Comparative Transcriptome Analysis Revealed Candidate Genes Potentially Related to Desiccation Sensitivity of Recalcitrant *Quercus variabilis* Seeds

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Abstract

Background: Chinese cork oak (*Quercus variabilis*) is a widely distributed and highly valuable deciduous broadleaf tree from both ecological and economic perspectives. Seeds of *Q. variabilis* are recalcitrant, i.e., sensitive to desiccation, which affects their storage and long-term preservation of germplasm. However, little is known about the underlying mechanism of desiccation sensitivity of *Q. variabilis* seeds.

Results: In this study, the seeds were desiccated with silica gel for 0 day (control, CK), one day (T1) to 15 days (T15). After desiccation, the transcriptomic profiles of these different desiccation stages were compared using the *Quercus suber* genome as a reference, as well four key stages (CK, T2, T4 and T11) of desiccation sensitivity of *Q. variabilis* seeds through germination test were identified. A total of 4405, 4441, and 5907 differentially expressed genes (DEGs) were identified in T2 *vs* CK, T4 *vs* CK, and T11 *vs* CK, respectively. Among them, 2219 DEGs were overlapped in the three comparison groups. KEGG (Kyoto Encyclopaedia of Genes and Genomes) enrichment analysis showed that these DEGs were enriched into 124 pathways, such as "plant hormone signal transduction" and "glycerophospholipid metabolism". DEGs related to hormone synthesis and signal transduction (*ZEP*, *YUC*, *PYR*, *ABI5*, *ERF1B*, etc), stress response proteins (*LEA D-29*, *HSP70*, etc), and phospholipase D (*PLD1*) were detected during seed desiccation. These genes and their interactions may regulate the desiccation sensitivity of *Q. variabilis* seeds. Finally, a possible work model was proposed to show the molecular regulation mechanism of desiccation sensitivity in recalcitrant *Q. variabilis* seeds

Conclusions: Our study is the first on the molecular regulation mechanism of desiccation sensitivity of *Q. variabilis* seeds by using RNA-Seq and propose a possible work model. Our findings could make a great contribute to seed storage and long-term conservation of germplasm resources of recalcitrant seeds in the future.

Background

Because vegetative propagation can not only capture the superior phenotypes of selected trees, but can also propagate uniform clones [1], it is an extremely useful method for tree propagation. However, many *Quercus* species have difficulties in vegetative propagation [2, 3], and their regeneration and reproduction mainly depend on seeds. Mature seeds are larger and can provide nutrition and metabolism reserve for germination and seedling growth [4]. Therefore, seed determines the quality of *Quercus* seedlings and their afforestation effect.

According to desiccation sensitivity, seeds are classified into orthodox, intermediate, and recalcitrant [5]. Unlike orthodox seeds, desiccation tolerance is not acquired during the late maturation stage of recalcitrant seeds, resulting in their sensitive to desiccation. Therefore, recalcitrant seeds will lose viability rapidly by using conventional storage methods (low moisture content and low temperature) [6, 7]. Numerous plant species, especially for those from tropical and subtropical area, produce recalcitrant seeds [7]. For example, seeds of *Quercus* have been demonstrated to all recalcitrant [8]. An important

reason that makes seed sensitive to desiccation is high metabolic activity [9]. It has been proved that the increases in raffinose can slow metabolism in seeds with desiccation tolerance [10]. In addition, reactive oxygen species (ROS) accumulation leads to loss of seed vigor, which is related to seed desiccation sensitivity [11]. Seed desiccation sensitivity is also considered as an important functional and secondary evolutionary characteristic of recalcitrant species with a series of molecular processes being involved, such as gene expression regulation at the transcriptional level, phytohormone signalling transduction, and accumulation of late embryogenic accumulating proteins (LEAs) [9, 12]. In recalcitrant seeds, genes related to abscisic acid (ABA) synthesis and signal transduction, as well as LEAs and heat shock proteins (HSPs) were found to be differentially expressed during desiccation [13–15]. Similarly, the acquisition of seed desiccation tolerance is closely related to multiple genes encoding ABA, LEAs, and HSPs [16]. Therefore, many ABA-related genes may play essential roles in seed desiccation tolerance/sensitivity acquisition. For example, *ABSCISIC ACID INSENSITIVE3* (*ABI3*) is one of four major regulators of seed desiccation tolerance acquisition in *Arabidopsis thaliana*, and its downstream transcription factors play crucial roles in the desiccation tolerance acquisition network [17].

Transcriptome can reflect the gene expression level and has been used for investigating molecular mechanism of plant responding to abiotic stress [18–20]. In order to explore the regulatory mechanism of recalcitrant seed desiccation sensitivity, RNA-Seq technology has been used [13–15]. In the majority of recalcitrant seeds, the average dry weight was more than 1000 mg, which may mean typical recalcitrant seeds are large and heavy [21]. And there are also some seeds, i.e., *Citrus limon* [13] and *Taxillusi chinensis* [14], that the average dry weight were much less than 1000 mg. There are few studies on large recalcitrant seeds by using RNA-Seq, and is little known about desiccation sensitivity in these species. In addition, the molecular mechanisms of desiccation sensitivity are different in recalcitrant species. Therefore, it is necessary to explore the related study of recalcitrant seeds, especially in large seeds. *Quercus variabilis* Blume is an important afforestation and economic timber species, with high ecological and economic value [22]. Its bark is widely used for making wine bottle stoppers, heat preservation, as well as environmental protection [23]. Its acorns are rich in nutrition and high in starch, which can be used not only as raw materials for food, medicine and wine making [24], but also for the production of the fuel ethanol. Nevertheless, *Q. variabilis* seeds are typical recalcitrant seeds. High sensitivity to water loss is the main factor affecting its seed storage and long-term preservation of germplasm resources. Hence, the purpose of this research was to explore the gene expression changes during desiccation of recalcitrant *Q. variabilis* seeds by RNA-Seq, then identify desiccation-responsive genes, and analyse the potential molecular mechanism of desiccation sensitivity regulation. The results will not only provide insights into the molecular regulation mechanism of recalcitrant *Q. variabilis* seeds, but will also contribute to seedling breeding, seed storage and long-term conservation of germplasm resources in *Q. variabilis*.

