Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2023.027231

ARTICLE

Comparative Non Participating Transcriptome Analysis Response to Low Phosphorus by *CmPht1;2* in Chrysanthemum

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Received: 20 October 2022 Accepted: 28 November 2022

ABSTRACT

Chrysanthemum morifolium Ramat is one of the four major cut flowers in the world. Pht1 family is focus on the uptake and transport of phosphate in plants. In our previous studies, *CmPht1;2* overexpression line (Oe1) had higher phosphate contents both in roots and shoots, and its root development was significantly enhanced than wild type (WT) at low phosphorus conditions in chrysanthemum. Metabolomics analysis showed that several metabolites had a change in pyruvate metabolism and tricarboxylic acid (TCA) cycle pathway. To explore the gene difference expression and the change of metabolic pathway between *CmPht1;2*-Oe1 and WT, we conducted the transcriptome analysis. A total of 617,681 and 207,271 unigenes were obtained from roots and shoots, respectively. They were classified into biological process, cellular component and molecular function by Gene Ontology (GO). In addition, 450 different expression genes (DEGs) were found in the roots after 2 d treatment, and 1,787 DEGs were identified in shoots after 7 d treatment under LP condition between Oe1 and WT. From the top 20 pathways of DEGs assigned by Kyoto Encyclopedia of Genes and Genomes (KEGG), TCA cycle and pyruvate metabolism pathways mostly affected by overexpression of *CmPht1;2* attracted our attention. This research will be helpful for elucidating the mechanism of effects by *CmPht1;2* overexpression on growth, development and stress tolerance.

KEYWORDS

Chrysanthemum; *CmPht1;2* overexpression line; transcriptome; pathways

1 Introduction

Phosphorus (P) is one of the 17 essential nutrients needed for plant growth which is involved in the entire life of plants [1]. Phosphorus efficient utilization plants can use more soil contact area to obtain phosphate (Pi) by producing a larger root system [2]. Some of them will release a variety of organic acids such as citric acid, malic acid and other organic anions, or phosphatase and phytase to increase the solubility of phosphorus [3]. To overcome low Pi availability, plants also evolved some complex mechanisms to maintain P homeostasis as remobilization of internal Pi within the plant [4].

PHOSPHATE TRANSPORTER1 (Pht1) family are plasma membrane proteins that play a pivotal role on the uptake of inorganic phosphate (Pi) from soil [5]. These family members were originally obtained by homologous cloning from the yeast PHO84 [6]. Most Arabidopsis PHT1 transporters are expressed in roots and induced by low phosphorus, consistent with their primary function of phosphorus uptake from the rhizome [7]. Pht1;1 or Pht1;4 played a significant role on the uptake of Pi both under Pi-sufficient and deficient soil in Arabidopsis [8]. Most of the OsPTs were induced by Pi-deprivation in rice roots. OsPT1;3 acts as an uptake and translocation protein in rice under low Pi conditions [9]. However, overexpression of *OsPT8* in rice under high phosphorus environment resulted in excessive accumulation of phosphorus and phosphorus toxicity symptoms [10]. Here in our previous researches, *CmPT1* [11] and *CmPht1;2* [12] were all involved in Pi uptake in chrysanthemum and up-regulated in the P-starvation roots. However, it is not clear the transcriptional regulatory network of phosphate absorption and low phosphorus adaptation by *CmPht1;2* in chrysanthemum.

Transcriptomic sequences were widely applied to functional genomics research in the non-model plant [13]. Transcriptome response to phosphorus starvation had already been analyzed in kinds of plants. Affymetrix Gene-Chip rice genome arrays were used to analyze the dynamics of rice transcriptome under P starvation [14]. Low P-tolerant line and the low P-sensitive line were used to generate strand-specific RNA libraries subjected to P stress in the roots and shoots of maize, respectively [15]. Transcriptome datasets were acquired from roots and leaves of the low-phosphorus tolerant soybean variety both under phosphorus-deficient and -sufficient conditions for further analysis [16]. Nevertheless, there were few reports about the transcription reprogramming after *Pht1* overexpression. How phosphate transporters regulate gene expression and metabolic changes after transformation is still unclear.

Chrysanthemums (*Chrysanthemum morifolium* Ramat.) is an important plant due to its ornamental, edible and medicinal value in the world's flower industry. *CmPht1;2* is involved in phosphorus starvation response and impacted the metabolism of chrysanthemum in our previous studies [12]. *CmPht1;2*-Oe1 showed a higher Pi content both in roots and shoots, and a higher root biomass compared to WT in the Pi-starvation conditions. Moreover, Oe1 may enhance glycolysis metabolic (pyruvate metabolism), TCA and other metabolic pathways in response to P starvation stress. However, the reason of metabolic changes still needs further study. Whether there were corresponding changes at transcriptional level? How did *CmPht1;2* overexpression cause these series of changes? To date, the molecular mechanisms of Pht1 transgene plants response to low phosphorus stress at the transcriptional level were rarely reported, especially in chrysanthemum. We describe here the transcriptome study of *CmPht1;2* overexpression line responses to phosphorus limitation in chrysanthemum, rather than investigating immediate responses to P availability of chrysanthemum. The effects of response to low phosphorus stress by *CmPht1;2* overexpression were discussed from the level of gene expression and metabolic pathways in chrysanthemum.

