Transcriptomic and proteomic responses to herbivory in cultivated, Bt-transgenic and wild rice

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Abstract

Background: Strategies are still employed to decrease insect damage in crop production, including conventional breeding with wild germplasm resources and transgenic technology with the insertion of foreign genes, while the insect-resistant mechanism of these strategies remains unclear.

Results: Under the feeding of brown planthopper (*Nilaparvata lugens*), cultivated rice (WT) showed less DEGs (568) and DAPs (4) than transgenic rice (2098 and 11) and wild rice CL (1990 and 39) and DX (1932 and 25). Hierarchical cluster of DEGs showed gene expression of CL and DX were similar, slightly distinct to GT, and clearly different from WT. DEGs assigned to the GO terms were less in WT rice than GT, CL and DX, and “Metabolic process”, “cellular process”, “response to stimulus” were dominant. Wild rice CL significantly enriched in KEGG pathways of “Metabolic pathways”, “biosynthesis of secondary metabolites”, “plant-pathogen interaction” and “plant hormone signal transduction”. The iTRAQ analysis confirmed the results of RNA-seq, which showing the least GO terms and KEGG pathways responding to herbivory in the cultivated rice.

Synthesize conclusions: This study demonstrated that similarity in the transcriptomic and proteomic response to herbivory for the wild rice and Bt-transgenic rice, while cultivated rice lack of enough pathways in response to herbivory. Our results highlighted the importance of conservation of crop wild species.

Background

Insect damage is one of main factors in reducing agricultural production [1], and the methods of controlling insect pests in agricultural systems include the application of chemical pesticides [2], biological control with releasing the natural enemies of insects [3], high crop diversity and good farm practices [4], breeding and growing resistant varieties [5]. The use of chemical pesticides contaminated food and the environment [2, 6]. Thus, germplasm and genomic resources from wild related species and have provided an opportunity for crop genetic improvement and are developed in controlling insect pests [1, 7].

In addition, since the genetically modified (GM) crops have experienced to a rapid development, insect-resistant Bt-transgenic crops from bacterium Bacillus thuringiensis have been cultivated worldwide [8]. However, the adoption of GM crops receives concerns on the risk to human health and ecological environment. There are no consensus results of risk assessment with the development of GM plants, although numerous studies on risks of GM plants have been done. Environmental risk assessments include gene flow from GM crops to their wild relatives [9, 10], potential risks on non-target organisms [11, 12], resistance evolution of target organisms [13–15]. Health risk assessments include substantial equivalence [16, 17], non-targeted organisms on food/feed consumption [18, 19], traceabiligy of transgenic proteins [20, 21]. However, cisgenic and intragenic plants, modified with genetic material derived from the species itself or sexually compatible species, are expected to be out of regulation [22]. Thus, detecting potential insect-resistant genes or related pathways in wild species are key in developing insect-resistance varieties. “Omic” methods, transcriptomic, proteomic and metabolomic analysis, are usually used to investigate functional genes in plants. Transcriptomic profiling by RNA sequencing is becoming an attractive method as it facilitates rapid generation, identification and quantification of large number of transcripts. Global analysis of transcriptome in a species under different stress conditions will facilitate identification of specific pathways and genes responsible for tolerance against a particular stress.

Therefore, the objectives of this study are 1) to explore the physiological and molecular basis of insect herbivory responsiveness in cultivated rice (Oryza sativa), insect-resistant transgenic rice expressing Bacillus thuringiensis (Bt) and wild rice (O. rufipogon), and 2) to detect the variation of genes related plant response to herbivory in rice domestication and the insertion of a foreign gene. Wild rice possesses a number of elite genes in resistance to insects, diseases, drought, and other stresses [23, 24]. Insect-resistant Bt-transgenic rice has been developed and granted biosafety

certificates in China [25]. Comparing cultivated and Bt-transgenic rice can understand a transcriptomic variation of the Bt transgene insertion, and comparative physiological studies can prove the superiority of wild rice genotypes over cultivated and Bt-transgenic rice in terms of insect resistance.

Results

Transcriptome profiling

Whole genome transcriptome sequencing was performed in leaves to obtain a global view on the herbivory induced changes at transcriptome level and responsive metabolic pathways in rice seedlings, collected from control and herbivore stressed plants. After removing low quality reads, a total of > 1.17×10^7 reads were obtained (Table 1). About 81.44–88.18% of the reads were mapped to the indica rice reference genome, and 67.57–73.55% of the reads were assigned to the indica rice reference genes (Table S2). A total of 18104–18907 genes were identified and quantified (Table 1).

Table 1
Results of gene expression summary in four rice types

Sample ID	Mapped Genes	Unique reads	Length	Coverage	RPKM
CL-0 h	18907	1-59431	160–5950	0.99–99.94%	0.06-18256.7
CL-72 h	18818	1-44585	160–6528	0.75–99.93%	0.04-14022.25
DX-0 h	18548	1-96633	160–6528	0.75–99.93%	0.04-16709.8
DX-72 h	18525	1-44845	115–5950	1.01–99.94%	0.06-10387.5
GT-0 h	18651	1-46650	160–5950	0.99–99.94%	0.05-15012.8
GT-72 h	18138	1-42610	160–5950	1.07–99.93%	0.06-16259.2
WT-0 h	18104	1-52643	160–5950	1.00-99.93%	0.05-15368.3
WT-72 h	18166	1-54839	160–5950	1.01–99.93%	0.05-16186.8

In response to herbivory, the transgenic rice GT (2098 DEGs) and the wild rice genotypes CL and DX (1990 and 1932 DEGs) showed more DEGs than the cultivated rice WT (568 DEGs). This indicates that the cultivated rice lost insect resistance after long-term rice domestication, and that the insertion of Bt gene may induce more genes to express in GT. The up- and down-regulated DEGs was almost equal for each rice genotype (Table 2). A total of 466, 610, 463 and 144 DEGs were up-regulated in CL, DX, GT and WT respectively, and the four genotypes showed 30 common DEGs (Fig. 1). Down-regulated DEGs were 444, 255, 636 and 125 in CL, DX, GT and WT respectively, with 39 common DEGs for the four genotypes (Fig. 1). Hierarchical cluster was generated to gain a global view of the DEGs for the four genotypes. Wild rice CL and DX first closed together, with the same expression patterns of DEGs, and then closed with GT and finally with WT (Fig. 2).

Table 2
Number of differentially expressed genes (DEGs) and differentially accumulated proteins (DAPs) and mapped to GO (GO terms) and KEGG (KEEG pathways) in four rice types under 72-h insect infestation.

