Comparative transcriptome analysis provides insights into seed germination in herbicide resistance Polypogon fugax

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Abstract

Background

Herbicide resistant mutations are predicted to exhibit fitness cost under herbicide-free conditions. Asia minor bluegrass (*Polypogon fugax*) is a common weed species in the winter crops. Our previous study established a *P. fugax* accession (LR) resistant to acetyl-CoA carboxylase (ACCase) inhibiting herbicides. Besides, LR also exhibited fitness cost, like lower germination, relative to the sensitive plants (LS). But little is known about the gene expression profile of seed germination process in herbicide resistance *P.fugax*.

Results

The maximum germination (gMAX) of LS reached 94%, while that of LR was 52%. Exogenous gibberellin and abscisic acid synthesis inhibitor fluridone could break the dormancy of LR, increasing gMAX to 78% and 94%, respectively. A comparative transcriptome was conducted to analyze the genes expression profile of LS and LR at two germination time points. A total of 11,856 and 23,123 differentially expressed genes (DEGs) were identified in LS and LR at two time points. Most of DEGs were involved in lipid metabolism, carbohydrate metabolism, amino acid metabolism and secondary metabolites biosynthesis. Four genes related to GA biosynthesis and signal transduction showed higher expression in LS and five genes related to ABA biosynthesis and signal transduction showed higher expression in LR during germination. Twenty-four genes involved in glycolysis, TCA cycle, pentose phosphate pathway and fatty acid metabolism had higher expression levels in LS than LR during germination.

Conclusions

Our results indicated that GA biosynthesis and transduction, carbohydrate and fatty acid metabolism was more actively expressed in LS plants, resulting in stronger germination ability of LS relative to LR. The highly expressed genes in ABA biosynthesis and transduction contribute to maintain the seed dormancy of LR plants. This study provided new insight into transcriptional changes and interaction in seed germination process of *P. fugax*, and compared the differential expression profile between the herbicides resistant and sensitive plants during seed germination.

Background

Herbicide resistant mutations were considered as pre-existed or arise spontaneously with very low rate within weeds populations [1–3]. With frequent herbicide application, herbicide resistant mutations are rapidly selected and enriched in weed populations [4]. Without herbicide stress, herbicide resistant mutations are predict to exhibit an adaptation cost (fitness cost) due to the impaired enzyme ability or altered feedback inhibition resulting in insufficient or excessive product biosynthesis [5–7]. Many

herbicide resistant weeds exhibited altered phenotypes, including seed germination ability, growth cycle, biomass production, competitive ability and reproduction [8, 9]. For instance, the acetolactate synthase (ALS) and ACCase herbicides multiple-resistance *Lolium rigidum* biotypes showed lower vigor and competitive ability against wheat than that of the sensitive biotype [10]. The *Alopecurus myosuroides ACCase* gene mutant showed a reduction of biomass, height and seed production [11]. Two *Hordeum glaucum ACCase* gene mutants exhibited lower growth rate, biomass and seed production [12].

Asia minor bluegrass (*Polypogon fugax*) is a common winter weed species distributed across China and other Asian countries. This annual grass has an extended period of emergence from early November to late December. Its life cycle is highly close to several winter crops, including wheat (*Triticum aestivum* L.), rapeseed (*Brassica napus* L.) and some vegetables [13, 14]. *P. fugax* is a competitive weed, especially in moist soil and has become an increasing problem in wheat or rapeseed fields in rotation with rice [15]. In our previous study, a *P. fugax* accession (LR) collected from Sichuan province in China was highly resistant to ACCase inhibiting herbicides. The resistance mechanism was conferred by an Ile-2041-Asn substitution in *ACCase* gene [16]. In addition, this herbicide resistance plants exhibited fitness cost, including lower germination and emergence potential, earlier flowering and tiller and panicle emergence and seed shedding, relaively to the sensitive plants [17, 18]. A transcriptome study of the *P.fugax* resistant and sensitive plants were performed at the flowering stage, which provide a genomic resource for understanding the molecular basis of early flowering. The study identified twelve genes exhibited different expression pattern in herbicide resistant plants and sensitive plants [19]. But the molecular basis of lower germination ability in the herbicide resistance plants was still unclear. Whether the altered phenotypes were a consequence of pleiotropic effects of resistance mutations was difficult to figure out. The fitness cost occurred in herbicide resistance mutations are not universal, which depends on the particular mutation[11, 20–22], genetic background [23], and environmental conditions [24, 25]. Thus, the sensitive plants used in this study were collected from the same habitat of resistant plants to minimize the genetic background variance. The resistance and sensitive plants reproduced for three generations under the same environmental condition to minimize the influence of environmental conditions. These limitations allowed us to focus on the effect of ACCase mutation on fitness cost.

Seed germination and dormancy are important components of plant fitness with complex regulation network, including energy production, protein metabolism, phytohormone control and transcription factors regulation. With the development of genomics, transcriptomic analysis was one of the most effective and economic approaches to investigate the difference of germination process between the tested samples. A transcriptome analysis of two maize inbred lines with different germination ability identified abundant genes associated with plant hormones, nutrient reservoir, amino acid metabolism, and ribsome in driving seed germination. Genes related to plant hormones and amino acid metabolism showed higher expression level in the maize with high germination ability [26]. A comparative transcriptome analysis between two sheepgrass (*Leymus chinensis*) germplasm with various germination percentages at three developing stages indicated that genes involved in starch and sucrose metabolism, phenylpropanoid biosynthesis, plant hormone signal transduction, amino sugar and nucleotide sugar metabolism, and photosynthesis were significantly changed [27]. The aim of this study

is to investigate the differential expression profile of seed germination process in the herbicide resistant and sensitive plants, and try to figure out the relevant biological pathways or function genes contribute to germination delay in herbicide resistant plants.

Results And Discussion

Characterization of seed germination in LS and LR

The maximum germination (gMAX) of LR was about 52% while that of LS reached 94% (Fig 1a), consistent with the previous study results [17], suggesting that the low germination character was steadily inherited in LR. According to the germination curve, the rapid growth period was from 3d to 8d after incubated (DAI). Seed germination is a process with complex physiological, biochemical and molecular biological basis [28]. With the absorption of water, the storage macromolecules such as starch, proteins and lipids were decomposed into small and soluble molecules that can be easily utilized and transformed to support the germination [29]. The content of soluble sugar in the 0 DAI seeds of LS was higher than LR. After incubation, the soluble sugar content deceased and then dramatically increased at 6 DAI in LS and LR seeds. The soluble sugar content of LR seeds was always lower than that of LS seeds until 8 DAI (Fig. 1b). Similarly, the content of soluble protein showed down first and then up, and the content in LR was lower than that in LS during the germination process (Fig. 1c). These results indicated that more soluble sugar and protein could directly participate carbon oxidation and enzyme catalysis to supply energy in LS in comparison with LR during germination. Combined with the dynamic change of germination rate and soluble sugar and protein content, we chose 3 DAI and 6 DAI seeds for transcriptome analysis to compare the difference of germination process between LS and LR.