Results

Seed germination percentage

The moisture content (MC) and germination percentage (GP) of *Q. variabilis* seeds decreased with the desiccation time (Fig. 1). The GP and MC of seeds without desiccation were 84.00% and 34.27%, respectively. After 12 days of desiccation, the MC reduced to 17.17%, when the GP dropped down to 0%. Furthermore, at the early stage of desiccation (T1-T2), the GP decreased most rapidly; after a slow decline stage (T2-T4), the GP decreased rapidly again (T4-T6), and gradually decreased to 0% (T12). In addition, the MC of seeds were significantly positively correlated with GP ($P<0.01$, $R=0.976$). These results indicate that *Q. variabilis* seeds are highly sensitive to desiccation.

ABA and IAA contents

The ABA content decreased from 20.93 ng/g (CK) to 18.53 ng/g (T2), then to 9.53 ng/g (T4), and finally increased to 27.40 ng/g (T11). Meanwhile, IAA content showed a rising trend from 1.34 ng/g (CK) to 25.70 ng/g (T11) (Fig. 2).

Transcriptome analysis during desiccation

A total of 629.44 million raw reads were obtained from 12 libraries. Ultimately, 596.16 million clean reads were obtained after filtering out low-quality reads, adaptor and N-containing sequences, with GC contents and Q30 values were ranged from 43.50% to 44.00%, and 97.85% to 98.28%, respectively (Additional File Table S1). To align the clean reads to the reference genome (GCF_002906115.1), 86.73%-88.33% of clean reads could be mapped, and 50.83%-52.61% were uniquely mapped (Additional File Table S1).

Identification DEGs during desiccation

To better investigate gene expression changes during seed desiccation, three comparison groups were established: T2 *vs* CK (C1), T4 *vs* CK (C2), and T11 *vs* CK (C3). In total, we identified 4405 (2724 up-regulated and 1681 down-regulated), 4441 (2839 up-regulated and 2052 down-regulated), and 5907 (3208 up-regulated and 2699 down-regulated) DEGs in these groups by using FPKM ($|\log_2 \text{fold change}| \geq 1$) and P-value<0.05, respectively (Fig.3a). Among these DEGs, 1125 were commonly up-regulated and 1094 were down-regulated between C1, C2, and C3 (Fig.3a; Additional File Table S2). In the further analyses, these DEGs shared in all comparison groups were used.

GO and KEGG analyses of DEGs during desiccation

To explore biological functions of the DEGs in *Q. variabilis* seeds during desiccation, GO enrichment analysis was used. As a result, these DEGs covered three GO categories: "molecular function (MF) ", "cellular component (CC) " and "biological progress (BP) ". Within the MF categories, the DEGs were mainly involved in "Molecular function", "Protein binding", and "DNA binding transcription factor activity". As for the GO terms of CC, the DEGs were mainly included in "Nucleus", "Membrane" and "Plasma membrane". In the BP categories, the DEGs were mainly enriched in "Biological process", "Secondary metabolite biosynthetic process" and "Regulation of transcription, DNA-templated" (Fig. 3b; Additional File Table S3).

To further characterize the DEG-associated pathways, the DEGs were subjected to KEGG enrichment analysis. Eventually, these DEGs were involved in 124 pathways. And most of them were enriched in the pathway of "Protein processing in endoplasmic reticulum" (ko04141), followed by "Plant hormone signal transduction" (ko04075) and "Plant pathogen interaction" (ko04626) (Additional File Table S4). The significantly enriched pathways were mainly involved in "Ether lipid metabolism" (ko00565), "Glycerophospholipid metabolism" (ko00564), "Carbon fixation in photosynthetic organisms" (ko00710) and others (Fig. 3c).

DEGs related to plant hormones

Genes encoding beta-carotene 3-hydroxylase (crtZ), 9-cis-epoxycarotenoid dioxygenase (NCED), and aldehyde oxidase (AO) were downregulated during *Q. variabilis* seeds desiccation in ABA synthesis. Two genes encoding abscisic acid 8'-hydroxylases (CYP707A) and 13 encoding ABA β- and UDP glucosyltransferase (AOG) related to ABA catabolism were downregulated during desiccation. Meanwhile, genes encoding zeaxanthin epoxidase (ZEP) and ten *AOG* genes were upregulated during desiccation (Fig. 4a and b). Furthermore, genes related to ABA signalling transduction were differentially expressed during seed desiccation. ABA receptor PYR/PYL family (*PYR*), Protein phosphatase 2C (*PP2C*), three Sucrose Non-fermenting 1-related protein kinase 2 (*SnRK2*), and ABA responsive element binding factor (*ABF*) genes were down-regulated, while one *SnRK2* gene was up-regulated (Fig. 5a and b).

At the same time, one gene (*LOC112010754*) encoding flavin containing monooxygenase (FMO) related to IAA synthesis was up-regulated continuously during *Q. variabilis* seeds desiccation (Fig. 4a). Furthermore, desiccation also interfered the IAA signal transduction pathway. During desiccation, one encoding auxin response (AUX/IAA) and one *SAUR* genes were upregulated. Meanwhile, three *AUX/IAA* and two encoding auxin response factor (ARF) genes were downregulated (Fig. 5a and c).

DEGs related to transcription factors and dehydrating proteins

Differentially expressed transcription factors (TFs) in *Q. variabilis* seeds under desiccation were mainly distributed in nine families: MYB, WRKY, ERF, NAC, bZIP, bHLH, Hsfs, GATA, and Trihelix (Fig. 6a). For example, the TFs of bZIP family (*TGA10*, *TGA9*) were up-regulated and *ABI5* were down-regulated during desiccation. In addition, ERF1B may represent the crucial protein mediating crosstalk between "MAPK signalling pathway-plant" and "Plant hormone signal transduction" in response to desiccation. And the downregulation of UNE10 and PIF3 may be important proteins mediating crosstalk between "Plant hormone signal transduction" and "Circadian rhythm-plant" during desiccation (Fig. 7).

Surprisingly, 36 and 14 genes encoding HSPs and LEAs were differentially expressed during *Q. variabilis* seeds desiccation, respectively (Fig. 6b). Additionally, except for HSF30-like (*LOC112016017*), HSPs related genes were all up-regulated, while LEAs-related genes, except for At1g64065-like (*LOC11202022025*, *LOC112028199* and *LOC112034431*), were all down-regulated (Fig. 6b).