Wild rice	DEGs				DAPs			
	up-regulated	down-regulated	Mapped to GO (GO terms)[a]	Mapped to KEEG (KEEG pathways) [b]	up-regulated	down-regulated	Mapped to GO (GO terms) [c]	Mapped to KEEG (KEEG pathways) [d]
CL	974	1016	1274 (36)	1549 (18)	21	18	27(117)	11(13)
DX	1180	752	1224 (8)	1501 (6)	15	10	16(75)	4(7)
GT	902	1196	1346 (17)	1670 (6)	5	6	7(72)	3(7)
WT	293	275	382 (23)	473 (1)	1	3	3(9)	0(0)

[a] GO terms of DEGs were significantly enriched (corrected P < 0.05);

[b] KEEG pathways of DEGs were significantly enriched (Q-value < 0.05);

[c] GO terms of DAPs were significantly enriched (P < 0.05);

[d] KEEG pathways of DAPs were mapped.

A total of 36, 23, 17 and 8 GO terms were significantly enriched in CL, DX, GT and WT rice, respectively (Fig. S2).WT rice assigned the lowest number of DEGs (3217) to the GO functional classification than GT (10859), CL (10595) and DX (9890) did. For GO functional categories, cellular component (44.3% on average) made up the majority, followed by biological process (37.9%) and molecular function (17.8%) in the four rice types (Fig. 3). The high number of DEGs involved in “metabolic process”, “cellular process” and “response to stimulus” in the biological process category indicated that rice leaves undergone exquisite metabolic activities and activated some protective mechanisms during the 72 h herbivory. “Catalytic activity”, “binding” and “transporter activity” in the category of molecular function were prominent classes, and “cell”, “cell part” and “organelle” dominant in the cellular component (Fig. 3).

Based on the criteria of Q-values < 0.05, a total of 21 KEGG pathways were significantly enriched in the four rice types (Fig. 4; Table S3). CL significantly enriched the most KEGG pathways (18 pathways), with the dominant pathway “metabolic pathways (536 DEGs)” followed by “biosynthesis of secondary metabolites (273 DEGs)”, “plant-pathogen interaction (123 DEGs)” and plant hormone signal transduction (87 DEGs). DX significantly enriched six KEGG pathways, including “biosynthesis of secondary metabolites (237 DEGs)”, “plant-pathogen interaction (96 DEGs)” and “amino sugar and nucleotide sugar metabolism (42 DEGs)”. Transgenic rice GT significantly enriched in six KEGG pathways, including “plant-pathogen interaction (119 DEGs)”, “plant hormone signal transduction (97 DEGs)” and “amino sugar and nucleotide sugar metabolism (45 DEGs)”. Cultivated rice WT only showed one significantly enriched pathway “biosynthesis of secondary metabolites (79 DEGs)” (Fig. 4). Two pathways “plant-pathogen interaction” and “amino sugar and nucleotide sugar metabolism” were enriched in both CL, DX and GT. The “biosynthesis of secondary metabolites” pathway was enriched in CL, DX and WT but not in GT (Table S3).

Validation Of Differential Expression Genes Through Qrt-pcr

Ten DEGs, enriched in the KEGG pathways of “plant hormone signal transduction” and “plant-pathogen interaction”, were confirmed by qRT-PCR, including three DEGs in ethylene (ET), two gibberellin (GA), one salicylic acid (SA), one auxin (AUX), one mitogen-activated protein kinases (MAPK), one brassinosteroid, one calmodulin (CALM). These regulatory elements involve in the networks of plants responding to herbivory [26]. The expression pattern of these DEGs in the qRT-PCR analysis showed the same trend as the RNA-Seq analysis did (Table 3).

Table 3

Expression results of selected ten genes in four types of rice under 72 h herbivory using RNA-Seq and qRT-PCR methods

Gene symbol	Function	Primer name	WT		GT		DX		CL	
			RNA-Seq	qRT-PCR	RNA-Seq	qRT-PCR	RNA-Seq	qRT-PCR	RNA-Seq	qRT-PCR
LOC4330189	ethylene-responsive transcription factor 2	ET-105940	+ 0.29	1.2	+ 0.55	1.3	+ 8.9	2.8**	+ 2.2	2.1**
LOC4330189	ethylene-responsive transcription factor 2	ET-073812	-1	0.6	+ 0.84	0.7	+ 3.2	4.7*	+ 3.5	7.6*
LOC4343433	probable carboxylesterase 3	GA-109058	+ 1.52	0.85	+ 0.82	0.82	-	4.12***	+ 8.9	3.22**
LOC4351217	scarecrow-like protein 9	GA-102199	-0.001	1.27	-0.02	0.75	+ 1.5	3.04**	+ 1.8	3.03*
LOC4335566	G-type lectin S-receptor-like serine/threonine-protein kinase At2g19130	MAPK-066063	-1.22	1.2	+ 3.1	1.6	+ 3.57	2.1**	+ 5.9	4.0**
LOC4334072	calmodulin-like protein 4	CALM-069620	+ 1.4	0.9	+ 2.2	0.75	+ 1.7	2.9*	+ 1.8	2.1**
LOC4324059	transcription factor TGA6	SA-106273	-0.001	1.49	+ 5.3	0.44	+ 1.2	1.65	+ 9.6	15.73***
LOC4324956	probable serine/threonine-protein kinase DDB_G0267514	ET-100268	-1.5	0.57	-1.5	0.35*	+ 0.03	1.2	+ 0.21	1.6
LOC4326694	cytochrome P450 72A13	Brass-074025	+ 2.2	7.6***	-0.78	0.84	+ 0.95	0.79	-0.6	0.36***
LOC4336419	auxin response factor 10	ARF-100795	-0.55	0.4***	-2.7	0.8*	+ 0.2	1.84**	-1.15	2.57**

Block indicates significantly enriched in KEGG pathways, which were consistent with results of qRT-PCR.

In RNA-Seq, differentially expressed genes (DEGs) were defined with the RPKM absolute value of log2Ratio ≥ 1 fold and false discovery rate (FDR) ≤ 0.001; “-” indicated down-regulated.

In qRT-PCR, > 1 indicated up-regulated and < 1 up-regulated. *, **, *** indicated significance at $P < 0.05$, 0.01 and 0.0001 respectively.

Protein Profiling Using Itraq Technique

After a 72 h feeding by brown planthopper (N. lugens), wild rice CL showed the most DAPs (39), followed by DX (25 DAPs), transgenic rice GT (11 DAPs), and cultivated rice WT (four DAPs) (Table 2). Venn diagram showed no common DAP for both the four genotypes, and one common DAP (gi|125544232) for CL, DX and GT (Fig. S3). Wild rice CL had four common DAPs with DX and two with WT respectively, and transgenic rice GT had one common DAP (gi|374277679) with DX (Fig. S3).

DAPs in wild rice CL were assigned to 117 GO terms, including 60 biological process, 41 cell component and 15 molecular function terms (Table S4). DAPs participated in response to stress (GO:0006950), oxidation-reduction process (GO:0055114) and organonitrogen compound metabolic process (GO:1901564) were most numerous (Fig. 5). Two KEGG pathways, ribosome and photosynthesis, were significantly enriched (Fig. 6).