Hormones regulate the seed germination of LS and LR

Gibberellin (GA) and abscisic acid (ABA) antagonistically regulate the transition of germination and dormancy, that GA promote germination and ABA promote dormancy [30] To investigate whether the germination delay of LR was related to the hormonal regulation, the seeds of LS and LR were treated with exogenous GA and ABA and their synthesis inhibitors, fluridone (FL) and paclobutrazol (PA), to observed the gMAX values (Fig. 1d). The application of GA significantly evaluated the gMAX of LR from 52% to 78%, suggesting that exogenous GA could promote the germination of LR. The application of ABA had no significant effect on the gMAX of LS but decrease that of LR to 17%, suggesting that the inhibitory by ABA was stronger in LR than LS. PA and FL inhibits the GA and ABA biosynthesis had been proved in many plant species [31]. The application of PA decreased the gMAX of LS to 73% and that of LR decreased to 25%, suggesting that the GA biosynthesis in LR was more strongly inhibited by PA. The application of FL dramatically increased the gMAX of LR to 94%, suggesting that the dormancy of LR completely removed when ABA biosynthesis was inhibited.

de novo assembly of *P.fugx* reference transcriptome

Two *P. fugax* accessions LS and LR were incubated for 3d and 6d, respectively, and three biological replicates were designed in each accession at two time points. Thus twelve samples were used to conduct the RNA-seq analysis. The number of total clean reads of each samples were shown in Table 1. A total of 476,220 transcripts and 149,330 genes were *de novo* assembled, and the distribution of length interval was shown in Fig. 2a.

The number of annotated unigenes of each database was show in Table 2. Basically, a total of 99,209 genes (66.43%) were at least annotated to one database, suggesting that the *de novo* transcriptome had relatively complete gene function information. Among them, 67,183 genes (44.98%) were annotated to NR database, and established that *P.fugax* were highly similar to *Aegilops tauschii* subsp. *tauschii* (30.4%), *Brachypodium distachyon* (L.) P. Beauv. (16.5%), *Hordeum vulgare* subsp. *vulgare* (8.8%), *Triticum urartu* (5.6%), and *Oryza sativa* Japonica Group (4.4%) (Fig. 2b). A total of 20,042 genes (13.42%) were annotated into 25 classifications of KOG database (Additional file 2). Among them, the highest number of categories were "posttranslational modification, protein turnover, chaperones" (2,701, 13.48%), "general function prediction only" (2,627, 13.11%) and "translation, ribosomal structure and biogenesis" (2,321, 11.58%) (Fig. 2c). Moreover, there were 61,384 genes (41.1%) annotated to GO database, 22,834 unigenes (15.29%) annotated to KEGG database, 68,540 (45.89%) to Nt database, and 52,642 (35.25%) to SwissProt database (Additional file 3, 4).

Identification of differentially expressed genes (DEGs)

To determine the molecular basis involved in the germination process of LS and LR, two comparison settings, LS_6d vs LS_3d and LR_6d vs LR_3d, were analyzed. The biological samples had good reproducibility due to the high correlation values (Fig. 3c). The distribution of the DEGs in LS and LR were directly exhibited in volcano plots (Fig. 3a and 3b). A total of 11,856 DEGs were identified and 8,936 were up-regulated and 2,920 were down-regulated in the LS comparison setting. A total of 23,123 DEGs were identified and 20,055 were up-regulated and 3,068 were down-regulated in the LR comparison setting. A venn diagram showed that 7,165 up-regulated DEGs and 1,167 down-regulated DEGs were common in LS and LR comparison settings (Fig. 3d).

GO and KEGG enrichment of DEGs

To understand the difference in biological function and processes related to germination between LS and LR from 3 DAI to 6 DAI, all the DEGs were enriched to GO and KEGG database. The up-regulated DEGs in LS were enriched to 109 GO terms and the down-regulated DEGs were enriched to 9 GO terms (Additional file 5). The up-regulated DEGs in LR were enriched to 224 GO terms and the down-regulated DEGs were enriched to 40 GO terms (Additional file 6). The up-regulated DEGs in LS and LR were highly enriched in "metabolic process", "single-organism process", and "catalytic activity". With regard to the down-regulated DEGs, the enriched GO terms were much less in LS than LR. These results indicated that most of the up-regulated DEGs in both LS and LR had similar biological function. All the up-regulated DEGs in LS and LR were enriched to KEGG database and the top 20 enriched pathways were shown in Fig. 4a and 4b. The majority of up-regulated DEGs were related to lipid metabolism, carbohydrate metabolism, amino acid

metabolism and secondary metabolites biosynthesis in LS and LR seed during germination. Here, we selected some pathways to investigate the difference of regulation network in LS and LR (Table 3).

DEGs related to hormones biosynthesis and signal transduction

Seeds germination and dormancy processes are regulated by diverse endogenous hormones. According to the results of exogenous hormones regulation, GA and ABA affected the seed germination of LS and LR at different levels. The genes (Cluster-37472.24032 and Cluster-37472.23672) coding two ent-kaurene synthases (KS) and gene (Cluster-37472.70387) encoding gibberellin 3beta-hydroxylase (GA3ox) were up-regulated in both LS and LR comparison settings, and the increasing was much higher in LS than that in LR. The first step in GA biosynthetic pathway is transformed geranylgeranyl pyrophosphate to ent-kaurene catalyzed by ent-kaurene synthetases. The *KS* deficient mutants of Arabidopsis showed strong seed dormancy and recovered germinate with exogenous GA treatment [32, 33]. GA3ox catalyzes the final biosynthetic step to produce bioactive GAs. Two Arabidopsis genes, *GA4* and *GA4H*, encoding GA3ox were expressed during seed germination [34]. These GA biosynthesis related genes with higher expression in LS suggested that GA biosynthesis was more active in LS than LR. Consistent with this result, the inhibition of GA biosynthesis by PA was more effective in LR than LS. The gene (Cluster-51396.0) encoding phytochrome-interacting factor 4 (PIF4) was down-regulated in LS but up-regulated in LR during germination. PIF4 regulated the gibberellin-signaling pathway via DELLA proteins, which blocked the GA signal pathway and resulting in germination delay [35]. Thus, the increased expression of PIF4 might repress seed germination via inhibiting the GA signal transduction in LR.

ABA is the major hormone that inducing seed dormancy. The gene (Cluster-37472.23110) coding 9-cis-epoxycarotenoid dioxygenase (NCED) was down-regulated in LS but up-regulated in LR during germination. The expression of gene (Cluster-37472.23992) coding phytoene synthase (PSY) and gene (Cluster-37472.76697) coding lycopene epsilon-cyclase (LcyE) were both elevated in LS and LR during germination, and the increasing was higher in LR than LS. The cleavage of 9-cis-epoxycarotenoids catalyzed by NCED is the key regulatory step of ABA biosynthesis. The Arabidopsis mutants of *NCED* genes, *Atnced6* and *Atnced9*, reduced ABA content level in seeds [36]. PSY is the limiting step of carotenoids synthesis, the upstream of ABA biosynthesis. The *PSY* gene overexpressed Arabidopsis mutant exhibited delayed germination, and the degree of delay was positively associated with the increased levels of carotenoids and ABA [37]. The gene (Cluster-37472.6593) encoding serine/threonine-protein kinase SRK2 (SNRK2) and gene (Cluster-48355.0) coding ABA responsive element binding factor (ABF) were up-regulated in LS and LR, and increasing were higher in LR than LS. It has established in rice and Arabidopsis that SNRK2 is activated by ABA and phosphorylate ABF, which is important for the activation of ABA signal transduction [38, 39]. Taken together, these genes associated with ABA biosynthesis and signal transduction pathways exhibited higher expression level in LR than LS, which might explain why the LR seeds were more sensitive to ABA and FL could restore the germination ability of LR.