DEGs related to glycerophospholipid metabolism

Glycerophospholipid is the most abundant phospholipid in the organism, and is an important component of membrane. Nine up-regulated and ten down-regulated DEGs in glycerophospholipid metabolism pathway were found (Fig. 8a). Among them, gene expression of *PLD1* was up-regulated and that of *CDS2* was down-regulated (Fig. 8b).

Validation of DEGs by qRT-PCR

In order to confirm our RNA-Seq data, the expression quantification of nine DEGs were validated by qRT-PCR. Although the $|\log_2$ fold change| values of these DEGs were different from RNA-Seq, the patterns were similar (Fig 9), suggesting that our RNA-Seq results were reliable.

Discussion

Effect of desiccation on seed germination

Consistent with other recalcitrant seeds [14, 15, 25], the GP of *Q. variabilis* seeds decreased along with the decrease of MC, and there was significantly positively correlated between MC and GP. The critical MC of recalcitrant seeds were determined when about 50% seeds became unviable, which can be used as a standard of seed desiccation sensitivity [7]. In the present study, the critical MC of *Q. variabilis* seed was about 28.20%. Furthermore, when the MC of seeds decreased to 17.17%, the seeds lost their vitality completely, which was similar to the recalcitrant seeds of *Aesculus chinensi* [26].

Phytohormone synthesis

In plant, hormones play important roles in coping with abiotic stress [27]. For instance, ABA can regulate the drought stress response and is also a vital hormone for seed desiccation tolerance acquisition [28]. ABA synthesis and catabolism in plants are mainly regulated by *NCED* and *CYP707A* [29]. Similar to previous study on recalcitrant *Camellia sinensis* seeds [15], genes related to ABA synthesis, such as *NCED*, *crtZ*, and *AO*, were down-regulated, while *ZEP* was up-regulated during *Q. variabilis* seeds desiccation. Furthermore, previous research also has pointed out that lack of ABA in the ZEP-deficient mutant of *Nicotiana plumbaginifolia* resulted in rapid water loss [30]. In this study, although the expression of *ZEP* was up-regulated, the down-regulation of *NCED*, *crtZ* and *AO* might lead to the decrease of ABA content during seed desiccation. ABA 8'-hydroxylases encoded by *CYP707A* can deactivate ABA and play an irreplaceable role in ABA catabolism [31]. ABA can also be reversibly inactivated by glucosylation. For instance, plants lacking UDP glucosyltransferase have fewer glucosyl esters and more free ABA, exhibiting stronger resistance to water deficit [32]. In our study, genes encoding ABA β-glucosyltransferase were up-regulated, and genes encoding UDP glycosyltransferase were both up- and down-regulated. Therefore, we speculate that the downregulation of genes encoding NCED, crtZ, AO, ABA β-glucosyltransferase, and upregulation of the UDP glycosyltransferase related genes might cause the decline of ABA content during seed desiccation.

It is widely known that indole pyruvate pathway is the most basic pathway for IAA synthesis in plants [33]. FMO encoded by YUC family genes has been confirmed to be able to directly convert indole pyruvate to IAA [34]. In this study, we found that YUC family gene (*LOC112010754*) was up-regulated, which may lead to the increase of IAA content during *Q. variabilis* seeds desiccation.

ABA and IAA are closely related to many physiological processes in plants and their interactions regulating to various developmental processes such as seed dormancy [35], lateral root development [36], and plant growth [37]. For instance, during the seed maturation of *Coffea canephora*, the up-regulated expression of many genes related to IAA may inhibit the mitochondrial retrograde response while ABA has the opposite effect, playing an important role in the acquisition of desiccation tolerance [38]. In this study, desiccation leads to the differential expression of genes related to hormone synthesis and catabolism, and changes the content of ABA and IAA. Thus, the balance between hormone is changed, which has been demonstrated to be able to induce desiccation tolerance in desiccation sensitive seeds, i.e., *Acer saccharinum* and *C. limon* seeds [13, 39]. Therefore, the desiccation sensitivity in recalcitrant *Q. variabilis* seeds might be a result of a broken balance between ABA and IAA.

Plant hormone signal transduction

Plant can respond to stress by regulating the expression of genes in the ABA signalling pathway, which is critical for plant response to water deficit [40]. ABA receptors PYR/PYLs, PP2Cs, SnRK2s, and ABF constitute the core network of ABA signal regulation [27, 40]. In plants, the perception of ABA through PYR/PYL receptors is necessary for ABA signal transduction. The PYR can interact with ABA, inactivate PP2C, activate SnRK2 and self-phosphorylation, activate ABF and then regulate the expression of related stress genes [41, 42]. During desiccation, the downregulation of *PYL*, *PP2C*, and *SnRK* may cause recalcitrant tea seeds sensitive to desiccation [15]. In our study, desiccation induced downregulation of *PYR*, *PP2C* and *ABF*, while *SnRK2* were both up- and down-regulated. These results indicate that desiccation interferes with the ABA signal transduction pathway, which eventually results in high desiccation sensitivity.

The TIR1/AFB-AUX/IAA/TPL-ARFs pathway is a widely accepted IAA signalling pathway. IAA can promote the formation of synergistic receptor complexes between TIR1 and AUX/IAA proteins, thereby activating TFs of auxin response factor (ARF) family and regulating IAA response genes [43]. In the present study, the expression of *AUX/IAA* genes were both up- and down-regulated, that of the *ARF* genes were down-regulated, and that of *SAUR* gene was up-regulated, similar to the findings in *C. canephora* seeds [38].

The *auxin response factor2* (*ARF2*) in Arabidopsis negatively regulates the homologous domain gene *HB33*, which is a regulator of ABA signalling pathway [37]. As previously reported, IAA can activate its response factor *ARF10/16* to induce the expression of *ABI3*, thus activating the ABA signal transduction pathway [35]. Moreover, the *PYL8* can enhance the expression of the IAA response genes by activating the *WRKY77* transcription factor [36]. In the present study, desiccation induced differential expression of

ARF and *PYL* in hormonal-signalling pathway in *Q. variabilis* seeds, indicating the potential interaction between them might contribute to regulate seed desiccation sensitivity.