2 Material and Methods

2.1 Plant Material and Growing Conditions

The wild type chrysanthemum cultivar 'Jinba' was obtained from the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. The *CmPht1;2* overexpression line-Oe1 was generated by our group [12]. The cuttings of chrysanthemum cultivated in a greenhouse at 25°C, a 16-h photoperiod with an irradiance of 2000 Lux, and a relative humidity of 70%. The rooting process was cultured in deionized water for 14 days. They were then transferred to hydroponic solutions consisting of diluted Hoagland solutions (1:4) and (1:2) for 3 d [17]. The nutrient solution renewal was carried out every 3 d. After a week of curing in total nutrient solution, the plants were respectively treated in normal phosphate concentration (HP, 300 μM Pi) or low phosphate concentration (LP, 15 μM Pi) hydroponic solution for phosphorus sufficient and deficiency treatment, respectively. The roots and shoots were harvested at 0, 2, and 7 d after the phosphate treatment, snap-frozen in liquid nitrogen, and kept at −80°C. The shoots selected here were the aboveground part including shoots and leaves. Experiments included three replicates. Each replicate contained nine seedlings.

2.2 Isolation of Total RNA and RT-PCR

The total RNA from the above samples was extracted using Quick RNA isolation Kit (Huayueyang Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. First strain cDNA was carried out by SPARKscript II RT Kit (With gDNA Eraser) (Shandong Sparkjade Biotechnology Co., Ltd., China). 500 $ng{\cdot}\mu L^{-1}$ RNA was used for cDNA synthesis.

2.3 Transcriptome Sequencing

After the RNA samples were qualified, eukaryotic mRNAs were enriched with Oligo(dT) magnetic beads. Then, mRNA was fragmented into short fragments by adding fragmentation buffer. The mRNA was used as template and 6 base random primer was used as random hexamers to synthesize first strand cDNA. Then buffer, dNTPs, DNA polymerase I and RNase H were added to synthesize two strands cDNA. Then last PCR amplification was performed after cDNA modification. AMPure XP beads were used to purify double strand cDNA to obtain the final product Library. After library construction, Qubit2.0 was first used for preliminary quantification, and the library was diluted to 1.5 $ng{\cdot}uL^{-1}$. Agilent 2100 was then used to detect the insert size of the library. The qPCR method was used after the insert size met the expectation. The effective concentration of the library was accurately quantified (Library effective concentration >2 nM) to ensure the quality of the library. The standard Illumina protocol was followed thereafter to develop mRNA-seq libraries. Subsequently, the library preparations were sequenced on an Illumina HiSeq2000 platform at Beijing Novogene Bioinformatics Technology Corporation and paired-end reads were generated. The screening criteria for the differential genes were: padj <0.05.

2.4 Differential Gene Expression Verification

The verification of differential expression genes was performed using SYBR Green I Real Time PCR. 2 × SYBR qPCR Mix (5.0 μL, Shandong Sparkjade Biotechnology Co., Ltd., China), forward/reverse primer (10 μM, 0.5 μL each), cDNA template (1.0 μL) and ddH_2O (3.0 μL) were added into a 384-well plate. The reference sequence, a 151 bp fragment of *EF-1α*, which amplified with the primers EF1α-F/-R and the specific primers of c137909_g1 and c183919_g1 were shown in Supplementary Table S1. The experiment was performed on ROCHE (LightCycler® 480 II, Switzerland) real time fluorescence quantitative PCR instrument with 94°C 2 min; 94°C 20 s, 55°C 20 s, 72°C 30 s, 40 cycles. Finally, add the dissociation curve stage. Relative transcription levels were estimated using the $2^{-\Delta\Delta Ct}$ method [18].

3 Result

3.1 Gene Annotation

The genome-wide research was investigated in the roots and shoots of Oe1 and WT under LP treatment for 0, 2, and 7 d. The reads from roots or shoots were pooled together for more comprehensive reconstruction of transcripts. In roots, 617,681 unigenes was compared and annotated in seven functional databases: 230,009 (NCBI non-redundant protein sequences (NR): 37.23%), 72,935 (NCBI nucleotide sequences (NT): 11.80%), 110,894 (euKaryotic Ortholog (KO): 17.95%), 234,503 (A manually annotated and reviewed protein sequence database (SwissProt): 37.96%), 271,994 (Protein family (PFAM): 44.03%), 276,138 (GO: 44.70%) and 144,621 (euKaryotic Ortholog Groups (KOG): 23.41%). In shoots, 207,271 unigenes were compared and annotated: 81,214 (NR: 39.18%), 40,853 (NT: 19.70%), 37,988 (KO: 18.32%), 72,736 (SwissProt: 35.09%), 74,950 (PFAM: 36.16%), 76,557 (GO: 36.93%) and 37,906 (KOG: 18.28%) (Table 1).

Table 1: Gene annotation by searching against public databases

Annotation	Roots		Shoots	
	Number of unigenes	Percentage (%)	Number of unigenes	Percentage (%)
Annotated in NR	230,009	37.23	81,214	39.18
Annotated in NT	72,935	11.8	40,853	19.7
Annotated in KO	110,894	17.95	37,988	18.32
Annotated in SwissProt	234,503	37.96	72,736	35.09
Annotated in PFAM	271,994	44.03	74,950	36.16
Annotated in GO	276,138	44.7	76,557	36.93
Annotated in KOG	144,621	23.41	37,906	18.28
Annotated in all databases	26,342	4.26	12,534	6.04
Annotated in at least one database	363,211	58.8	109,113	52.64
Total unigenes	617,681	100	207,271	100

GO assignments were used to predict the functions of unigenes obtained above by classifying them into various biological processes. The unigenes annotated in the GO database were categorized into 56 functional groups which belong to Biological processes, Cellular component, and Molecular function. Among them, there were 25 subclasses of biological processes, 21 cellular components and 10 molecular functions. Most of these unigenes were classified into the cellular process, metabolic process and binding both in roots and shoots (Fig. 1). The unigenes were aligned to the 26 KOG classifications to classify potential functions in roots, while 25 KOG classifications in shoots. Both in roots and shoots, assignments to (J) translation, ribosomal structure and biogenesis, (O) posttranslational modification, protein turnover, and chaperones, and (R) general function prediction were all over 10.0% (Fig. 2). (T) Signal transduction mechanism was also accounted for a large proportion in roots rather than shoots (Fig. 2A). To further identify the biological pathways of Oe1 and WT, the unigenes were annotated by KEGG database. The unigenes were mainly focused on translation, folding sorting and degradation, carbohydrate metabolism, transport and catabolism, and amino acid metabolism both in roots and shoots (Fig. 3).