DEGs related to fatty acid metabolism

ACCase catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, acting as the initial step of fatty acid biosynthesis [40, 41]. ACCase has three subunits: biotin carboxylase carrier protein, biotin carboxylase, and carboxyltransferase domains [42]. Molecular and biochemical studies have proved that the carboxyltransferase domain is the target site of ACCase-inhibiting herbicides [43]. LR plants had an amino acid substitution in carboxyltransferase domain that caused the resistance to ACCase-inhibiting herbicide [16]. Unfortunately, the transcriptome data did not identify any DEGs annotated as ACCase carboxyltransferase domain in LS or LR. But gene (Cluster-39490.0) coding ACCase biotin carboxylase (ACACA) was identified to up-regulated with the higher increasing level in LS than LR. ACCase is the rate-limiting enzyme in fatty acid metabolism, the different regulation level between LS and LR might influence the downstream reactions as well. The gene (Cluster-31226.0) coding 3-oxoacyl-[acyl-carrier protein] reductase (FabG), gene (Cluster-37472.4287) coding long-chain acyl-CoA synthetase (ACSL) showed higher regulation level in LS compared with LR during germination. These enzymes were respectively involved in the elongation cycle and termination of fatty acid synthesis. Besides, the genes involved in fatty acid degradation were also up-regulated higher in LS than LR, such as the gene (Cluster-33548.0) coding acetyl-CoA acyltransferase 1 (ACAA1), gene (Cluster-37472.85335) coding acyl-CoA dehydrogenase (ACADM), gene (Cluster-30044.0) coding acetyl-CoA C-acetyltransferase (ACAT). The fatty acid forms triacylglycerol (TAG) that degrades to provide carbon and energy during germination and early seedling growth. Some ACCase herbicide resistant weeds also showed higher level of dormancy, absence of germination in dark conditions, and delayed seed germination [44-46]. We assumed that the mutated ACCase might alter normal fatty acid metabolism and impact the germination.

DEGs related to carbohydrate metabolism

Acetyl-CoA, acts as the precursor of fatty acid biosynthesis, is also the product of glycolysis and TCA. According to the dynamic change of soluble sugar and protein content, more soluble sugar and protein could directly participate carbon oxidation and enzyme catalysis in LS than LR during germination. Thus, the genes involved in carbohydrate metabolism were selected to compare the differential expression pattern. The gene (Cluster-37472.6545) coding glucose-6-phosphate isomerase (GPI)、gene (Cluster-37472.71503) coding fructose-bisphosphate aldolase (FBA), gene (Cluster-35171.2) coding phosphoenolpyruvate carboxykinase (PCKA), gene (Cluster-40718.0) coding pyruvate dehydrogenase E1 component alpha subunit (PDHA), genes (Cluster-37472.39187, Cluster-37472.47005) coding alcohol dehydrogenase (ADH), were up-regulated in LS and LR comparison settings, and the increasing levels were higher in LS than LR. All of these genes were involved in glycolysis, which produces energy by utilizing endosaccharides [47]. PCKA catabolizes the storage lipid and protein to produce soluble sugar via glycolysis. The PCKA deficient mutants of Arabidopsis and tomato were observed growth suppression of germinated seedlings [48].

The genes (Cluster-37472.85108, Cluster-15133.3) coding malate dehydrogenase (MDH), gene (Cluster-37472.21027) coding citrate synthase (CS), gene (Cluster-33485.0) coding 2-oxoglutarate dehydrogenase E1 component (OGDH), genes (Cluster-38121.0, Cluster-34010.0) coding succinyl-CoA synthetase alpha subunit and beta subunit (LSC1 and LSC2), genes (Cluster-37472.82495, Cluster-39676.0) coding

succinate dehydrogenase (ubiquinone) iron-sulfur subunit (SDHB), and gene (Cluster-41938.0) coding pyruvate carboxylase (PYC) were up-regulated in LS and LR at 6 DAI compared with 3 DAI, and the increasing levels were higher in LS than LR. All of these genes were involved in TCA cycle, which produced high-energy phosphate compounds as the main source of cellular energy [49]. MDH catalyze the interconversion of malate and oxaloacetate in TCA cycle. The Arabidopsis *MDH* gene mutants showed slowly germination rate, higher content of free amino acids, different sugar levels, and lower content of 2-oxoglutarate [50]. The seeds of the *CS* deficient Arabidopsis were dormant and did not metabolize triacyglycerol [51].

The gene (Cluster-37472.72264) coding 6-phosphogluconate dehydrogenase (PGD), gene (Cluster-37472.83492) coding transketolase (TKT), gene (Cluster-37472.237) coding gluconokinase (gntK) were highly up-regulated in LS compared with LR during germination. Pentose phosphate pathway takes substrate from glycolysis and feeds its products back into glycolysis, which is important in determining the flux through glycolysis [52].

One of the major changes during germination is a rapid increase in respiration, which involves glycolysis, oxidative pentose phosphate pathway, TCA cycle and oxidative phosphorylation. All of the DEGs mentioned above were related to TCA cycle, glycolysis or pentose phosphate pathway that producing energy during seed germination. These genes were up-regulated in LS and LR during germination and had higher expression in LS, indicating that more energy were supplied to promote germination in LS in comparison with LR.

Validation of DEGs by qRT-PCR

A total of sixteen DEGs were randomly selected to verify the accuracy and reproducibility of the transcriptome results by qRT-PCR (Fig. 5). The correlation between transcriptome results (FC) and qRT-PCR results ($2^{-\Delta\Delta Ct}$) were calculated using $\log_2$ fold variation measurements to produce a scatter plot. The results showed that the expression profiles of these DEGs were consistent with the transcriptome results, with relative R^2 = 0.8751 and 0.7376 in LS and LR, respectively.

Conclusion

In this study, we investigated the expression profile during seed germination in two *P.fugax* accessions at two time points. GA and FL promote the germination of LR plants, while ABA and PA showed greater inhibiting effect on germination of LR plants. Consistent with this, the transcriptome results identified four GA signal related genes with higher expression in LS and five ABA signal related genes with higher expression in LR during germination. LS showed higher content of soluble sugar and protein than LR during germination. The expression of the genes related to carbohydrate metabolism and fatty acid metabolism were highly increased in LS than LR, suggesting that more energy was supplied during seed germination in LS (Fig. 6). This study provided novel insight into the gene expression profile of seed germination in *P. fugax* and identified abundant function genes involved in germination delay in herbicide

resistant plants. It should stress that this study could not summarize the effect of herbicide resistance mutation on seed germination. Because the differential expression profile of seed germination was described only in two accessions. The pathways or function genes identified in this study should be verified in other resistant plants with germination delay.

Methods

Plant materials and growth condition

The *P.fugax* accessions were originally collected in 2012 in or nearby a grower's field in Qingshen County, Sichuan Province, China, and identified to be resistant and sensitive to ACCase inhibiting herbicides, respectively. No specific permission is required for the *P. fugax* accessions collection, and the collected location are not privately owned or natural protected areas. The plant materials were identified by Prof. Xiaogang Zhou from Sichuan Academy of Agriculture Sciences. The voucher specimens were deposited at State Key Laboratory of Rice Biology, China National Rice Research Institute. The F2 seeds collected from the resistant plants (LR) had lower germination rate compared with the sensitive plants (LS) under various environment conditions [17]. The seeds used in this study were F3 seeds collected from the F2 plants of LR and LS that separately cultivated in December 2018 screen house (an 8 m by 20 m chamber framed with 2-cm iron mesh and covered overhead with a transparent plastic cover to prevent rain damage) at the China National Rice Research Institute (CNRRI; 30.04°N, 119.55°E), Hangzhou, Zhejiang Province, China. The seeds were collected in Match 2019 and dried at 35°C for 7 days and stored at 4 °C under darkness for six months until use. The seed viability were tested by bromothymol blue and reached almost 100%.