In wild rice DX, DAPs were significantly enriched in 31 biological process, 19 cell component and 25 molecular function terms (Table S5). DAPs mainly participated in single-organism metabolic process (GO:0044710), oxidation-reduction process (GO:0055114) and photosynthesis (GO:0015979) (Fig. 5). Photosynthesis and tryptophan metabolism KEGG pathways were significantly involved in DX plants responding to herbivory (Fig. 6).

DAPs in transgenic rice GT were significantly enriched in 74 GO terms, including 58 biological process, 4 cell component and 10 molecular function terms (Table S6). The top GO terms for biological processes were cellular biosynthetic process (GO:0044249), single-organism biosynthetic process (GO:0044711) and organonitrogen compound metabolic process (GO: 1901564) (Fig. 5). Selenocompound metabolism and porphyrin and chlorophyll metabolism were two significant KEGG pathways in transgenic rice under non-target insect herbivory (Fig. 6).

DAPs in cultivated rice WT were significantly enriched in nine GO terms, with three terms of biological processes in response to stress (GO:0006950), response to biotic stimulus (GO:0009607) and response to oxidative stress (GO:0006979) (Fig. 5); one GO term in cell component, ribosome (GO:0005840); and five GO terms in molecular function (Table S7). No significant KEGG pathway was found.

Comparison Of Rna-seq And Itraq

RNA-seq and iTRAQ analysis showed 33 common items in wild rice CL, three in wild rice DX, three in Bt-transgenic rice GT and four in cultivated rice WT. Among these items, based on DAPs (> 1.5 fold change) and DEGS (log2Ratio ≥ 1 fold and FDR ≤ 0.001), two common items (gi|115489014 and gi|53791994) in CL, one item in DX (gi|115450521), GT (gi|75248671) and WT (gi|115489014), were found (Table S8). The same pattern was found between RNA-seq and iTRAQ for gi|115489014 (up-regulated) in CL, gi|115450521 (up-regulated) in DX and gi|75248671 (down-regulated) in GT. The other two items were opposite between RNA-seq and iTRAQ for gi|53791994 (up-regulated in DAP vs. down-regulated in DEG) in CL and gi|115489014 (down-regulated in DAP vs. up-regulated in DEG) in WT. These items involved in GO terms, including response to biotic stimulus, defense response, oxidation-reduction process, hydrogen peroxide catabolic process, chlorophyll biosynthetic process, photosynthesis and response to ethylene stimulus.

Discussion

Our results showed genes and proteins involved in plant response to brown planthopper (BPH, N. lugens) in four rice genotypes. A total of 19 genes resistance to BPH have been reported in cultivated and wild rice species and been used for rice breeding and production [1]. Although we here did not find the bph genes in the identified DEGs or DAPs, wild rice showed lots of DEGs or DAPs related to plant-pathogen interaction and plant hormone signal transduction, including JA,

SA, ABA, GA, auxin, brassinosteroid and ethylene signaling, which are the important pathways in response to herbivory (Figs. 4, 6 and Supplemental Table S9, S10).

We found that the DEGs and DAPs in response to herbivory were mostly distinct from each other, which indicates that RNA-seq and proteomic methods need to be considered together in studying gene regulatory networks during the process of plants responding to biotic stresses. In fact, the expression of genes does not mean the related proteins will express [27]. An alternative reason is the low number of DAPs in this study, which resulted from the lack of multiple fractionation or separation methods that were usually used to improve signal-to-noise and proteome coverage and to reduce interference between peptides in quantitative proteomics [28].

The insertion of Bt gene may promote the response of plants to herbivory because GT showed more DEGs and DAPs than cultivated rice. Foreign genes can induce plants to motivate the expression of many other genes. Li et al. (2010) found genes related with defense response upregulated after induction of a foreign protein in tobacco [29]. However, it is difficult to understand the induction mechanism of foreign gene to other genes.

Our results support crop domestication weakened the response of plants to herbivory. We found that cultivated rice showed less DEGs and DAPs than wild rice. In addition, the plant-pathogen interaction process was specially enriched in the DEGs and DAPs of wild rice CL and DX, but not the cultivated rice (Figs. 4, 6). Number of those genes were much less in cultivated rice than in wild rice (Table S9, S10). Furthermore, wild rice showed strong change in the expression of genes involved in plant hormone signaling, but cultivated rice did not (Fig. 4). JA signaling plays a central role in the herbivory response. We detected that several JAZ genes (OsJAZ5, OsJAZ6, OsJAZ8, OsJAZ9 and OsJAZ12) were differentially expressed in wild rice during herbivory, but not in cultivated rice (Supplemental Table S9). Ethylene signaling appears to be also particularly important for the herbivory response of wild rice as many genes involved in this pathway were specially differentially expressed in wild rice (Supplemental Table S9) It is widely known that cultivated rice (O. sativa) was domesticated from common wild rice [30], during which lots of functional genes were lost [31]. Crop domestication is considered to cause a genetic bottleneck and reduced genetic diversity throughout the genome, and genes that influence desirable phenotypes experiences a drastic loss of diversity [32]. The genetic diversity of cultivated rice is less than that of wild rice [33, 34]. Contrast to wild rice, cultivated rice suffered less herbivory pressure because of huge pesticide application in fields [2]. The target genes of selection (e.g. herbivory) may decrease in nucleotide diversity. In addition, genomic recombination affects the influence of selection on genomic regions via genomic hitchhiking.

Within wild rice, the CL ecotype showed more DEGs, GO terms and KEGG pathways than the DX ecotype, which means the latter was less sensitive to the herbivory of N. lugens than the former. This could be explained by the difference in habitat conditions. The CL population locates in a remote hill with high plant diversity while the DX population was normally grown together with crops including the cultivated rice [35]. Plant-insect interaction depends on environmental abiotic and biotic factors [36]. Herbivore history, a strong driver of plants growth, affects not only plant response to herbivore attack but plant community parameters [37]. Our results indicate growing environment plays an important role in regulating gene networks of plant response to herbivory. Thus, the strategy of in situ conservation is key in the biological conservation of wild germplasm resources.

Conclusions

In conclusion, our results support that rice domestication lost some genes related plant response to herbivory, and the insertion of a foreign gene and plant growing environment can affect the regulation networks of gene expression. It is urgent to pay more attention on the conservation of crop wild species, particularly on the strategy of in situ conservation that highlight the habitat conservation.

