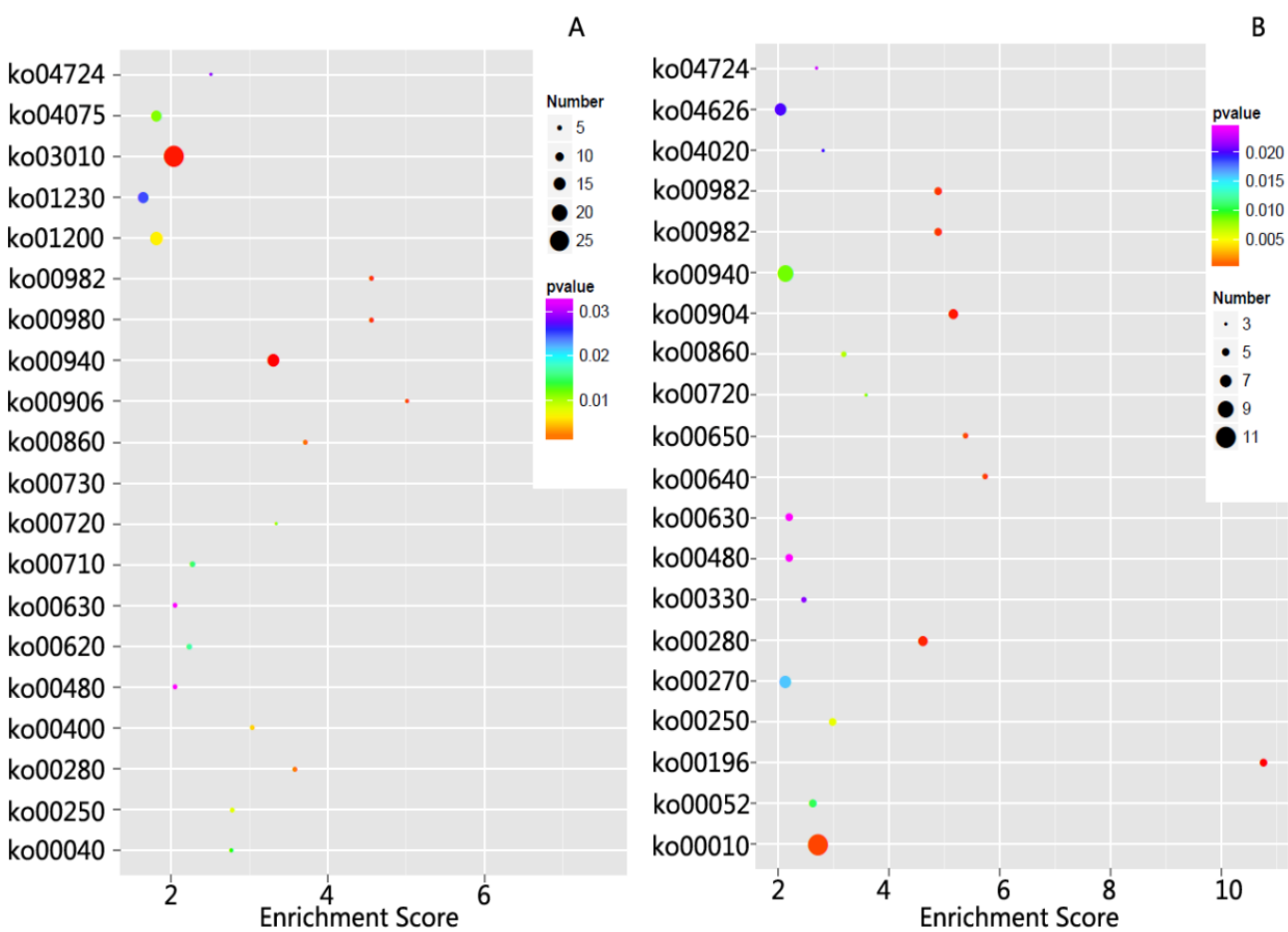

Fig. 3. The bubble graph of top20 enriched pathways of differentially expressed genes by KEGG analysis.

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