Dynamic changes in germination percentage, soluble sugar and protein content

The peeled seeds of LS and LR were surface sterilized with 70% ethanol for 1 min and 10% sodium hypochlorite for 10 min and then washed with sterile water for three times. Sterile seeds placed on the 9 cm petri dishes with one layer of filter paper that moistened with sterile water. The seeds were incubated at 20/10 °C day/night cycles with a 12 h photoperiod in the growth chamber. The seed was considered as germinated when the radicle emergence to the seed length. The germinated seeds were observed by 40× microscope (Olympus) at 1d, 2d, 3d, 5d, 6d, 8d, 9d, 11d after incubated. The non-germinated seeds were tested the viability by bromothymol blue and the dead seeds were excluded from calculation. The germination curve was built based on the percentage of germination. Each sample had five dishes (30 seeds per dish) as replications.

0.1 g sterile seeds from each sample were collected after incubated for 0d, 4d, 6d and 8d, immediately frozen with liquid nitrogen and stored at -80°C. All the samples were homogenized in 1 ml cold distilled water and centrifuged at 12,000 rpm for 5 min and then collected the supernatants for measurement. The soluble protein content was measured as Bradford methods [53]. The soluble sugar concentration was determined by anthrone method [54]. Three replicates were analyzed for each treatment.

Effect of exogenous hormones on seed germination of AS and AR

The peeled seeds of LS and LR were surface sterilized and placed on the petri dishes with one layer of filter paper that moistened with 5 ml waters or tested solutions, including 100μM gibberellin$_{4+7}$ (GA), 1μM abscisic acid (ABA), 100μM fluridone (FL) and 1μM paclobutrazol (PA). The seeds were incubated under the same condition as mentioned above. The maximum germination (gMAX) was calculated the number of the germinated seeds divided with the total number of the viable seeds. Each treatment had five dishes (30 seeds per dish) as replications.

RNA isolation and transcriptome sequencing

The peeled seeds of LS and LR were incubated for 3d and 6d and collected for RNA extraction. Each sample had three biological replications named as LS_3d_1, LS_3d_2, LS_3d_3, LR_3d_1, LR_3d_2, LR_3d_3, LS_6d_1, LS_6d_2, LS_6d_3, LR_6d_1, LR_6d_2, LR_6d_3. RNA extraction and RNA-seq was performed by Novogene Corporation (Beijing, China). RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 1.5 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations.

Transcriptome assembly and functional classification

Raw reads of fastq format were firstly processed through in-house perl scripts. In this step, clean reads were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analysis were based on clean data with high quality. All clean reads of the twelve libraries were obtained using Trinity software to assemble the full-length transcript sequences [55]. Gene function was annotated based on the following databases: NR (NCBI non-redundant protein sequences)、NT (NCBI non-redundant nucleotide sequences)、Pfam (Protein family)、KOG/COG (Clusters of Orthologous Groups of proteins)、Swiss-Prot (A manually annotated and reviewed protein sequence database)、 KO (KEGG Ortholog database)、GO (Gene Ontology). The transcription factors were identified by iTAK program [56].

Differential gene expression and enrichment analysis

Gene expression levels were calculated using the fragments per kilobase per million fragments (FPKM) method [57]. Differential expression analysis was conducted at 6d compared to 3d in LS and LR using the DEGseq R package [58]. Q-value < 0.05 and | log2 (fold change) | > 1 as the threshold for significantly differential expression. All the DEGs were enriched to GO and KEGG database by GOseq and KOBAS software [59, 60]. The significant enriched GO terms and KEGG pathways were filtered by padj values < 0.05.

Quantitative real-time PCR validation

The quantitative real-time PCR (qRT-PCR) was performed to verify the DEGs identified from the transcriptome results. Total RNA was extracted from the LS and LR seeds under the same condition as the RNA-Seq samples using RNAprep Pure Plant Extraction Kit (Tiangen, China). Then, reverse transcription was performed with 1 μg of each RNA sample using cDNA Synthesis kit (TAKARA, China). qRT-PCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad, USA) on QuantStudio^{TM} 1 real-time PCR System and with the following thermal cycle conditions: denaturing at 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 1 min. The RT-PCR primers were designed based on the coding sequences (CDS) of the tested genes (Additional file 1. Table S1). *UBQ* was used as the housekeeping genes. Each treatment had three biological replications and the average values from three technical replications were used for calculating the relative expression level by $2^{-\Delta\Delta Ct}$ methods.

Abbreviations

ACCase
acetyl-CoA carboxylase
DEGs
differentially expressed genes
P.fugax
Polypogon fugax
GA
gibberellin
PA
paclobutrazol
ABA
abscisic acid
FL
fluridone
gMAX
maximum germination
DAI
day after incubated
TCA
tricarboxylic acid
NR
NCBI non-redundant protein sequences
NT
NCBI non-redundant nucleotide sequences

Pfam

Protein family

KOG

Clusters of Orthologous Groups of proteins

Swiss-Prot

A manually annotated and reviewed protein sequence database

KEGG

Kyoto Encyclopedia of Genes and Genomes

FPKM

fragments per kilobase per million fragments

qRT-PCR

quantitative real-time PCR

Declarations

Ethics approval and consent to participate

There were no specific permits required for plant collection in the selected locations. The sampling locations are not privately owned or natural protected areas. The plants used for the experiments are not considered an endangered or protected species. The collection is legal in China.

Consent for publication

Not applicable.

Availability of data and materials.

The raw reads have been deposited in the NCBI Sequence Read Archive (SRA) database (BioProject: PRJNA625626).

Competing interests

The authors declare that they have no competing interests.

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Authors' contribution

X Y contributes to the experiment design and writing; W T contribute to the plant materials preparation; Y Y and J Z contribute to data analysis and revise the paper; Y L contributes to supervise the experiments

and revise the paper. All authors reviewed the results and approved the final version of the manuscript.

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Tables

Table 1 Overview of the sequencing reads obtained from each sample.

Sample	Raw Reads	Clean Reads	Clean Bases	Error (%)	Q20 (%)	Q30 (%)	GC Content (%)
LR_3d_1	46947070	46112196	6.92G	0.03	97.95	94.2	55.51
LR_3d_2	57819080	56883382	8.53G	0.03	97.89	94.03	55.32
LR_3d_3	61713628	60389070	9.06G	0.03	97.87	93.97	55.07
LR_6d_1	55934224	55303162	8.3G	0.02	98	94.24	55.02
LR_6d_2	53631602	52984034	7.95G	0.02	98.03	94.3	55.09
LR_6d_3	63912876	63221906	9.48G	0.03	97.92	94.08	55.1
LS_3d_1	56261812	55216600	8.28G	0.03	97.83	93.9	54.98
LS_3d_2	56163918	55050700	8.26G	0.03	97.84	93.88	54.59
LS_3d_3	56540874	55577868	8.34G	0.03	97.89	94.03	54.98
LS_6d_1	57849026	56878644	8.53G	0.03	97.88	93.98	54.88
LS_6d_2	48963372	48180318	7.23G	0.03	97.92	94.1	54.63
LS_6d_3	49585538	48901900	7.34G	0.02	98.01	94.28	53.51

Table 2 Functional annotations of unigenes in the databases

	Number of Unigenes	Percentage (%)
Annotated in NR	67183	44.98
Annotated in NT	68540	45.89
Annotated in KO	22834	15.29
Annotated in SwissProt	52642	35.25
Annotated in PFAM	61384	41.1
Annotated in GO	61384	41.1
Annotated in KOG	20042	13.42
Annotated in all Databases	8625	5.77
Annotated in at least one Database	99209	66.43
Total Unigenes	149330	100

Table 3 DEGs significantly up- or down- regulated in LS and LR comparison settings.