Methods

Plants

Insect-resistant transgenic rice (GT) expressing Bacillus thuringiensis (Bt) and its non-transgenic counterpart rice Minghui 63 (WT, an indica rice type, Oryza sativa L.), and two genotypes (DX, CL) of wild rice O. rufipogon Griff. were employed to investigate their physiological responses against brown planthopper (Nilaparvata lugens). Seeds of GT and WT were provided by Prof. Lin, from Huazhong Agricultural University. Seedlings of Dongxiang (DX) common wild rice were taken from the Zhangtang population in Jiangxi province, China. The Zhangtang population locates around paddy fields and ponds with abundant water supplement [35]. Seedlings of CL common wild rice was sampled from the Chaling village in Hunan province, China. Seedlings of DX and CL were cultivated in one research base of Jiangxi Agricultural Academy in 2013, and their seeds were collected to manipulate next trials in 2016.

Greenhouse Trials

Seeds of four rice genotypes (GT, WT, DX and CL) were germinated in petri dishes with 10 seeds per dish in a growth chamber (a 16 h photoperiod, an irradiance of 340 mol m^{-2} s^{-1}, day/night temperatures around 30/25 °C, and a relative humidity of 70%). Seedlings were transplanted to plastic boxes (20 cm width × 20 cm length × 30 cm height), with three rice seedlings per box. At the stage of three to four true leaves, eight boxes (two boxes each rice type) were put in eight insect-free cages (30 cm width × 30 cm length × 80 cm height, protected by a 1 mm mesh nylon net). Brown planthopper (BPH, N. lugens) was put on rice leaves in one half of cages, with a density of 20 BPHs per box, and the remainder half of cages were not put BPHs as the control. After 72 hours of feeding, two leaves per rice seedling were sampled and stored in liquid nitrogen preservation immediately, one leaf for RNA extraction and the other one for protein extraction trials. Leaves of three replicated seedlings per box were treated as one sample.

Rna-seq

Total RNAs from rice leaves were extracted using ethanol precipitation protocol and CTAB-PBIOZOL reagents. RNA-Seq profiling was conducted at the Beijing Genomics Institute in Shenzhen. The total RNA samples' concentration, RIN, 28S/18S and size were detected by using Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit). The purity of the samples was tested by NanoDrop™.

Total RNA sample was digested by DNaseI (NEB), and purified by oligo-dT beads (Dynabeads mRNA purification kit, Invitrogen), then poly (A)-containing mRNA were fragmented into 130 bp with First Strand buffer. First-strand cDNA was generated by N6 primer. First Strand Master Mix and Super Script II reverse transcription (Invitrogen) (Reaction condition: 25℃for 10 min, 42℃for 40 min, 70℃for 15 min). Then Second Strand Master Mixto was added to synthesize the second-strand cDNA (16℃for 1 h). The cDNA was purified with Ampure XP Beads (AGENCOURT) and combined with End Repair Mix, then incubated at 20℃ for 30 min. Purified and add A-Tailing Mix, incubated at 37℃ for 30 min. We combined the Adenylate 3'Ends DNA, Adapter and Ligation Mix, incubating the ligate reaction at 20℃for 20 min. Several rounds of PCR amplification with PCR Primer Cocktail and PCR Master Mix were performed to enrich the cDNA fragments. The PCR products were purified with Ampure XP Beads (AGENCOURT).

The final library was quantitated in two ways: determining the average molecule length using the Agilent 2100 bioanalyzer instrument (Agilent DNA 1000 Reagents), and quantifying the library by real-time quantitative PCR (QPCR) (TaqMan Probe). The Qualified libraries was amplified on cBot to generate the cluster on the flowcell (TruSeq PE Cluster Kit V3–cBot–HS,Illumina), and the amplified flowcell was sequenced pair end on the HiSeq 2000 System (TruSeq SBS KIT-HS V3Illumina), reading length 50.

The resulting clean reads were mapped to the reference genomes of indica rice 9311, using SOAPaligner/SOAP2 [38]. No more than 2 mismatches were allowed in the alignment. Data was normalized by calculating the read per kilobase per million mapped reads (RPKM = total exon reads/mapped reads in million X exon length in kb) for each gene. The differentially expressed genes (DEGs) were defined with the RPKM absolute value of log2Ratio ≥ 1 fold and false discovery rate (FDR) ≤ 0.001 (Fig. S1). We performed cluster analysis of gene expression patterns with cluster software and Java Treeview software.

The DEGs were annotated according to molecular function, biological process, and cellular component by Blast2GOv2.5 with Nr and Pfam annotation in Gene Ontology (GO) database (http://geneontology.org/), and/or were annotated at Kyoto Encyclopedia of Genes and Genomes Pathway database (KEGG, http://www.genome.jp/kegg/) Automatic Annotation Server [39]. After getting GO annotation for DEGs, we used WEGO software to do GO functional classification for DEGs. Pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways in DEGs comparing with the whole genome background. The calculating formula was the same as that in GO analysis. Q-value was corrected p-value ranging from 0 ~ 1, and its less value means greater intensiveness.

Validation Of Degs Using Qrt-pcr

Based on the results of significantly enriched in KEGG pathway, ten genes that related with plants respond to herbivory were selected for validation through real-time quantitative PCR (qRT-PCR). RNA of sampled leaves (100 mg) were isolated using RNA plant kits (Tiangen Biotech, Beijing, China). Copy DNAs were synthesized from 2 μg of total RNA using PrimeScript™ RT Master Mix (Takara, Shuzo, Japan) in a 20 μL reaction mixture. The cDNA was diluted to 1:50 by adding distilled water. Each sample that was used to perform qRT-PCR included 10 μL of a 2 × SYBR solution, 0.05–0.15 μL of a forward/reverse primer, 2 μL of cDNA, and 7.70–7.90 μL of distilled water. Real time qPCR was performed with a Takara SYBR Premix Ex Taq kit on a CFX 96 machine (Bio-Rad, Hercules, USA). The PCR was triplicated for each cDNA sample. Gene-specific primers were listed in Table S1. OsActin1 mRNA was used to normalize the expression of each gene [40]. Amplification conditions were following: 95 °C for 2 min followed by 40 PCR cycles of 95 °C for 15 s, 57–60 °C for 30 s, and 72 °C for 20 s. Changes in expression were calculated using the $2^{-\Delta\Delta Ct}$ method [41].