3.2 Gene Different Expression Analysis between Oe1 and WT

The significant DEGs were then identified with the applied criteria ($p < 0.05$) (Fig. 4). In roots, 94 DEGs (88 up-regulated genes and 6 down regulated genes) were identified between Oe1 and WT at 0 d (Fig. 4A). After 2 d LP-treatment, the number of DEGs (450 DEGs, 359 up-regulated genes and 91 down regulated genes) was increased immediately (Fig. 4B). However, only 27 DEGs (20 up-regulated genes and 7 down regulated genes) were identified between Oe1 and WT at 7 d (Fig. 4C). Meanwhile, in shoots, only 19 genes (11 up-regulated genes and 8 down-regulated gene) and 9 genes (2 up-regulated genes and 7 down-regulated gene) showed significant difference in expression levels in 0 and 2 d, respectively (Figs. 4D, 4E). In addition, reach up to 1,787 DEGs (716 up-regulated genes and 1,071 down regulated genes) were identified between Oe1 and WT at 7 d (Fig. 4F).

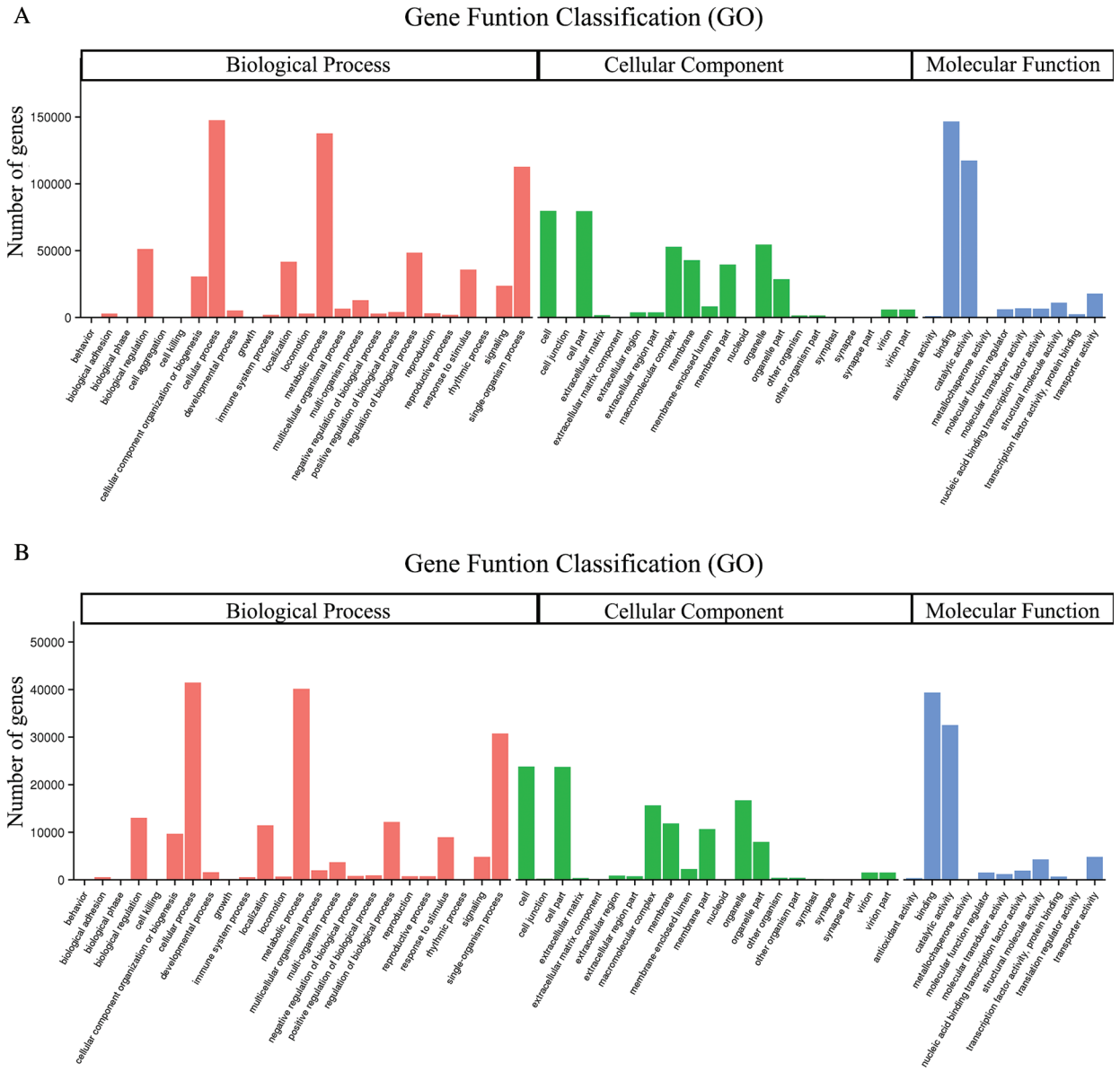

Figure 1: Gene ontology classifications of assembled unigenes. (A) Roots; (B) Shoots