Gene ID	log$_2$FC (LS_6d vs LS_3d)	log$_2$FC (LR_6d vs LR_3d)	Encoded protein	Description
Carbohydrate metabolism				
Cluster-37472.6545	7.36	3.84	GPI	glucose-6-phosphate isomerase
Cluster-37472.71503	5.65	2.84	FBA	fructose-bisphosphate aldolase
Cluster-40718.0	5.96	4.88	PDHA	pyruvate dehydrogenase E1 component alpha subunit
Cluster-35171.2	6.98	5.61	PCKA	phosphoenolpyruvate carboxykinase (ATP)
Cluster-37472.39187	3.80	1.85	ADH	alcohol dehydrogenase class-P
Cluster-37472.47005	4.19	2.04	ADH	alcohol dehydrogenase class-P
Cluster-37472.85108	8.43	6.07	MDH	malate dehydrogenase
Cluster-15133.3	6.95	3.19	MDH	malate dehydrogenase
Cluster-37472.21027	9.41	4.35	CS	citrate synthase
Cluster-33485.0	6.88	5.55	OGDH	2-oxoglutarate dehydrogenase E1 component
Cluster-38121.0	7.15	5.66	LSC1	succinyl-CoA synthetase alpha subunit
Cluster-34010.0	5.12	3.97	LSC2	succinyl-CoA synthetase beta subunit
Cluster-37472.82495	9.25	6.45	SDHB	succinate dehydrogenase (ubiquinone) iron-sulfur subunit
Cluster-39676.0	7.51	4.56	SDHB	succinate dehydrogenase (ubiquinone) iron-sulfur subunit
Cluster-41938.0	7.25	4.02	PYC	pyruvate carboxylase
Cluster-37472.72264	8.37	3.93	PGD	6-phosphogluconate dehydrogenase
Cluster-37472.83492	6.66	5.71	TKT	transketolase
Cluster-37472.237	7.72	4.88	gntK	gluconokinase
Fatty acid metabolism				
Cluster-39490.0	7.28	4.20	ACACA	acetyl-CoA carboxylase / biotin carboxylase 1
Cluster-31226.0	7.10	4.82	FabG	3-oxoacyl-[acyl-carrier protein] reductase
Cluster-37472.4287	9.16	5.52	ACSL	long-chain acyl-CoA synthetase
Cluster-33548.0	9.20	5.34	ACAA1	acetyl-CoA acyltransferase 1
Cluster-37472.85335	9.90	5.52	ACADM	acyl-CoA dehydrogenase
Cluster-30044.0	7.77	4.75	ACAT	acetyl-CoA C-acetyltransferase
Hormones biosynthesis and signal transduction				
Cluster-37472.24032	9.69	3.87	KS	ent-copalyl diphosphate synthase
Cluster-37472.23672	6.42	4.78	KS	ent-kaurene synthase
Cluster-37472.70387	3.31	2.02	GA3ox	gibberellin 3-beta-dioxygenase
Cluster-51396.0	-1.69	3.01	PIF4	phytochrome-interacting factor 4
Cluster-37472.23110	-1.97	1.81	NCED	9-cis-epoxycarotenoid dioxygenase
Cluster-37472.23992	2.73	3.36	PSY	phytoene synthase
Cluster-37472.76697	1.64	2.53	LcyE	lycopene epsilon-cyclase

Cluster-37472.6593	1.32	2.78	SNRK2	serine/threonine-protein kinase SRK2
Cluster-48355.0	1.63	2.58	ABF	ABA responsive element binding factor

Supplementary Information

Additional file 1: RT-PCR primers

Additional file 2: KOG classification

Additional file 3: GO classification

Additional file 4: KEGG classification

Additional file 5: Go function enrichment of up- or down-regulated DEGs at 3DAI compared with 6 DAI in LS

Additional file 6: Go function enrichment of up- or down-regulated DEGs at 3DAI compared with 6 DAI in LR