Itraq

Preparation of protein samples

Three replicates of leaf samples (about 0.2 g) were homogenized in 0.6 ml precooled extraction buffer composed of 50 mM TEAB, 10 mM DTT, 2% SDS, 1% insoluble PVPP, 1% PMSF. After washing the mortar with 0.4 ml extraction buffer, the total 1 ml homogenate was first centrifuged at 16 000 g for 10 min and then 25 000 g for 10 min. The supernatant was mixed with 6 volume of ice-cold 10% TCA acetone and incubated at -20 ^{o}C for protein precipitation by at least 6 h. The resultant pellet was rinsed four times with ice-cold acetone by centrifugation at 16 000 g for 5 min. The acetone was decanted and surface-dried for 10–15 min. Protein samples were stored immediately at -70^{o}C until used.

iTRAQ labeling

Protein samples (450 μg) were dissolved by 300 μl dissolution buffer containing 0.1 M TEAB, 8 M Urea. After a two-step centrifugation (16 000 g for 10 min plus 32 000 g for 20 min), 100 μg protein was reduced in 5 mM tris-(2-carboxyethyl) phosphine at 37^{o}C for 4 h and then alkylated in 10 mM methyl methanethiosulphate (MMTS) at 56^{o}C for 10 min. The alkylated protein samples were cleaned by loading on a 500 μl centrifugal concentrator (Vivaspin® 500, Sartorius, Goettingen, Germany) and rinsing three times with 0.5 M TEAB via centrifugation at 12 000 g for 20 min. After digestion

overnight with 4 μg trypsin in 100 μl 1 M TEAB at 37^{o}C, the peptides were collected by centrifugation at 12 000 g for 20 min using a centrifugal concentrator and rinsed twice with 100 μl water. Peptides were dried in a SpeedVac and then redissolved in 50 μl 1M TEAB for iTRAQ labeling. Peptide samples were labeled with iTRAQ 4 plex (114, 115, 116 and 117) according to the manufactruer's protocol (AB SCIEX, Framingham, MA, USA).

LC-MS/MS analysis

After desalted, the labeled peptides were subjected to the MS analysis using a commercial 5600 TripleTOFTM coupled with an Eksigent Nano LC-Ultra 1D plus HPLC system (Eksigent; Dublin, CA, USA). Nano LC was performed via a "trap and elute" configuration and the mobile phase included A (0.1% FA and 100% CAN) and B (100% water). Both trap column and analytical column were filled with MAGIC C18AQ 5 μm 200 Å phase (michrom BIORESOURCES, Inc). The peptides were separated over 75 min by a gradient of 5–30% of mobile phase B at a flow rate of 300 nl/min. The ions of mass range of 350–1500 were selected as precursor ions using 250 ms accumulation time per spectrum. With 100 ms accumulation time for per MS/MS, a 25 product ion per cycle from each MS spectrum were selected for later MS/MS analysis and dynamic exclusion time for 18 s. Tandem mass spectra were recorded in high sensitivity mode (resolution > 15000) with rolling collision energy on and adjust CE when using iTRAQ reagent. Fragment ion spectra produced via high energy collision dissociation was acquired in the TOF 5600 analyzer with ion spray voltage in the range of 2.5 kV.

Protein identification and quantification

Protein identification and quantification were performed using ProteinPilot Software v. 4.2 (AB Sciex). The Paragon algorithm in the ProteinPilot software was used for the protein identification which was further processed by Pro Group algorithm where isoform-specific quantification was adopted to trace the differences between expressions of various isoforms. Parameters of protein searching were defined as following: sample style – iTRAQ 4plex; cysteine alkylation – MMTS; digestion – trysin; instrument – Triple TOF 5600; database – NCBInr Oryza sativa protein database (NCBInr 20150104). A cutoff of unused socre higher than > 1.3 was applied for protein identification. For iTRAQ quantitation, the peptide for quantification was automatically selected by Pro Group algorithm to calculate the ratio between different iTRAQ tags and p-value. Proteins varied significantly ($P < 0.05$) and more than 1.5-fold in abundance were considered to be differentially accumulated proteins (DAPs). DAPs were identified (http://www.uniprot.org/) and the GO annotation (http://www.geneontology.org/) and KEGG pathway analysis (http://www.kegg.jp/) of DAPs between the control and 72-h herbivory groups was analyzed.

Abbreviations

Bt: *Bacillus thuringiensis*; CL: common wild rice in Chaling; DX: common wild rice in Zhangtang; GT: Bt-transgenic rice; WT: cultivated rice; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; qPCR: Real time quantitative PCR; RNA-seq: RNA-sequencing.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Author's Contributions

YL and JL designed the experiments. YL, WW, YHL, FL and WH performed the experiments, analysed data and prepared the manuscript. JL contributed with valuable discussions. YL and WW read and revised the manuscript. All authors provided helpful discussions and approved its final version.

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Figures

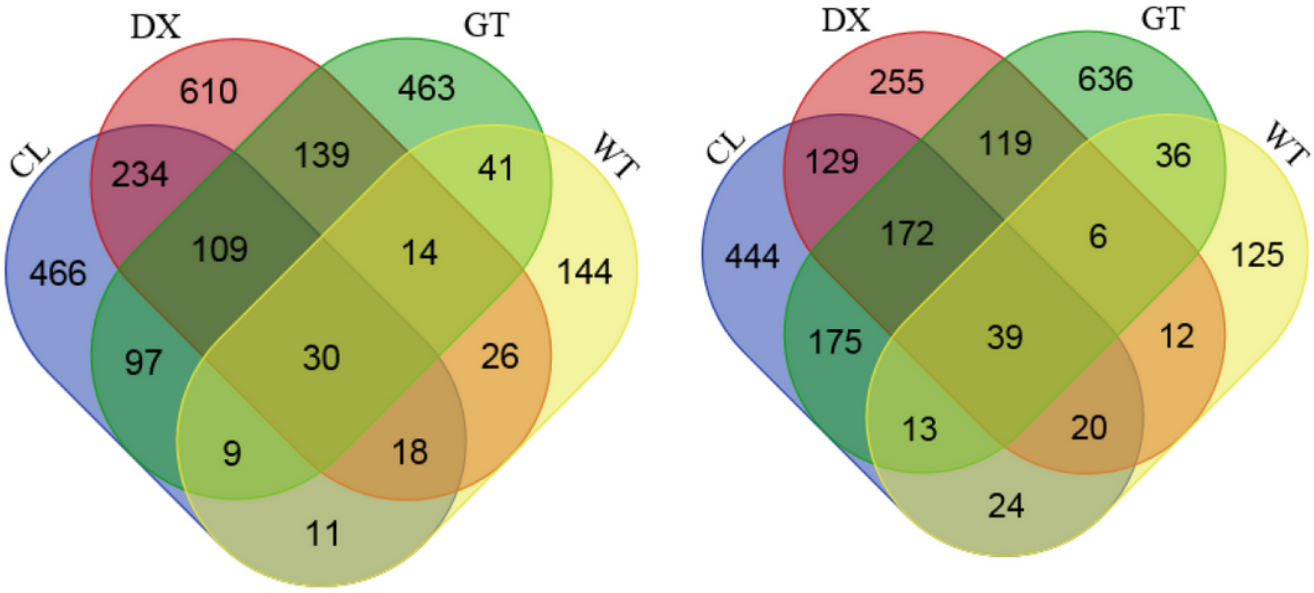

Figure 1

Venn diagram showed the number common and genotype specific differentially expressed genes (DEGs) in wild rice (DX, CL), Bt-transgenic (GT) and cultivated (WT) seedlings after a 72h feeding by brown planthopper (BPH, N. lugens). Left subfigure shows the number of up-regulated DEGs and right subfigure shows down-regulated DEGs.