3.3 Pathways Influenced by LP Stress between Oe1 and WT

The influence of LP stress on biological pathways was evaluated via enrichment analysis of DEGs in KEGG between Oe1 and WT. The DEGs were assigned into 14 pathways in 0 and 7 d in roots, respectively. However, only 1 or 2 DEGs were found in every pathway (Figs. 5A, 5C). After 2 d LP-treatment, the DEGs were assigned into 63 pathways in roots. We selected the top 20 pathways that were most significant in the enrichment process to be shown in this diagram (Fig. 5B). Among these pathways, the ribosome (37 DEGs), phenylpropanoid biosynthesis (6 DEGs), Glycolysis/Gluconeogenesis (6 DEGs), oxidative phosphorylation (6 DEGs) and plant-pathogen interaction (5 DEGs) pathway showed a significant degree of enrichment by DEGs. In addition, it was important to note that phenylpropanoid biosynthesis (6 DEGs) and flavonoid biosynthesis (2 DEGs) showed a significant degree of enrichment by DEGs in roots (Supplementary Table S2).

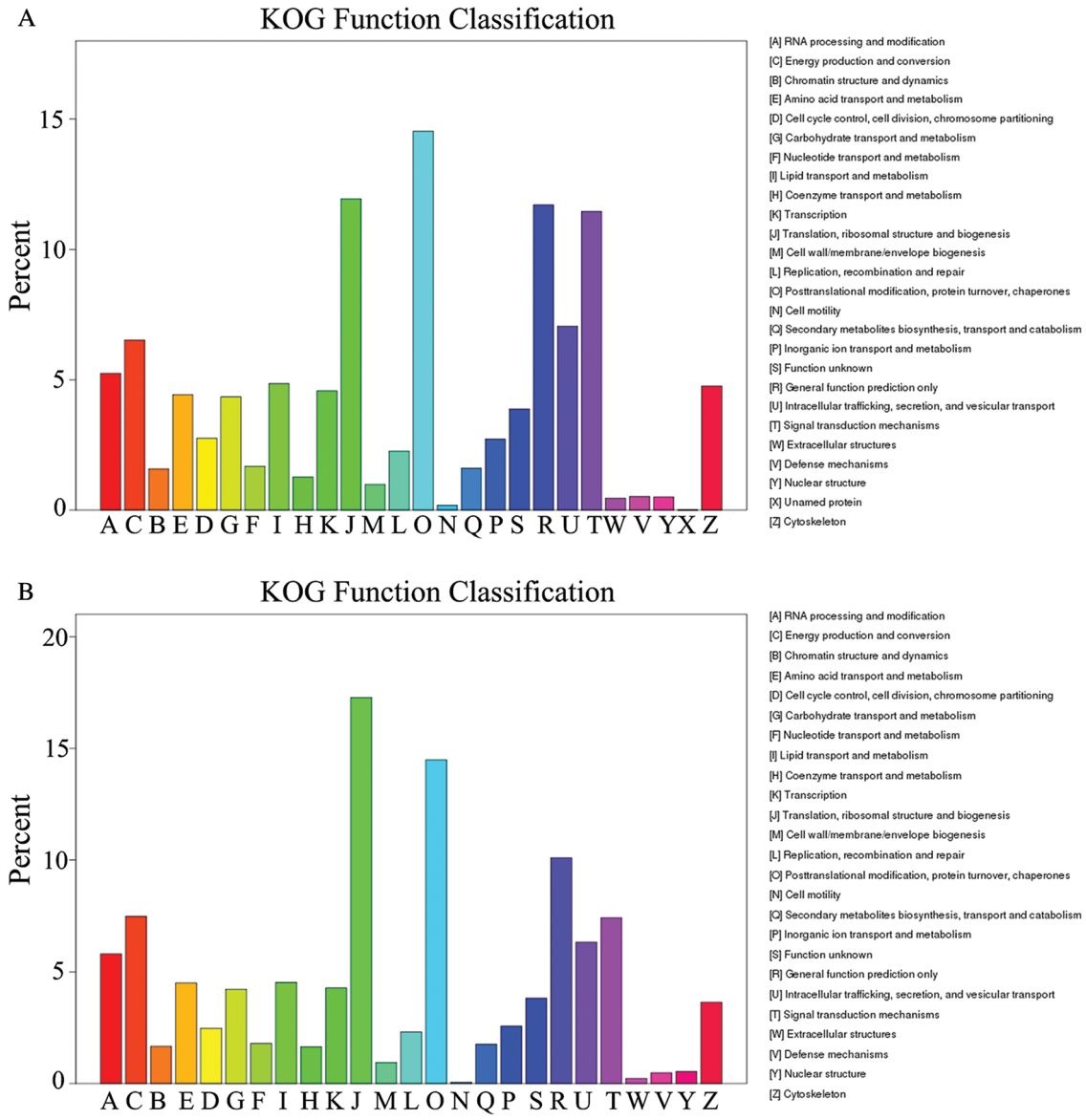

Figure 2: KOG classification of assembled unigenes. (A) Roots; (B) Shoots

In shoots, only 5 pathways were enriched in 0 d (Fig. 5D). While in 2 d, there was not any pathway enriched by KEGG. Suffering from phosphorus deficiency for 7 d, the DEGs were assigned into 74 pathways in shoots (Fig. 5E). Among the top 20 pathways, the starch and sucrose metabolism (36 DEGs), photosynthesis (31 DEGs) and Photosynthesis-antenna proteins (21 DEGs) showed a significant degree of enrichment by DEGs. Phenylpropanoid biosynthesis (17 DEGs) and flavonoid biosynthesis (5 DEGs) also showed a significant degree of enrichment by DEGs in shoots (Supplementary Table S3).