It has been demonstrated that members of MYB, WRKY, ERF, NAC, bZIP, and bHLH TF families are key regulatory factors in plant response to water stress [44]. In this research, the screened DEGs contain ERF, bZIP, bHLH and other TF families. And the *ERF1B*, *ABI5*, *TGA9*, *TGA10*, *UNE10*, *PIF3* and *PIF5* from screened families were involved in "plant hormone signal transduction" pathway. Among them, ERF1B protein play an important role in tolerance to abiotic stress [45]. Furthermore, it has been reported that *ABI3* is essential for acquisition of seed desiccation tolerance [16, 28]. The *ABI3* can interact with *ABI5* to regulate the expression of downstream genes and mediate ABA signal transduction [46]. In our research, the *ERF1B* was up-regulated and *ABI5* was down-regulated, which may change the expression of downstream stress-related genes and result in seed desiccation sensitivity. In addition, our study also found that GATA and Trihelix TFs were differentially expressed during seeds desiccation, which were reported to play crucial roles in plant response to abiotic stress [47, 48]. These TFs need to be further explored, in order to provide reference for understanding the molecular regulatory mechanism of recalcitrant *Q. variabilis* seeds desiccation sensitivity.

Protective proteins related to desiccation

HSPs play irreplaceable roles in protecting plants from abiotic stress [49] and are particularly related to the acquisition of seed desiccation tolerance [13]. There is a 22 kDa HSPs in recalcitrant *Acer saccharinum* seeds, and its content increases significantly after desiccation, so as to reduce the damage [50]. In this research, we identified eight kinds of HSPs with molecular weight of 15.7, 17.5, 18.1, 18.2, 18.5, 22.0 and 22.7 kDa, as well as HSP70, which were mainly up-regulated during *Q. variabilis* seeds desiccation. This result is consistent with recalcitrant *T. chinensis* seeds [14]. And we also identified that heat shock transcription factors (Hsfs) were differentially expressed during desiccation, which play crucial roles in plant response to abiotic stress [51]. In addition, LEAs and the transcripts encoding LEAs were reported to accumulate in large amounts during acquisition desiccation tolerance of orthodox seeds [52], which can prevent protein aggregation during desiccation by folding [53]. While recalcitrant seeds have fewer LEAs or the absence of specific LEAs [54]. In *Castanospermum australe* and *Medicago truncatula* seeds that are sensitive to desiccation, the seeds are unable to accumulate enough LEAs, which may contribute to their sensitive to desiccation [46]. In this study, LEA-related genes were mainly down-regulated during seed desiccation, which may inhibit the capability of *Q. variabilis* seeds to accumulate LEAs to resist the damage. In addition, *ABI3* indirectly induced the desiccation tolerance of seeds by regulating the expression of *HSPA9* to the increase of HSPs content, both *ABI5* and *ABI3* also connect many LEAs as well as desiccation tolerance related genes in *M. truncatula* seeds [55]. In the present study, *ABI5* may regulate the expression of HSPs and LEAs related genes to cope with the adverse effects of desiccation. However, the specific regulatory relationship between them needs further experimental analysis.

Glycerophospholipid metabolism

Membrane phospholipids proportions are different between recalcitrant and orthodox seeds [56, 57]. Phospholipase D (PLD) can hydrolyse phospholipids into phospholipid acid (PA) and is closely related to desiccation sensitivity, i.e., the increased of PA content and decreased seed survival under desiccation [58]. Furthermore, PA also can be induced by ABA, and as the second messenger to participate in plant responses to abiotic stress [40]. In this study, several ABA-related genes were differentially expressed, and we also found that PLD related genes were up-regulated. Therefore, PLD may become more abundant and increase PA content during desiccation, resulting in reduction of the membranes fluidity, leading to the desiccation sensitivity of *Q. variabilis* seeds.

Conclusions

In the present study, the DEGs associated with seed desiccation sensitivity were identified by performing the transcriptome changes during desiccation of recalcitrant *Q. variabilis* seeds. KEGG enrichment analysis of DEGs showed that desiccation resulted in changes of plant hormone signal transduction and glycerophospholipid metabolism, which was further confirmed by the ABA and IAA contents assessment. Genes related to hormone synthesis and signal transduction (*ZEP*, *YUC*, *PYR*, *ARF, ABI5*, *ERF1B*, etc.) were differentially expressed during seeds desiccation. Protective proteins including LEAs (*LEA D-29* etc.) and HSPs (*HSP70* etc.) were also enriched upon desiccation. Additionally, *PLD1* may play an important role during seeds desiccation. Finally, a possible work model was proposed to show the molecular regulation mechanism of desiccation sensitivity in recalcitrant *Q. variabilis* seeds (Fig. 10). Our findings make a great contribute to seed storage and long-term conservation of germplasm resources of recalcitrant seeds, such as *Q. variabilis*.

Methods

Plant materials

Mature seeds were collected from one *Q. variabilis* tree in 16-September 2019 located in Louguantai Forest Farm (Shanxi, China) and no specific permissions were required to collect these seeds. Then, they were confirmed by Prof. Fang Du and were tested in laboratory of Chinese Academy of Forestry. The impurities and inferior seeds were first removed by visual check. Then, seeds were immersed in water for 5min to separate viable seeds from floating seeds [59]. The viable seeds were air-dried in the shade for 12-15h. During that period, the seeds were turned every 2-3 h. Finally, the healthy, uniform, and non-germinating seeds were used for desiccation experiments.

Determination of seed moisture content

A random of 20 seeds × 3 replications were selected for computation of initial moisture content. The initial moisture content of *Q. variabilis* seeds (control) was determined as described in [25]. The moisture content of seeds was calculated by initial moisture content minus that of desiccation.

Desiccation treatments

45 seeds with three replications were selected for per desiccation treatment. These seeds and silica gel (the mass ratio of seeds to silica gel was about 1:3) were placed in ziplock bag for 0 day (control, CK), one day (T1) to 15 days (T15). Then, the desiccation test was conducted in laboratory conditions with a temperature of 20-25°C and relative humidity of 40-45%. Seed samples were collected on ten seeds with three replications per desiccation treatment. The samples were frozen in liquid nitrogen and stored at -80°C for further analyses.

Seed germination

After desiccation, seed germination was tested. There were four replicates in each treatment, with 25 seeds in each replicate. We placed seeds on top of two pieces of filter paper and a layer of gauze moistened with deionized water in 12 cm diameter Petri dishes. Afterwards, Petri dishes were transferred to cyclically alternating temperature (30/20°C for 8/16 h, light/dark photoperiod) in a light incubator [25]. The seeds were considered to have germinated when the emerging radicle was at least 2 mm [60]. Germination was recorded every day and tallied for up to 28 days [61]. The germination percentage was calculated according to the following formula:

$GP=\Sigma D_t/N\times100\%$

where D_t denotes the number of germinations on t day, and N is the total number of seeds.