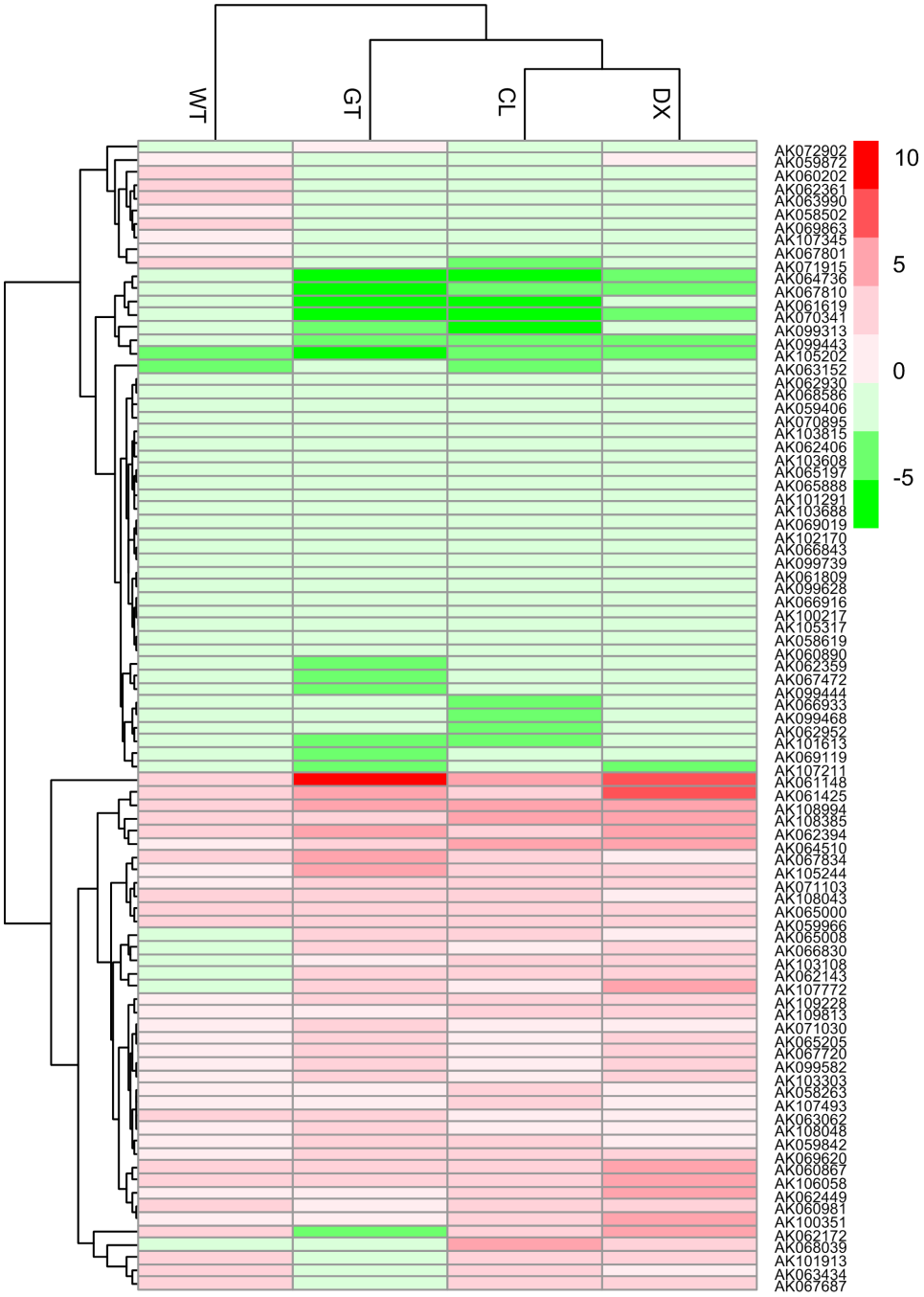

Figure 2

Cluster of differentially expressed genes (DEGs) in four genotypes of rice (CL, DX, GT and WT) after a 72-h insect herbivory. Expression changes and cluster analysis of 89 genes that were differentially expressed between any two of the four rice types. Each row represents a differentially expressed gene, while each column represents a rice type. Color gradients the log2ratio of transcript abundance in four rice types.