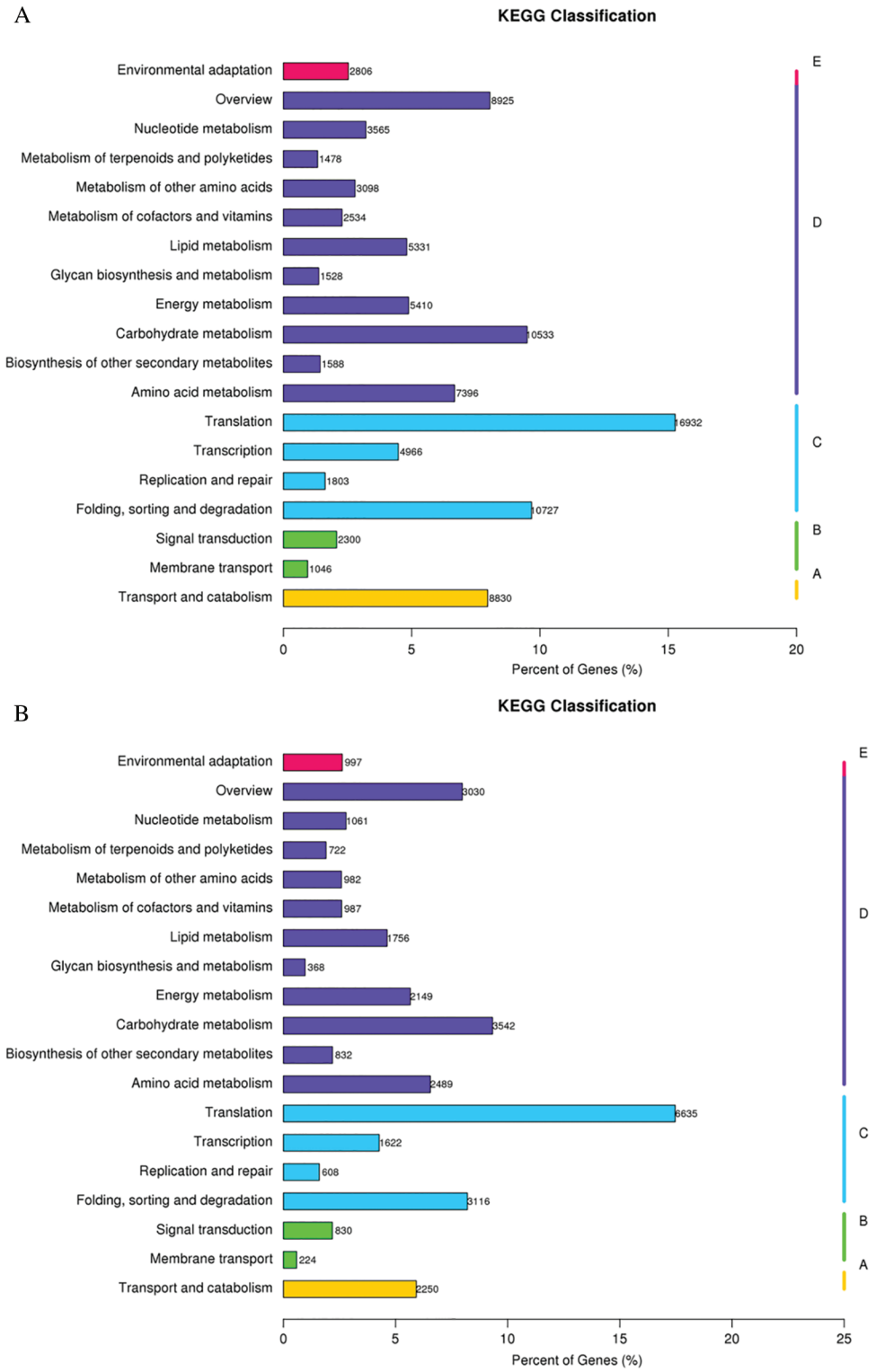

Figure 3: KEGG classification results. (A) Roots; (B) Shoots

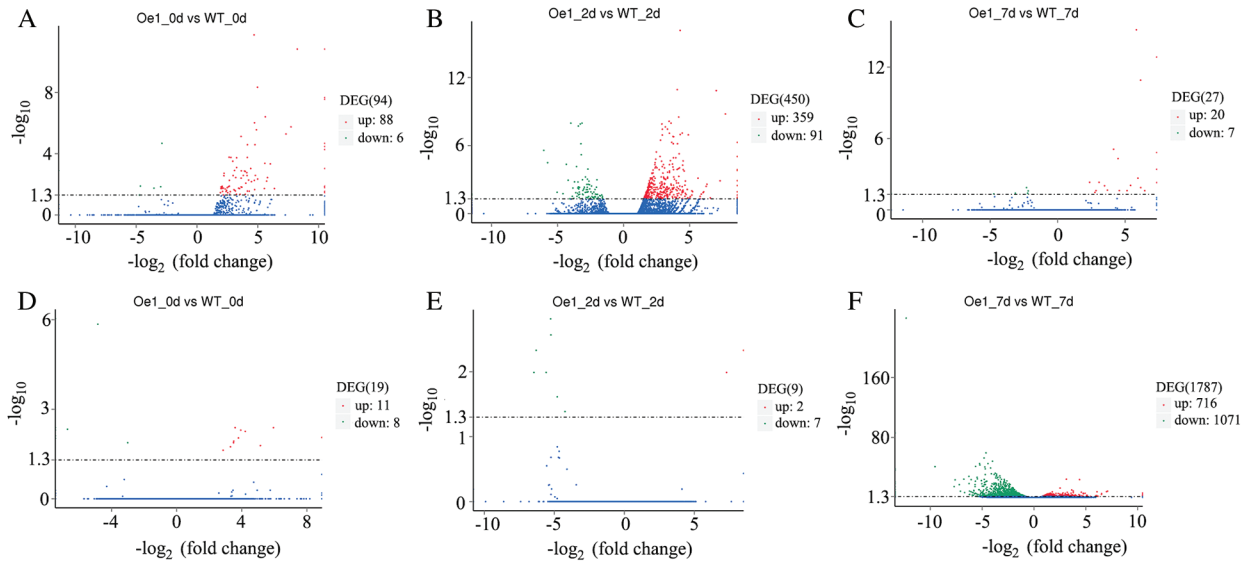

Figure 4: Genes expressed differentially in Oe1 and WT. The parameters ($p < 0.05$) were used as thresholds to determine the significance of the difference in gene expression. Red dots represent transcripts more prevalent in the Oe1 library compared with WT. Green dots show those present at a lower frequency in the Oe1 library and blue dots indicate transcripts that did not change significantly. (A–C) Roots; (D–F) Shoots

3.4 Analysis of DEGs in the Key Metabolic Pathways

As the remarkable differences at the metabolomics level, pyruvate metabolism and TCA cycle pathways had mainly discussed in our previous research. Here, these two pathways were also showed difference after 2 d LP-treatment in roots between Oe1 and WT. Part of the genes were identified in these key metabolic pathways. c137909_g1 in pyruvate metabolism and c183919_g1 in TCA cycle were up-regulated 18.25- and 3.62-folds more than those of WT under LP stress, respectively (Fig. 6).

4 Discussion

4.1 Low Phosphorus Affects Transcription Diversity of a Range of Genes

Multi-transcriptome database and candidate genes were provided for further study of genotypes with and without low-phosphorus tolerance in maize. The P deficiency-responsive genes common to low P-tolerant and the low P-sensitive line were associated with acid phosphatase (APase) activity and a series of metabolic pathways [15]. Transcriptome analysis reveals candidate genes related to low-P stress in sorghum. GO enrichment analyses showed that the candidate genes were involved in oxidoreductase activity. These will form the molecular network of sorghum responding to low P stress, and then contribute to the high-quality breeding with low P tolerance [19]. GO analysis showed that apple trees responded to metabolic processes, cell proliferation, biological process regulation, reactive oxygen metabolism and flavonoid metabolism under phosphorus stress. Pathway analysis in KEGG further showed that DEGs acted on mitogen-activated protein kinase (MAPK) signaling pathway, flavonoid biosynthesis, phenylpropanoid biosynthesis, and ATP binding box (ABC) transporters [20].