Finally, according to the germination assay, four critical periods (CK, T2, T4, and T11) of *Q. variabilis* seeds desiccation sensitivity were used for hormone measuring and transcriptome sequencing.

Determination of ABA and IAA concentration

Seed samples (50 mg fresh weight) were dissolved in 1 mL mixed solution with methanol/water/formic acid (15:4:1, V/V/V). The combined extracts were evaporated until dryness under a stream of nitrogen, reconstituted in 80% methanol (V/V), and filtrated (PTFE, 0.22 μm; Anpel). Phytohormones contents were detected and analysed by Metware Biotechnology Co., Ltd. (Wuhan, China, http://www.metware.cn/) based on the AB Sciex QTRAP 6500 LC-MS/MS platform. Three replicates were performed for measurement.

Extraction, transcriptome sequencing and analysis of total RNA from seeds

Total RNA was extracted from seeds using total RNA purification kit (TRK1001, LC Science, Houston, TX) following the manufacturer's procedure. The total RNA quantity and purity were analyzed by Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip Kit (Agilent, CA, USA), with RIN number >8.0. Approximately, 10 μg of total RNA representing a specific adipose type was subjected to isolate Poly (A) mRNA with poly-T oligo attached magnetic beads (Invitrogen). Following purification, the mRNA was fragmented into small pieces using divalent cations under elevated temperature. Then the cleaved RNA fragments were reverse-transcribed to create the final cDNA library in accordance with the protocol for the mRNA-Seq sample preparation kit (Illumina, San Diego, USA). The average insert size for the paired-end libraries was 300 bp

(± 50 bp). And then the paired-end sequencing was performed on an IlluminaHiseq4000 at the (LC Sciences, USA) following the vendor's recommended protocol. The Cutadapt was used to remove low-quality reads and adaptor sequences. The clean data was aligned to the *Q. suber* genome [62] using HISAT2. The mapped reads of each sample were assembled using String Tie, and the Bioconductor edgeR was used to identify DEGs. Ultimately, GO and KEGG enrichment analyses were performed for DEGs. The $P<0.05$ were defined as significantly enriched items and pathways.

Quantitative real-time PCR analysis

Nine DEGs with potential functions in regulating desiccation sensitivity of *Q. variabilis* seeds were selected for qRT-PCR validation. Total RNA was reversely transcribed into cDNA by using Prime Script™ Reagent Kit with gDNA Eraser (Takara, Dalian, China). The qRT-PCR assay was performed with KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, USA), according to the manufacturer's instructions. The primer sequences used for qRT-PCR are listed in Additional File Table S5. *ACTIN* (*ACT*) was used as the reference gene [63] and the relative expression data was calculated by using the $2^{-\Delta\Delta Ct}$ method [64].

Abbreviations

DEGs: Differentially expressed genes; ROS: Reactive oxygen species; LEAs: Late embryogenic accumulating proteins; ABA: Abscisic acid; HSPs: Heat shock proteins; MC: Moisture content; GP: Germination percentage; GO: Gene ontology; KEGG: Kyoto Encyclopaedia of Genes and Genomes; MF: Molecular function; CC: Cellular component; BP: Biological progress; Aldehyde oxidase: AO; Zeaxanthin epoxidase: ZEP; Protein phosphatase 2C: PP2C; Sucrose Non-fermenting 1-related protein kinase 2: SnRK2; Flavin containing monooxygenase: FMO; Auxin response factor: ARF; Transcription factor: TF; Phospholipase D: PLD; Phospholipid acid: PA

Declarations

Ethics approval and consent to participate

No specific permits were required for the described field studies. The location is not privately-owned, and the field studies did not involve endangered or protected species. The filed studies have been carried out in accordance with the relevant provisions of the Seed Law and other laws.

Consent for publication

All authors agreed to publish.

Availability of data and materials

The datasets generated in this study are included in its additional files. The raw RNA-Seq data for the four desiccation stages with three replicates are uploading in the NCBI Sequence Read Archive (SRA) repository via submission number SUB8556540.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HYY, LBW, DXL and YCL designed the experiments. DXL, YCL and JLQ performed the experiments. DXL, GWZ and JJR collected the materials. HHX and XJL coordinated the studies. DXL wrote the manuscript. LZ and DXL revised the manuscript. All authors read and approved the final manuscript.