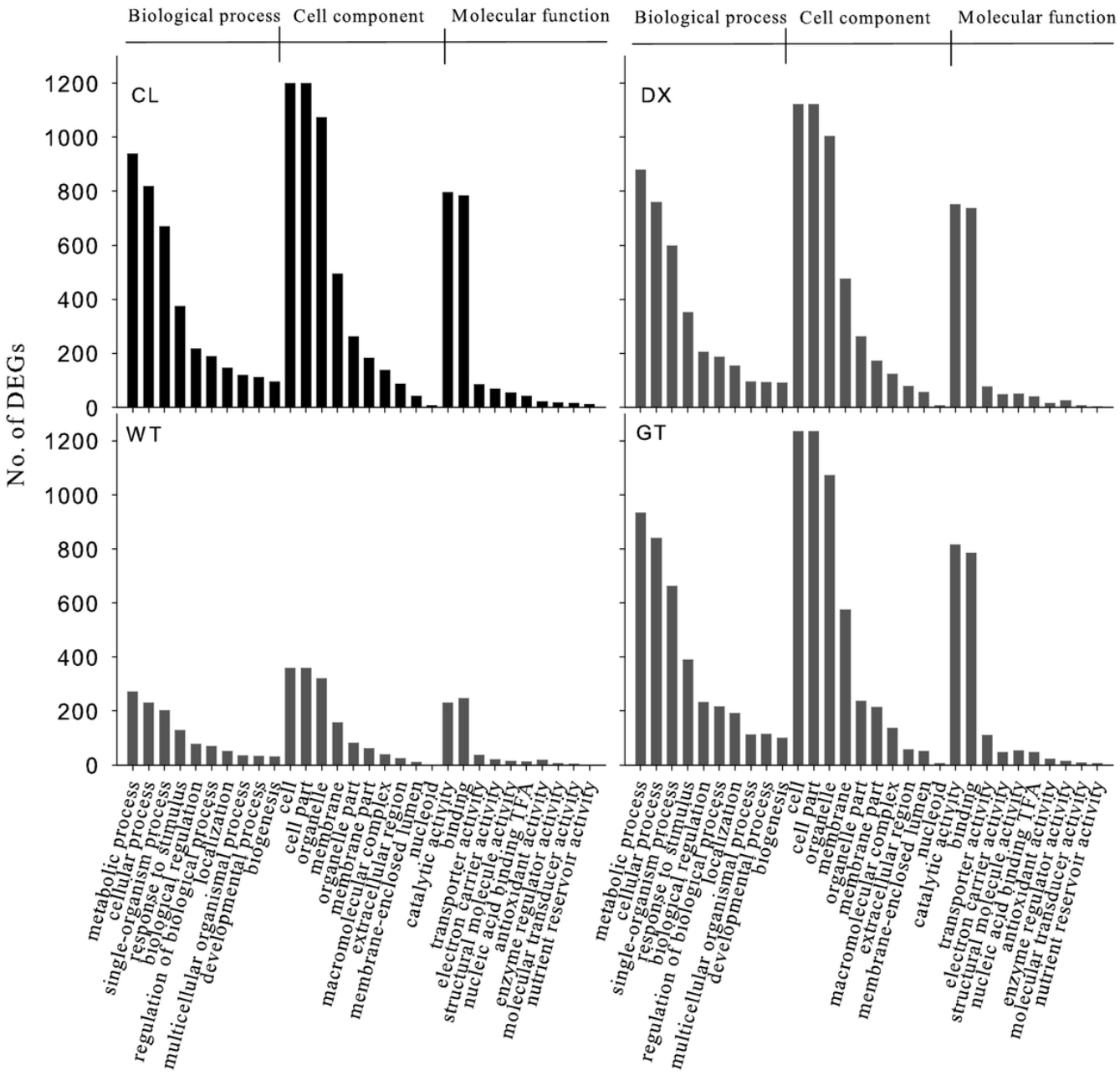

Figure 3

Histogram presentation of top 10 GO classes of DEGs significantly enriched in four rice types, CL, DX, GT and WT.

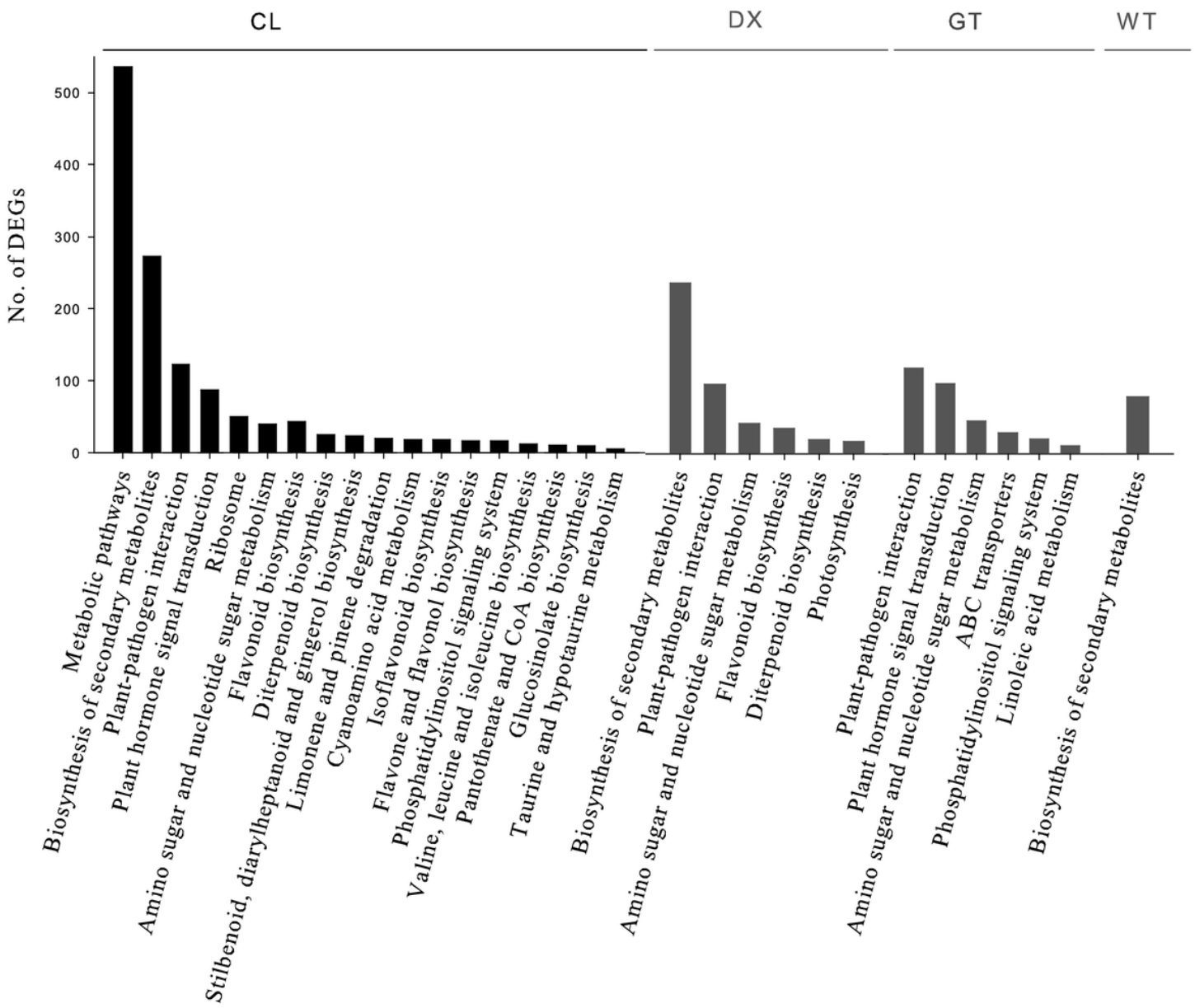

Figure 4

Histogram presentation of KEGG pathways of DEGs significantly enriched in four rice types, CL, DX, GT and WT.