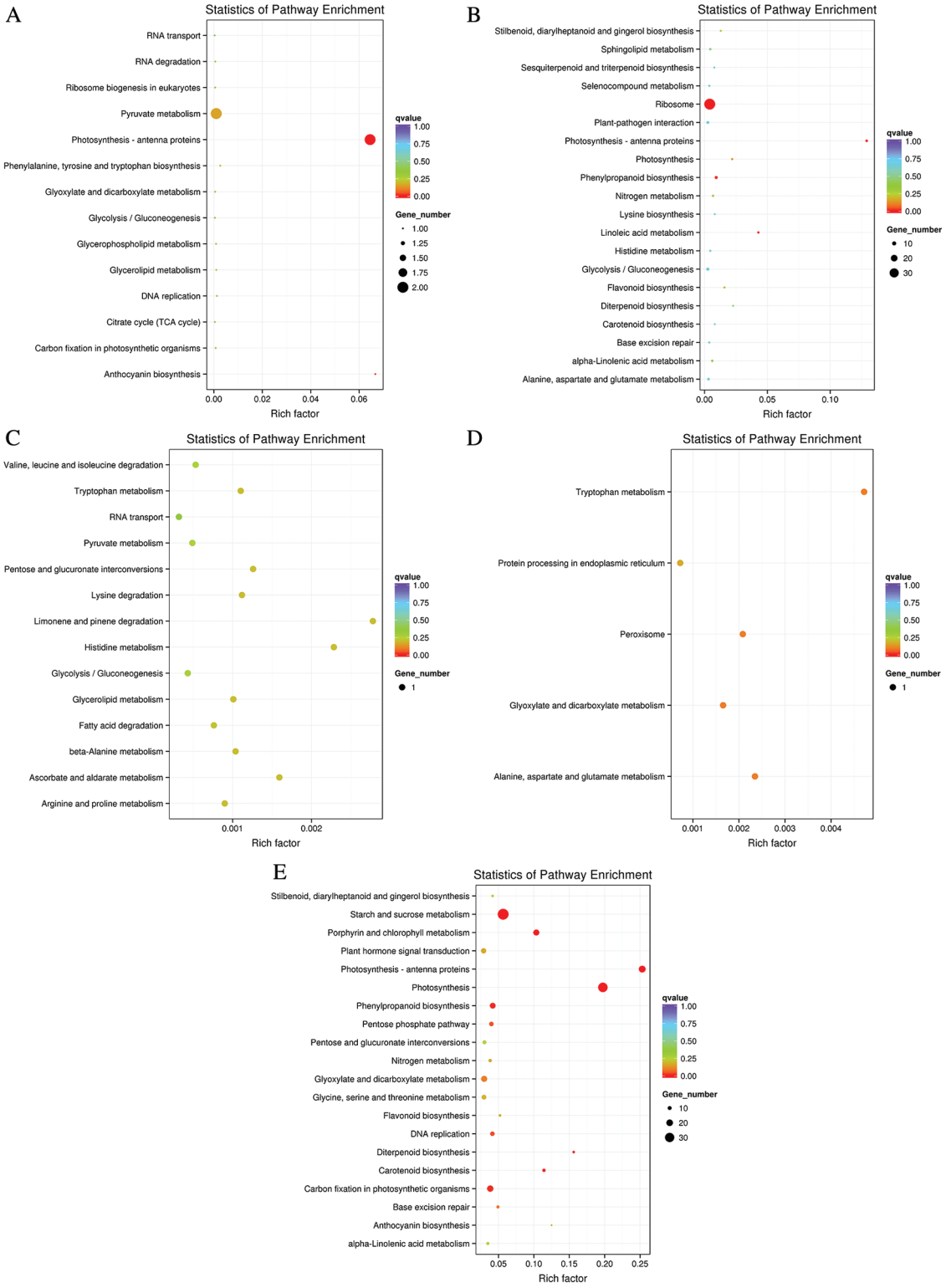

Figure 5: The top 20 pathways affected by DEGs according to the *p* value in KEGG. (A–C) Roots; (D–E) Shoots

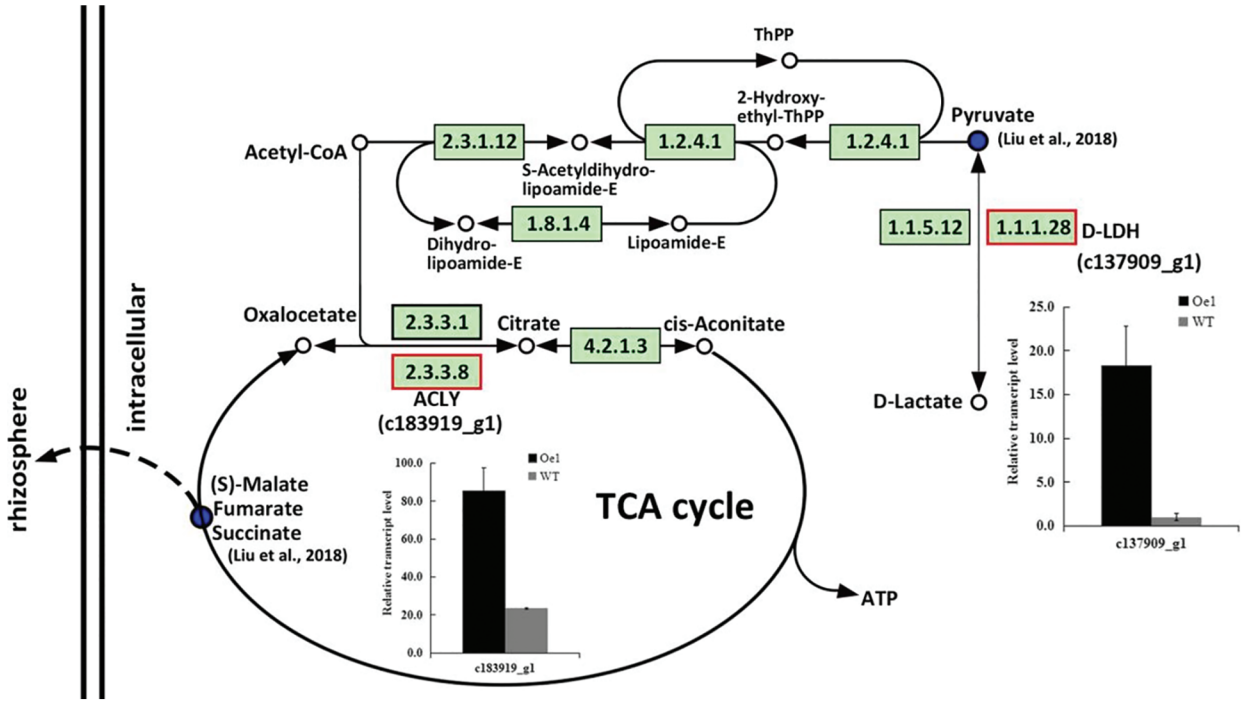

Figure 6: DEGs enriched by KEGG pathway in roots between Oe1 and WT under 2 d LP-stress. The metabolic pathway maps refer to Arabidopsis in KEGG database. The Up-regulated KEGG Orthology (KO) nodes were labeled with red frames. The bar chart shows the relative expression differences of adjacent genes

4.2 CmPht1;2 Affected Biological Processes at the Transcriptional Level

The majority of unigenes excavated in the taproot of Radish were predominately involved in basic physiological and metabolic processes, catalytic, binding, and cellular processes [21]. Gene ontology analysis revealed that salt stress-related categories involved in catalytic activity, binding, metabolic processes and cellular processes of the prunellae spica transcriptome under salt stress [22]. Similarly, here in our work, by the GO database, cellular process, metabolic process and binding were in the majority. These processes may indicate the endurance to abiotic stress of chrysanthemum. Meanwhile these unigenes were mainly annotated as translation and posttranslational modification both in roots and shoots by KOG, which contribute on the production of proteins. Nearly 200 different types of Post-translational modifications (PTMs) have been identified in previous studies, and most of them are critical regulators of protein function [23]. Under low phosphorus conditions, plant roots receive stress signals and transport them to above-ground tissues [24]. Similar to the results of the difference of MAPK signaling pathway genes in apple under LP stress, signal transduction mechanism performed noticeably in roots than shoots here [20]. The numbers of DEGs and difference pathways in roots were significantly higher than shoots under 2 d LP-treatment. While these numbers in shoots were significantly higher than those in roots under 7 d LP-treatment. This change might owe to the difference of signal transduction mechanism in roots which transmitted low phosphorus signal from roots to shoots. Carbohydrate metabolism, transport and catabolism were specially separated by KEGG in our data. These results checked with the data on many plant stress in several species. Carbohydrate metabolism focuses on biosynthesis, catabolism and biological function of the major storage carbohydrates during growth and development of the plant [25]. Soluble sugars, an important part of carbohydrate metabolism, provide important protection to plant stress. The accumulation of sorbitol and sucrose in the apple roots was higher, and the activities of