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Figures

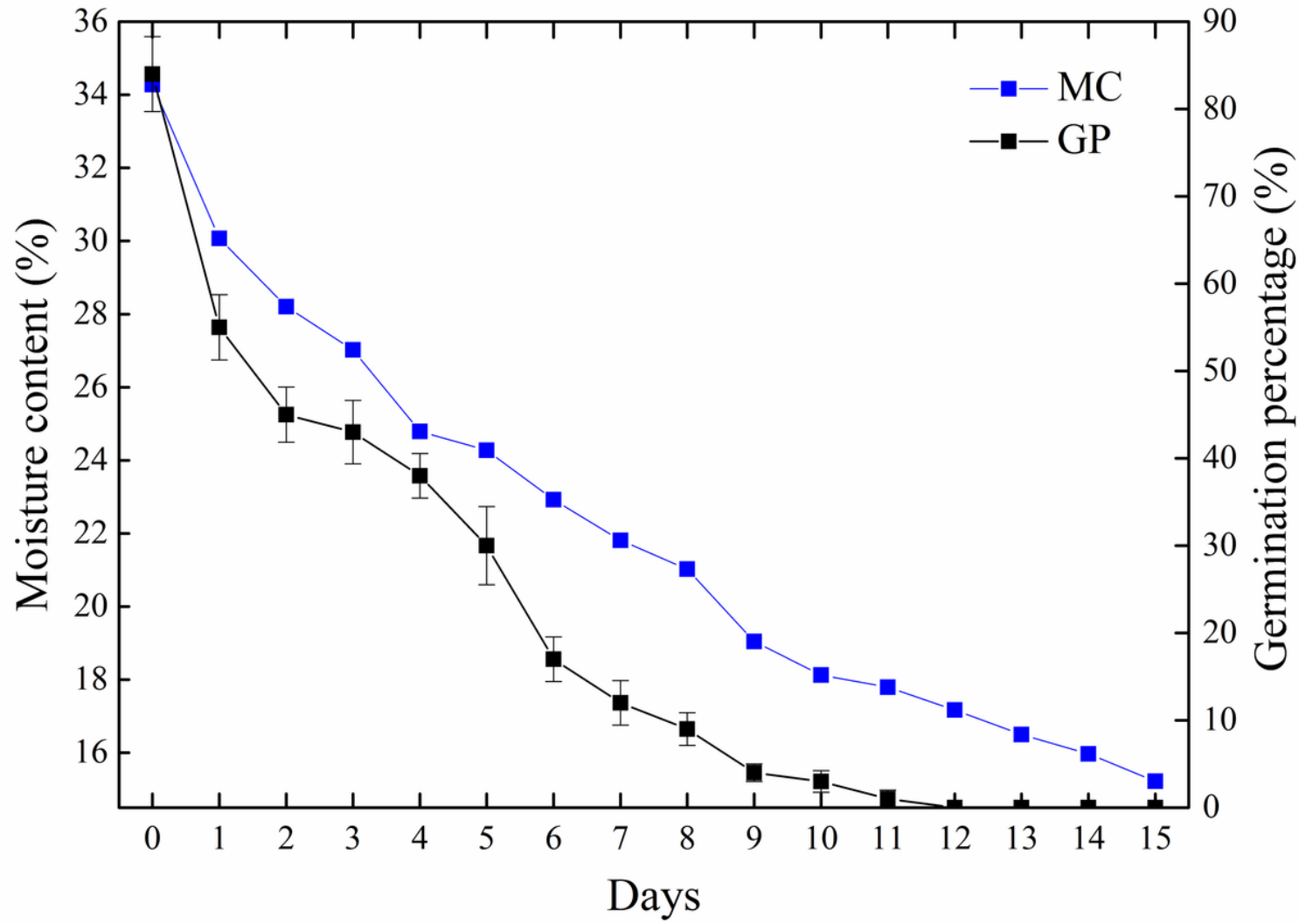

Figure 1

Changes of GP and MC of Q. variabilis seeds during desiccation. Vertical lines represent ± SE of the means. 0, 1 ,…, 15 represent desiccation time in days.

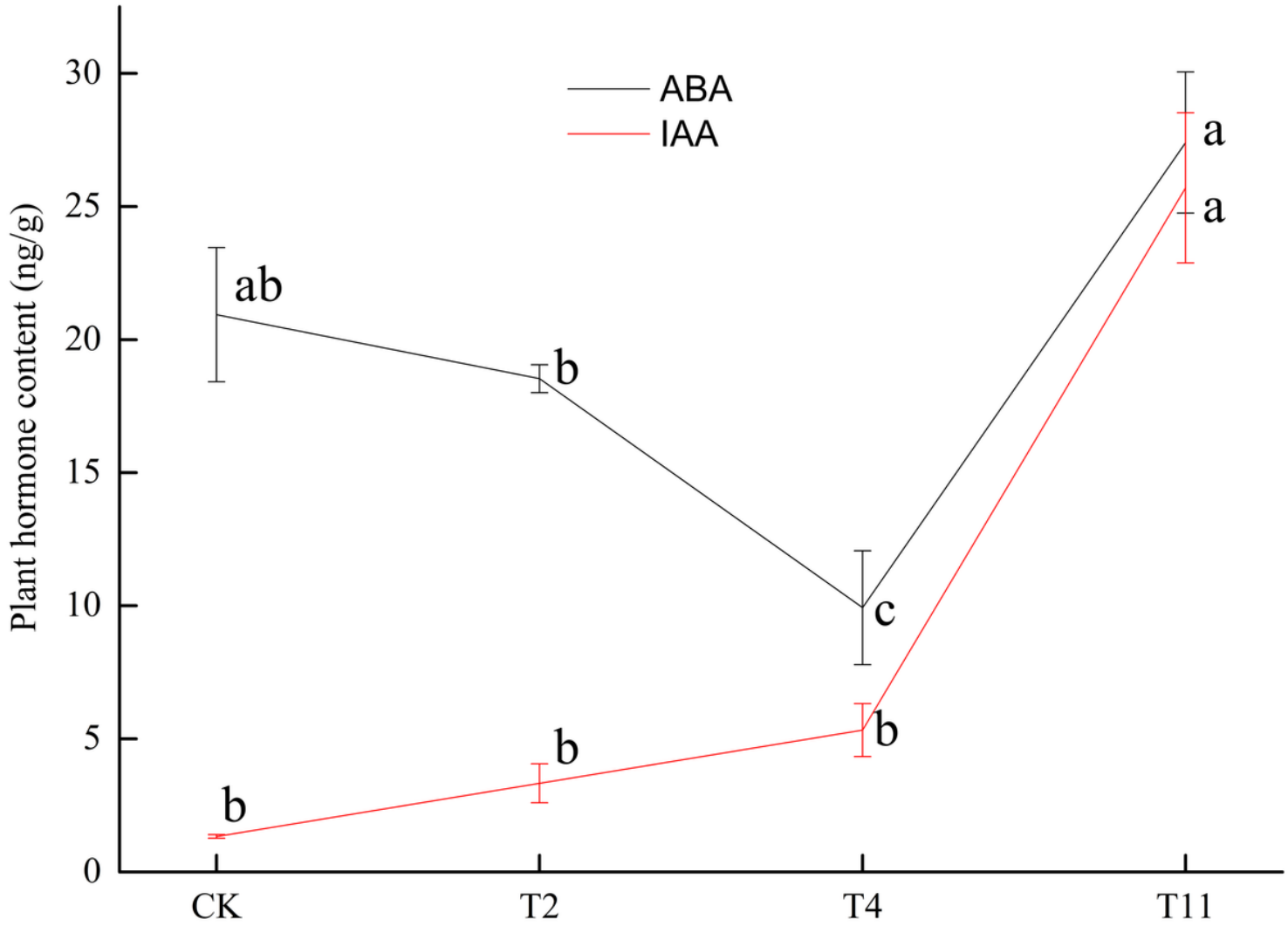

Figure 2

Changes of plant hormone content in Q. variabilis seeds during desiccation. Vertical lines represent ± SE of the means. Different lowercase letters represent statistically significant differences at the 0 .05 probability level.