Figures
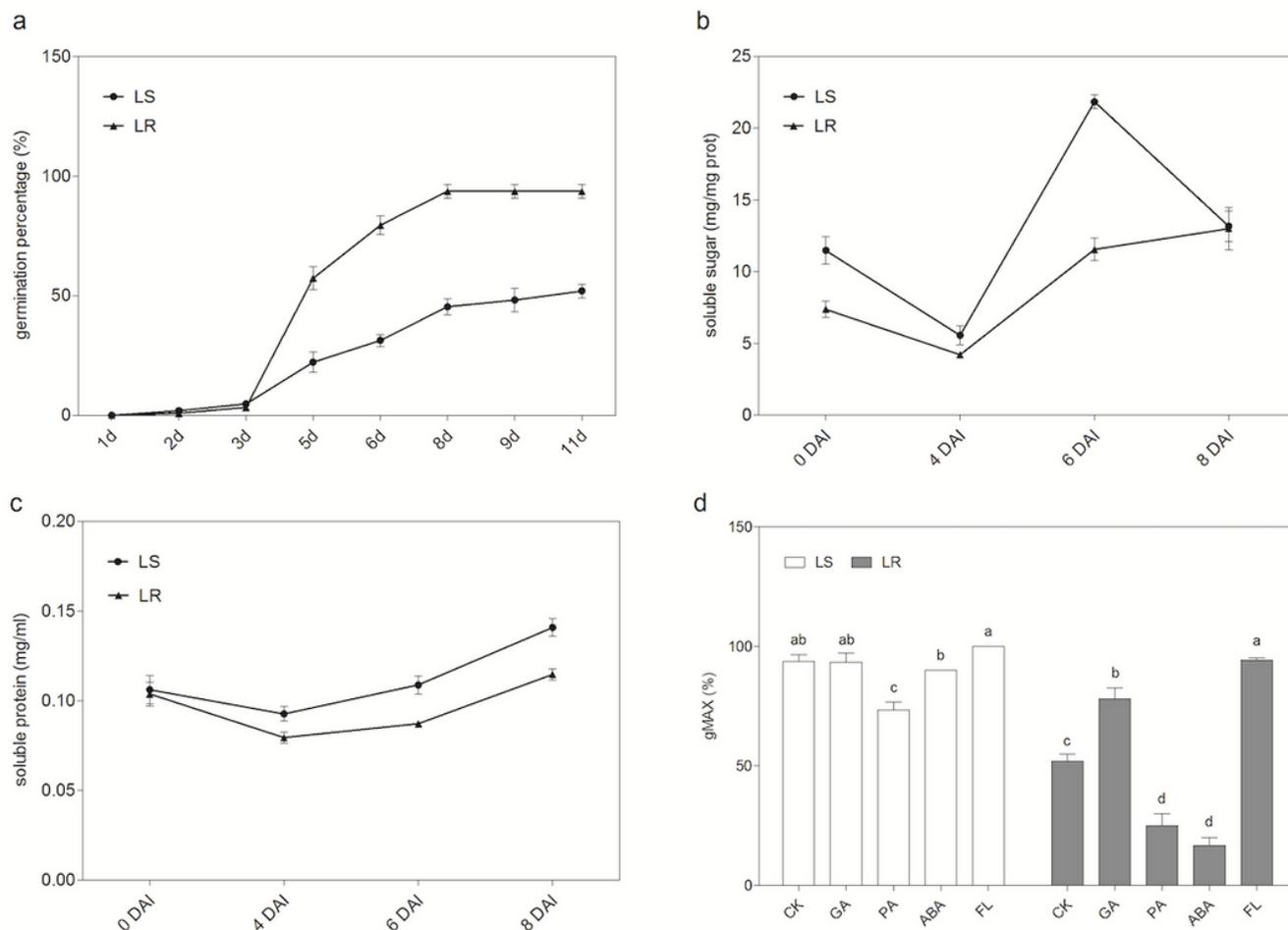
Figure 1

Seed germination characters in LS and LR. a Dynamic change of germination percentage of LS and LR seeds; b and c Dynamics change of soluble sugar and protein content of LS and LR seeds. d. The maximum germination (gMAX) of LS and LR with exogenous hormones application. CK: water, GA: gibberellin, PA: paclobutrazol, ABA: abscisic acid, FL: fluridone. Bars are mean ± standard error (n = 5). Values in a column followed by the different letters means significant differences (P ≤ 0.05).
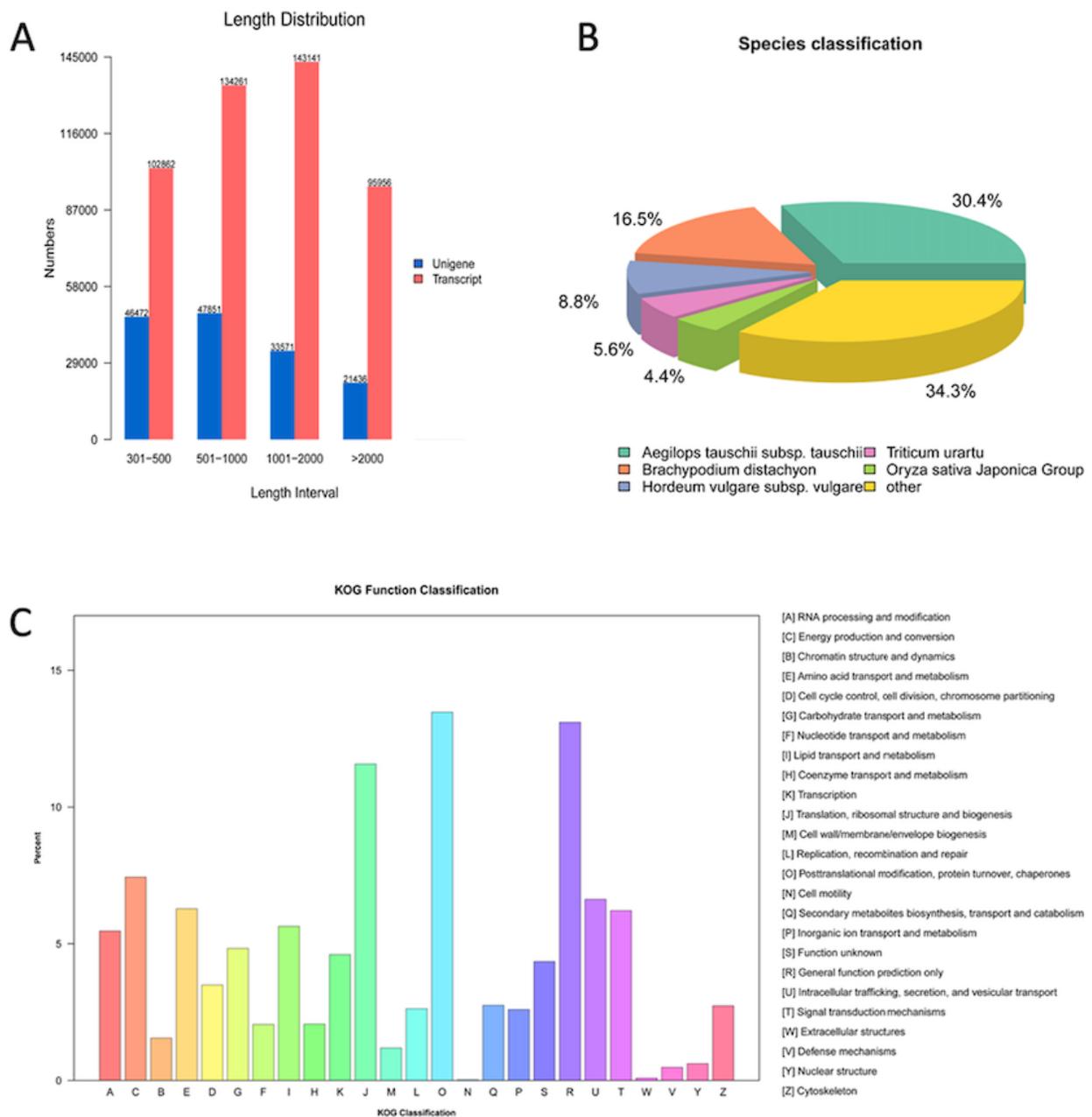
Figure 2