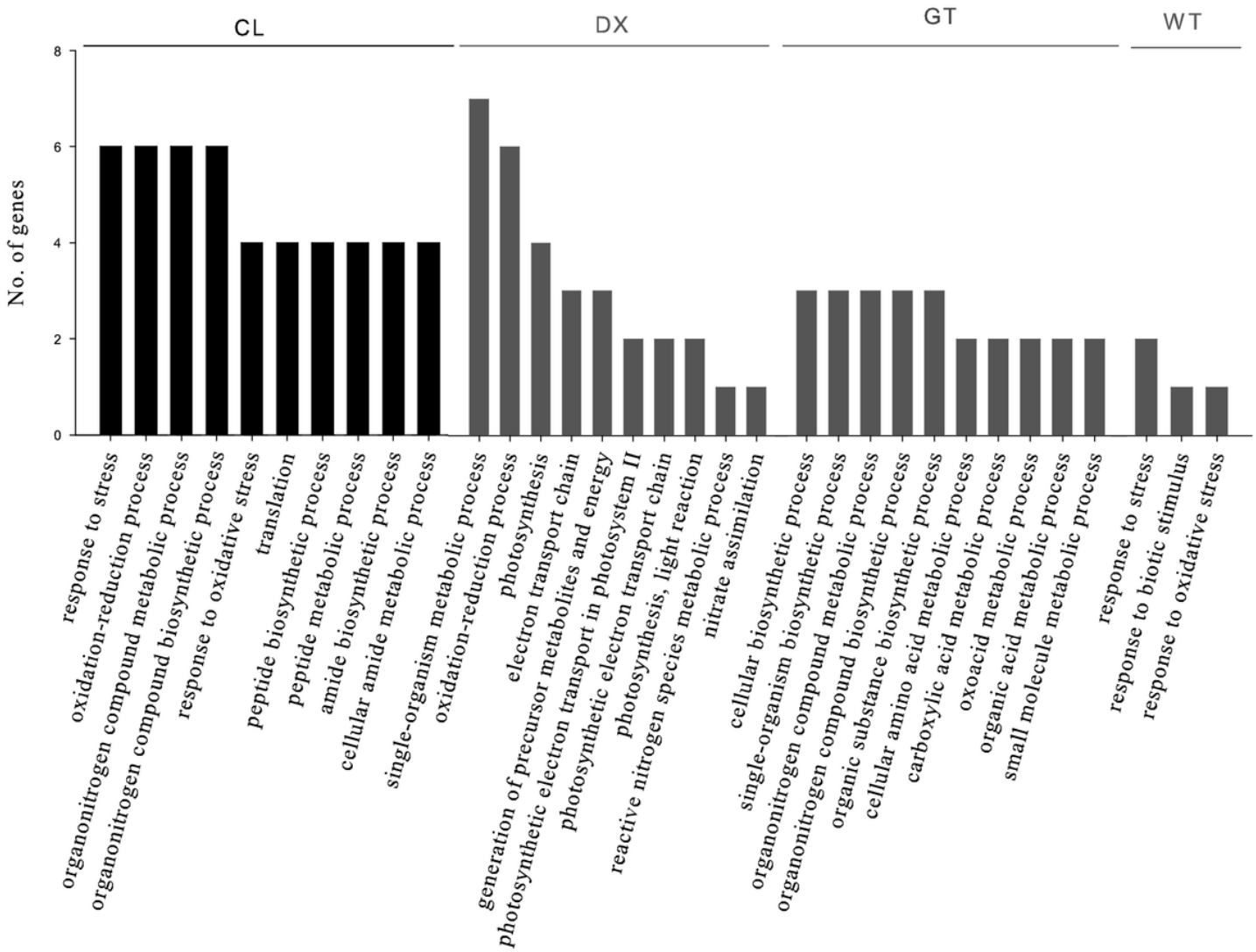

Figure 5

Histogram presentation of top 10 biological process in GO terms of DAPs significantly enriched in four rice types, CL, DX, GT and WT. Only three biological process terms were enriched in WT.

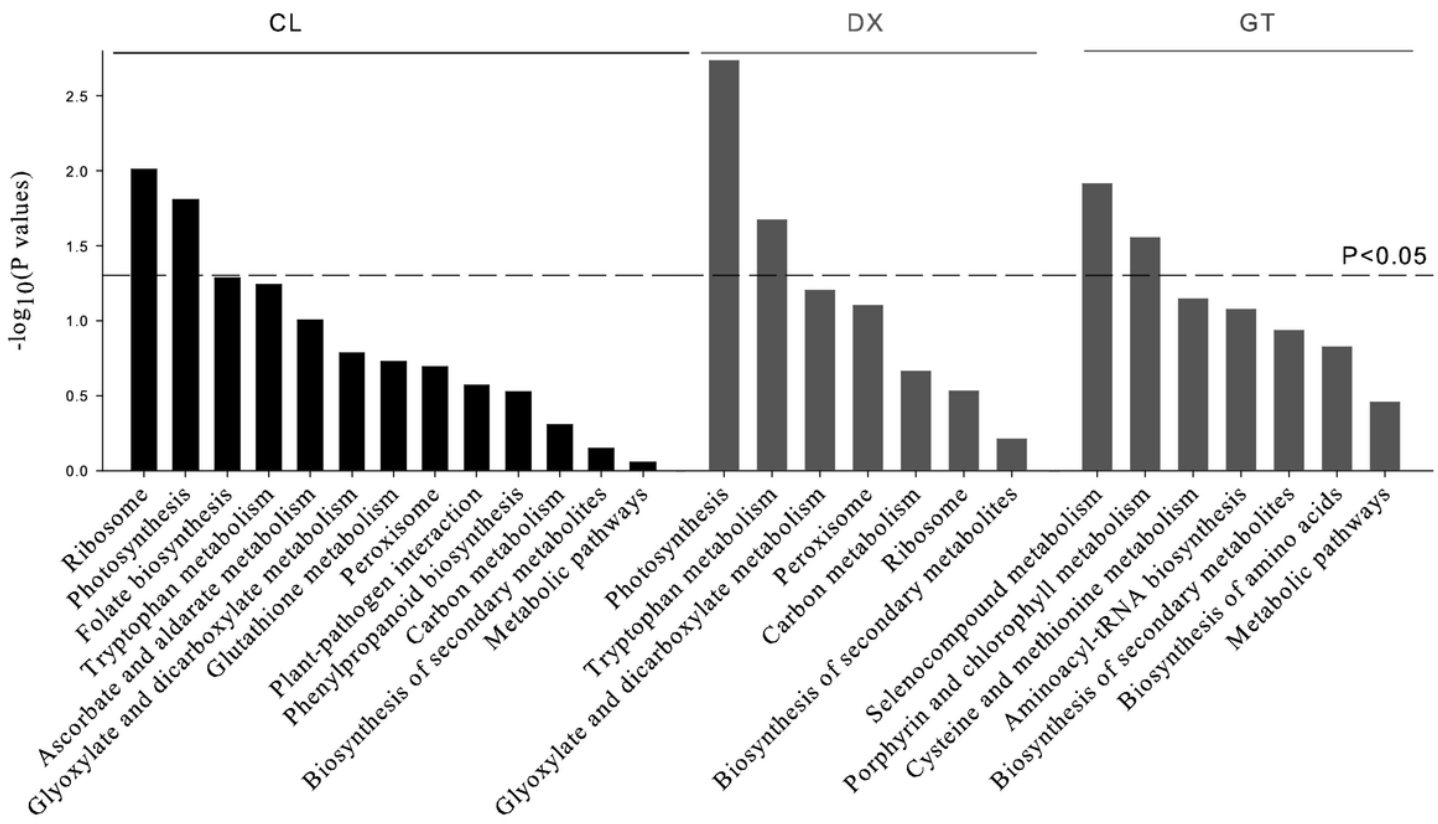

Figure 6

Histogram presentation of KEGG pathways of DAPs in rice CL, DX and GT. No KEGG pathway of DAPs was found in WT. P <0.05 indicates KEGG enriched significantly.

Supplementary Files

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