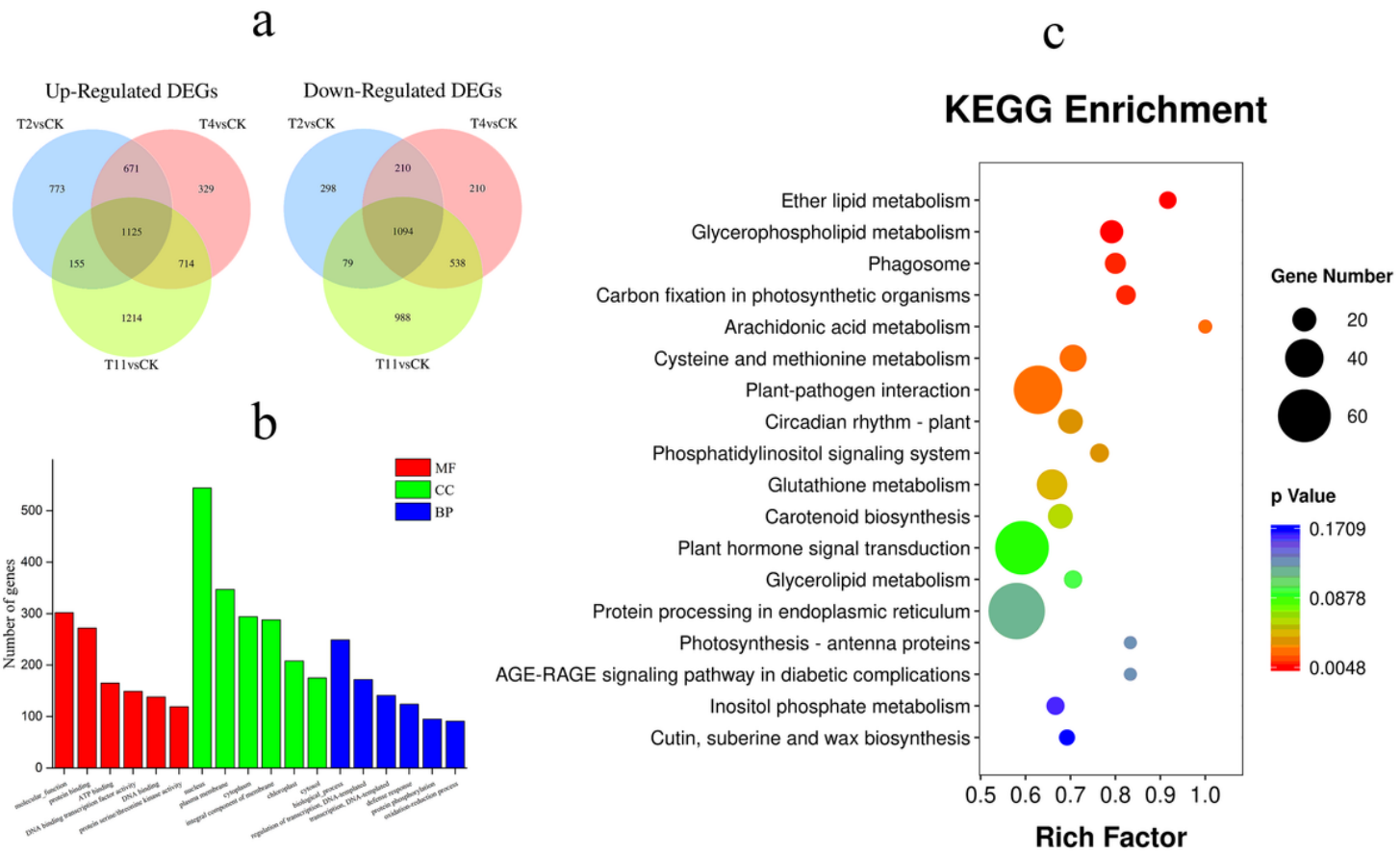

Figure 3

The number of DEGs identified by comparisons and enrichment analysis. (a) Venn diagrams show the number of up and down-regulated DEGs in three comparison groups, (b) GO enrichment analysis of DEGs, including molecular function, cellular component, and biological process, (c) KEGG enrichment analysis of DEGs.