Transcriptome assembly and unigenes annotation of P.fugax. a The length distribution of unigenes and transcripts; b Species classification of P. fugax transcriptome annotated to NR database. The numbers near the pie charts indicate the percentage of unigenes in each class; c KOG function classification of unigenes.
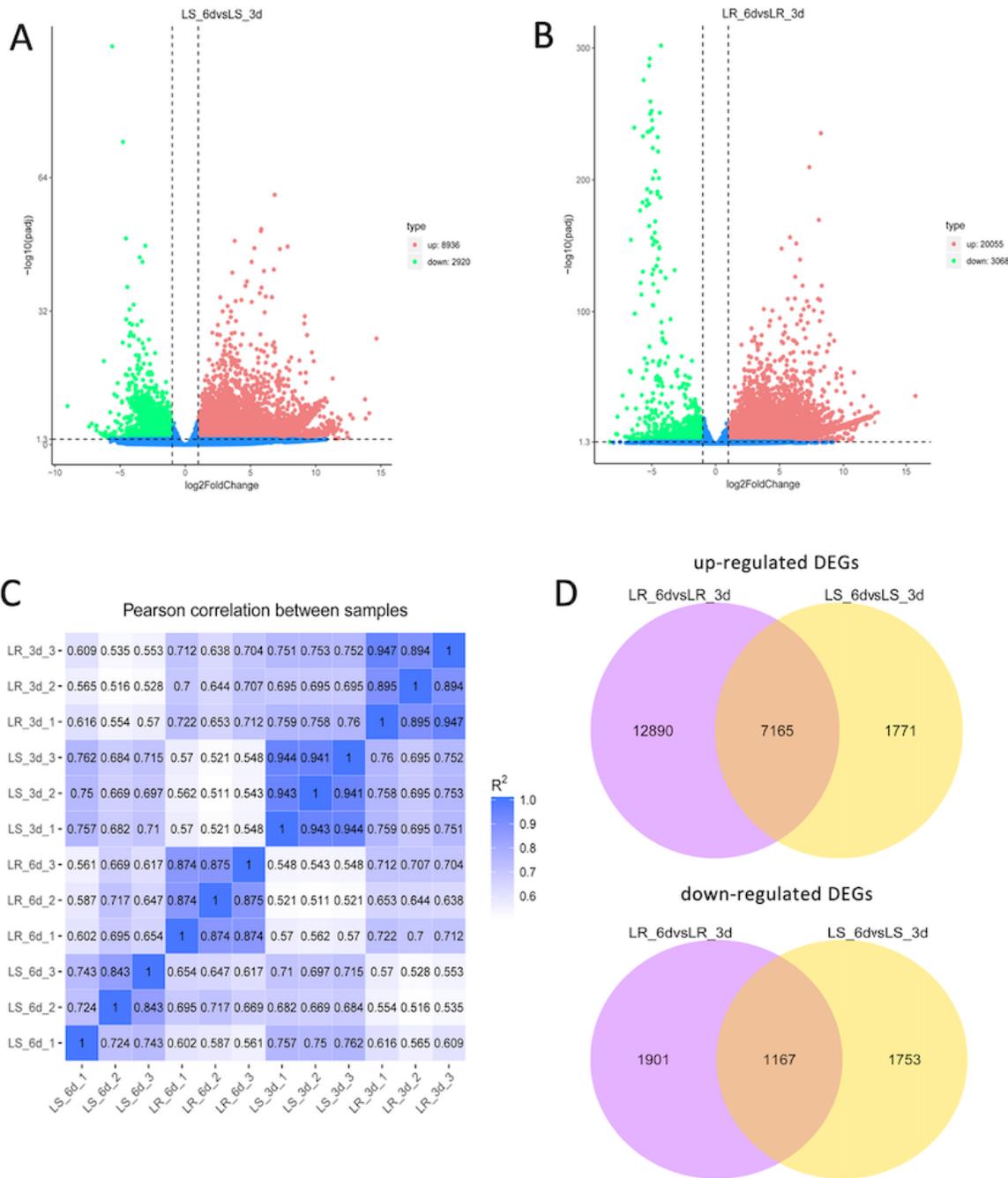
Figure 3

Analysis of different expression genes (DEGs) in LS comparison setting (LS_6d vs LS_3d) and LR comparison setting (LR_6d vs LR_3d). a, b the volcano plot of DEGs in LR and LS. Green dots represent the significant down-regulated DEGs. Red dots represent the significant up-regulated DEGs. c Correlation cluster between three biological replicates of LS_3d, LS_6d, LR_3d and LR_6d; d Venn diagram of up-regulated and down-regulated DEGs in LS and LR comparison settings.
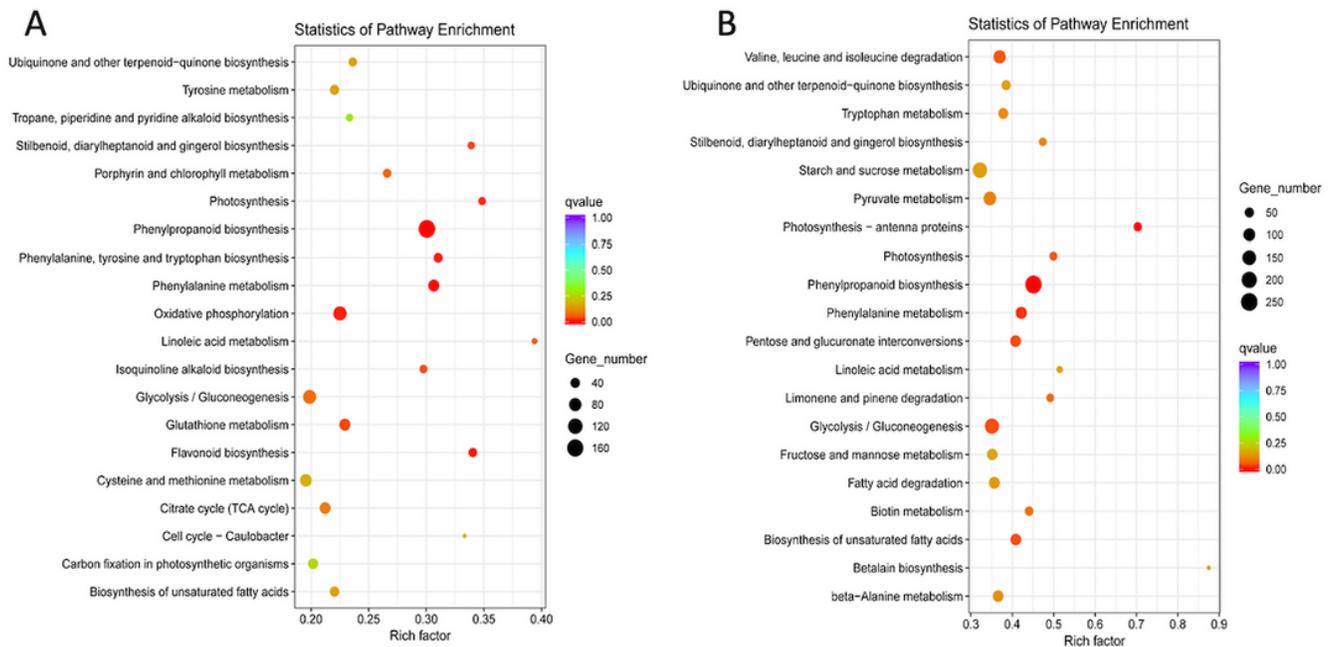
Figure 4