sucrose synthase, invertase and sorbitol dehydrogenase, which are involved in the degradation of sucrose and sorbitol, were significantly increased under a low nitrogen supply [26]. However, the influence by the overexpression of phosphate transporter genes had rarely reported before. Therefore *CmPht1;2*-overexpression or high phosphate concentration of Oe1 might make an effect on the biological processes after LP treatment both in roots and shoots [12,27].

4.3 Overexpression of CmPht1;2 Might Promoted Several Pathways Directly or Indirectly

TCA cycle is one of the iconic pathways in terms of energy metabolism, being responsible for the oxidation of respiratory substrates to drive ATP synthesis [28]. Mitochondrial TCA cycle signalling involved in the regulation of DNA and histone modifications to enable dynamic control of expanded genome during eukaryogenesis. And TCA cycle remodeling is also used to coordinate stress responses and gene expression programmes in eukaryotic organisms [29]. Intermediates of glycolytic and TCA cycle were crucial players response to oxidative stress in Arabidopsis [30]. Pyruvate is the key node connecting glycolysis and TCA cycle. Genome-wide association study in wheat indicated that 6 genes involved in pyruvate metabolism and TCA cycle were upregulated significantly in the P efficient variety under LP stress [31]. D-lactate is the end product of glyoxalase system. D-Lactate dehydrogenase (D-LDH) catalyzes the oxidation of D-lactate to pyruvate using flavin adenine dinucleotide as a cofactor in Arabidopsis [32]. D-LDH completes the detoxification process of methylglyoxal and diverts the stress induced toxic metabolites D-lactate towards energy production in rice [33]. Thereby D-LDH could protect the cells from their deteriorating effects and enhance resistance to multiple abiotic stresses of plants. Here. in our results, c137909_g1 in pyruvate metabolism which was commented to D-LDH had a significantly higher expression in the Oe1 roots compared with WT under LP condition. These were able to provide a detoxification process of methylglyoxal and a further transformation from D-lactate to energy. While instead of increase, pyruvate went down as the increase of D-LDH in the metabolism data of our previous research [12]. We hypothesized that this is due to the rapid transformation of pyruvate into the TCA cycle by way of acetyl-CoA. ATP-citrate lyase (ACLY) could catalyze the transformation among acetyl-CoA, oxaloacetate and citrate based on the KEGG database. So ACLY is the key node of pyruvate metabolism going into the TCA cycle. Here c183919_g1 (ACLY) also showed a higher expression in the Oe1 roots than WT and perhaps play a key role in promoting the TCA cycle. Malate affected the length of the primary root and the number of tips in sorghum suffering from low-P stress [19]. Root-exuded organic acids will simultaneously and disproportionately increase to facilitate the Pi mobility under P deficiency [34]. In the early work, we found a prominent decrease of malate, fumarate and succinate in TCA cycle, and speculated an enhanced secretion of organic acids into the rhizosphere [12]. Therefore, the up-regulation of D-LDH and ACLY in the Oe1 plants compared with WT might enhance the growth potential and root development through promoting the pyruvate metabolism and TCA cycle in chrysanthemum. The general phenylpropanoid metabolism can generate an enormous array of secondary metabolites to result in an organ and developmentally specific pattern of metabolites in plants [35]. Salt interferences to metabolite accumulation and flavonoid biosynthesis in *Tetrastigma hemsleyanum* might reveal the response to salt stress by flavonoid biosynthesis in plants [36]. Here, phenylpropanoid biosynthesis and flavonoid biosynthesis were shared by both roots and shoots, but with different number of DEGs. These results might indicate the different organ and development specific pattern between roots and shoots, and the resistance response to LP stress in chrysanthemum.

5 Conclusion

This study found that *CmPht1;2* affected a series of biological processes in the transcriptional level to surface the LP stress. *CmPht1;2* might promote the TCA cycle and pyruvate metabolism pathway directly or indirectly which enhance the growth potential and root development. These processes may contribute to the mechanism of tolerance under LP stress in chrysanthemum.

Acknowledgement: We thank to the support of a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, PAPD, and Central laboratory of College of Horticulture, Nanjing Agricultural University.

Funding Statement: This research was funded by the National Natural Science Foundation of China (31902061), CL. https://www.nsfc.gov.cn.

Author Contributions: The authors confirm contribution to the paper as follows: study conception and design: Sumei Chen, Fadi Chen, Jiafu Jiang; data collection, analysis and interpretation of results: Chen Liu, Xiaowei Tang, Xiaohui Qu; draft manuscript preparation: Chen Liu. All authors reviewed the results and approved the final version of the manuscript.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Supplementary Materials

Supplementary Table S1: Primers used in the manuscript

Supplementary Table S2: Pathways influenced by 2 d LP stress between Oe1 and WT in roots

Supplementary Table S3: Pathways influenced by 7 d LP stress between Oe1 and WT in shoots