KEGG enrichment analysis of the up-regulated DEGs. a Top 20 enriched pathways in LS comparison setting (LS_6d vs LS_3d); b Top 20 enriched pathways in LR comparison setting (LR_6d vs LR_3d); The colors are shaded according to the q-values level as shown in the color bars; the size of the circle indicates the number of DEGs.
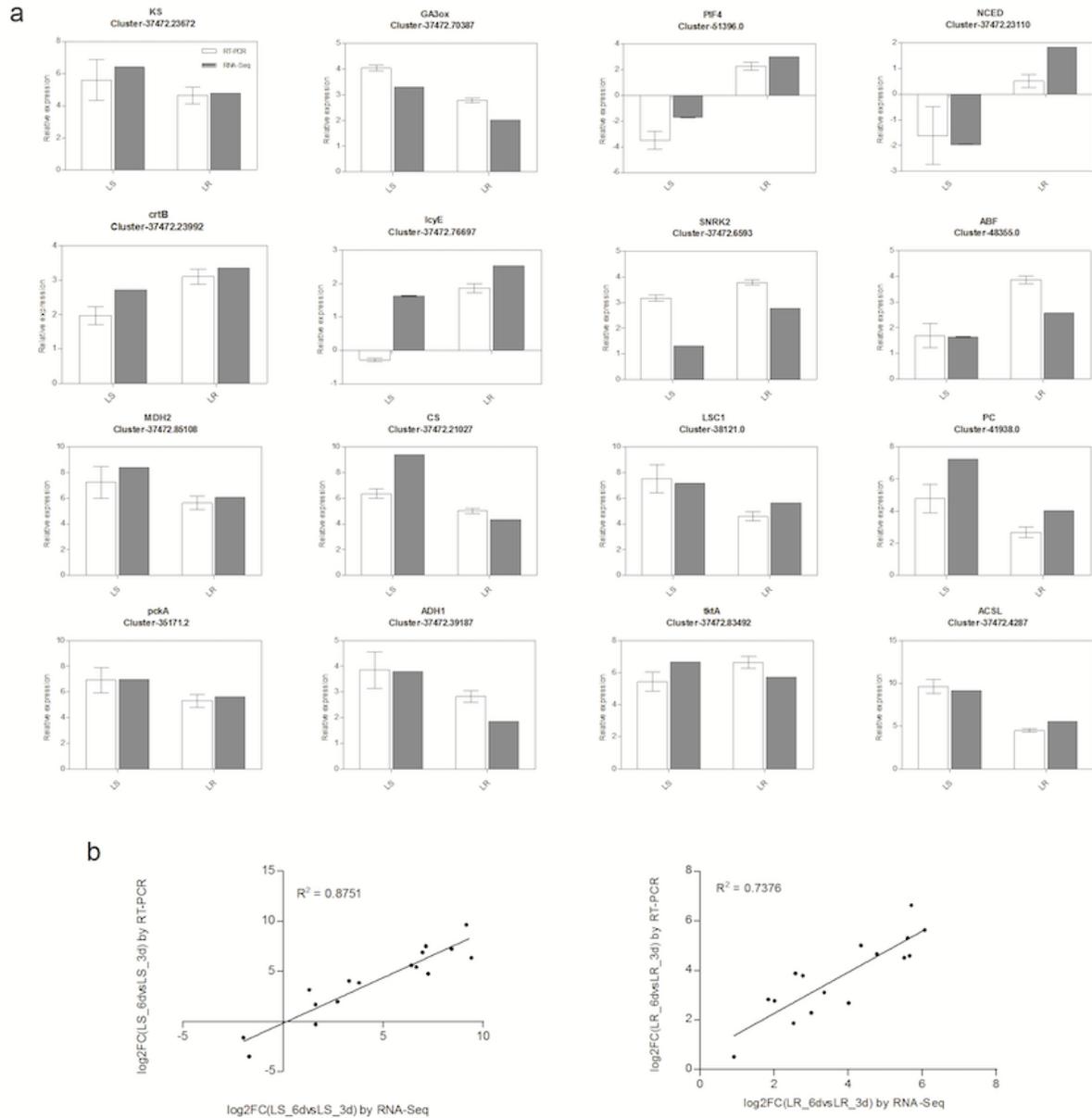
Figure 5

The validation of RNA-seq results using qRT-PCR. a The relative expression levels of 16 DEGs determine by qRT-PCR (white column) and RNA-Seq (grey column). RNA-Seq results (Fold change) and qRT-PCR results (2-ΔΔCt) were transformed to log2 forms. Bars are mean ± standard error (n = 3). b Linear regression analysis of the RNA-Seq and qRT-PCR results. The R2 values represent the correlation between RNA-Seq and qRT-PCR results.
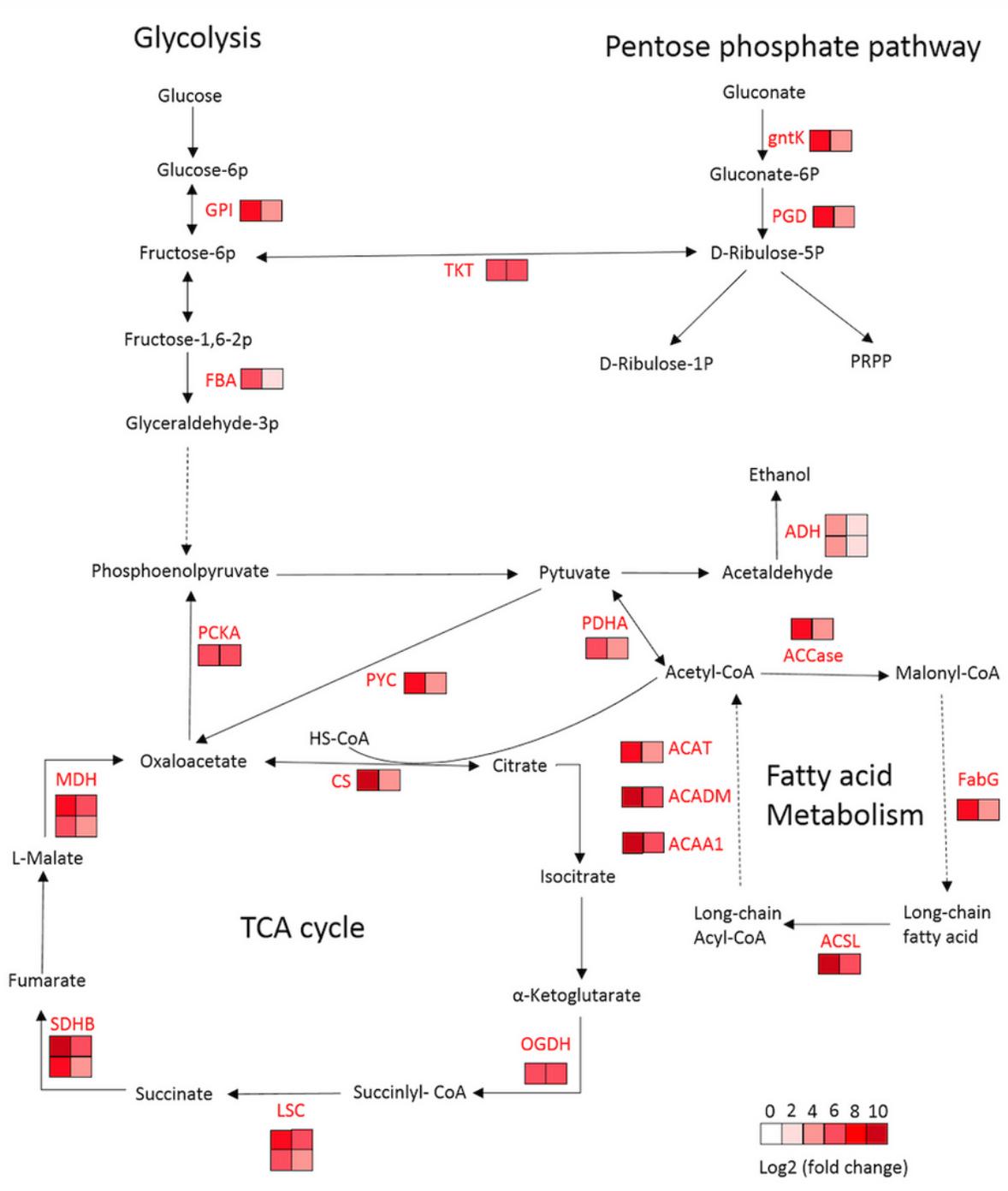
Figure 6

Visualization of DEGs involved in carbohydrate metabolism and fatty acid metabolism. Substrates are in black font and enzymes in red font. The color of boxes represents Log2 (fold change) values based on the transcriptome results. The values are shown by a color gradient from low (white) to high (red). The left column of boxes represents the Log2 (fold change) value obtained from LS comparison setting, and

the right column represents that from LR comparison setting. Each row of boxes represents one DEG. Solid arrows represent direct processes, and dashed arrows represent indirect processes.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1RTPCRprimers.xlsx
- Additionalfile2KOGclassification.xlsx
- Additionalfile3GOclassification.xls
- Additionalfile4KEGGclassification.xls
- Additionalfile5LS6dvsLS3dDEGGOenrichmentsig.xlsx
- Additionalfile6LR6dvsLR3dDEGGOenrichmentsig.xlsx