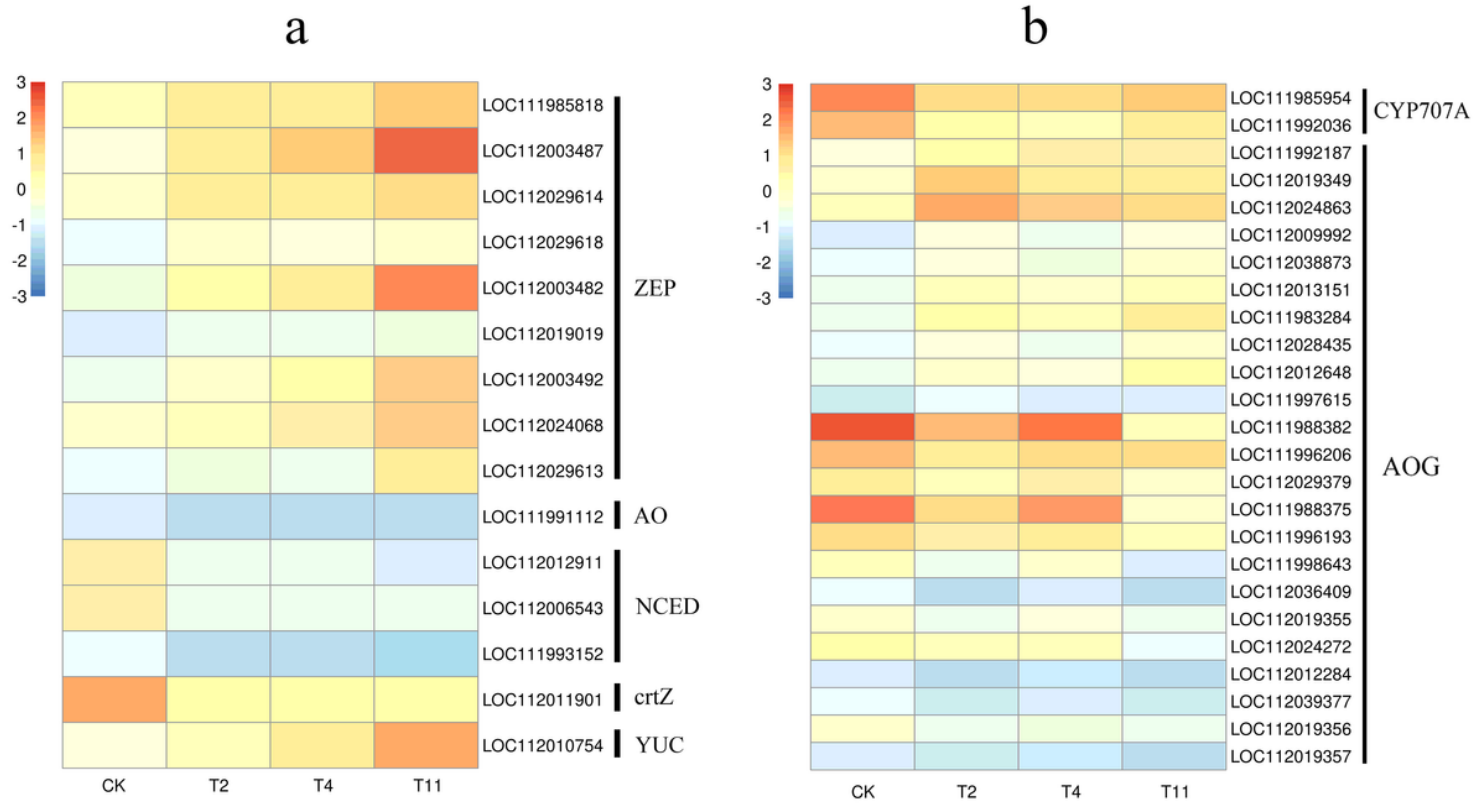

Figure 4

Heat maps show the effects of desiccation on plant hormone biosynthesis and catabolism in Q. variabilis seeds. (a) DEGs might be involved in “ABA and IAA biosynthesis”, (b) DEGs might be involved in “ABA catabolism”.

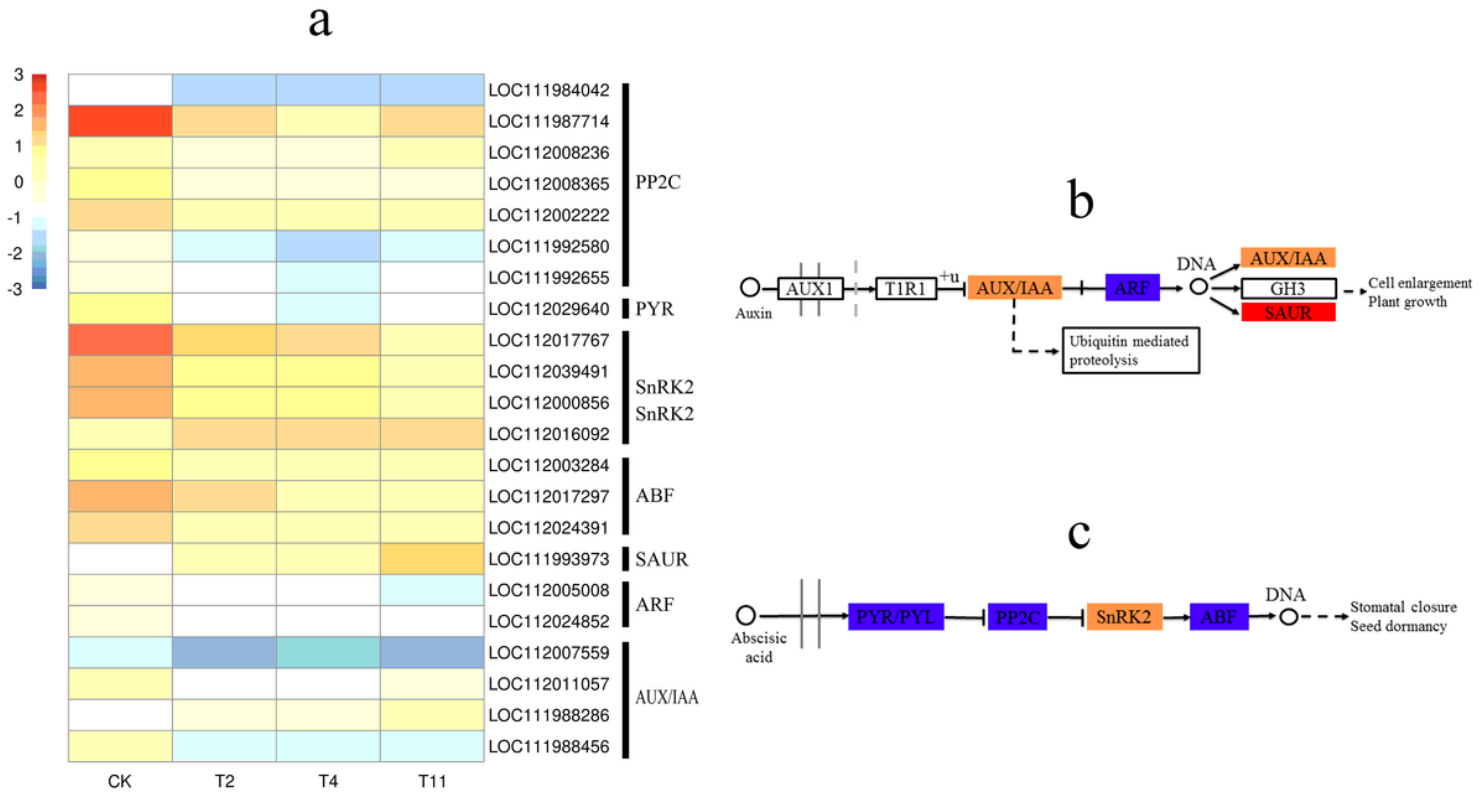

Figure 5

Effects of desiccation on gene expression in ABA and IAA signal transduction pathway. Heat maps shows DEGs related to ABA and IAA signal transcription pathway (a). (b) and (c) shows the pathways of ABA and IAA signaling. The boxes represent regulatory genes, and circles represent metabolites. Red indicates that the gene expression is up-regulated, blue indicates that the gene expression is down-regulated, while orange indicates that there are both up-regulated genes and down-regulated genes in the gene family.

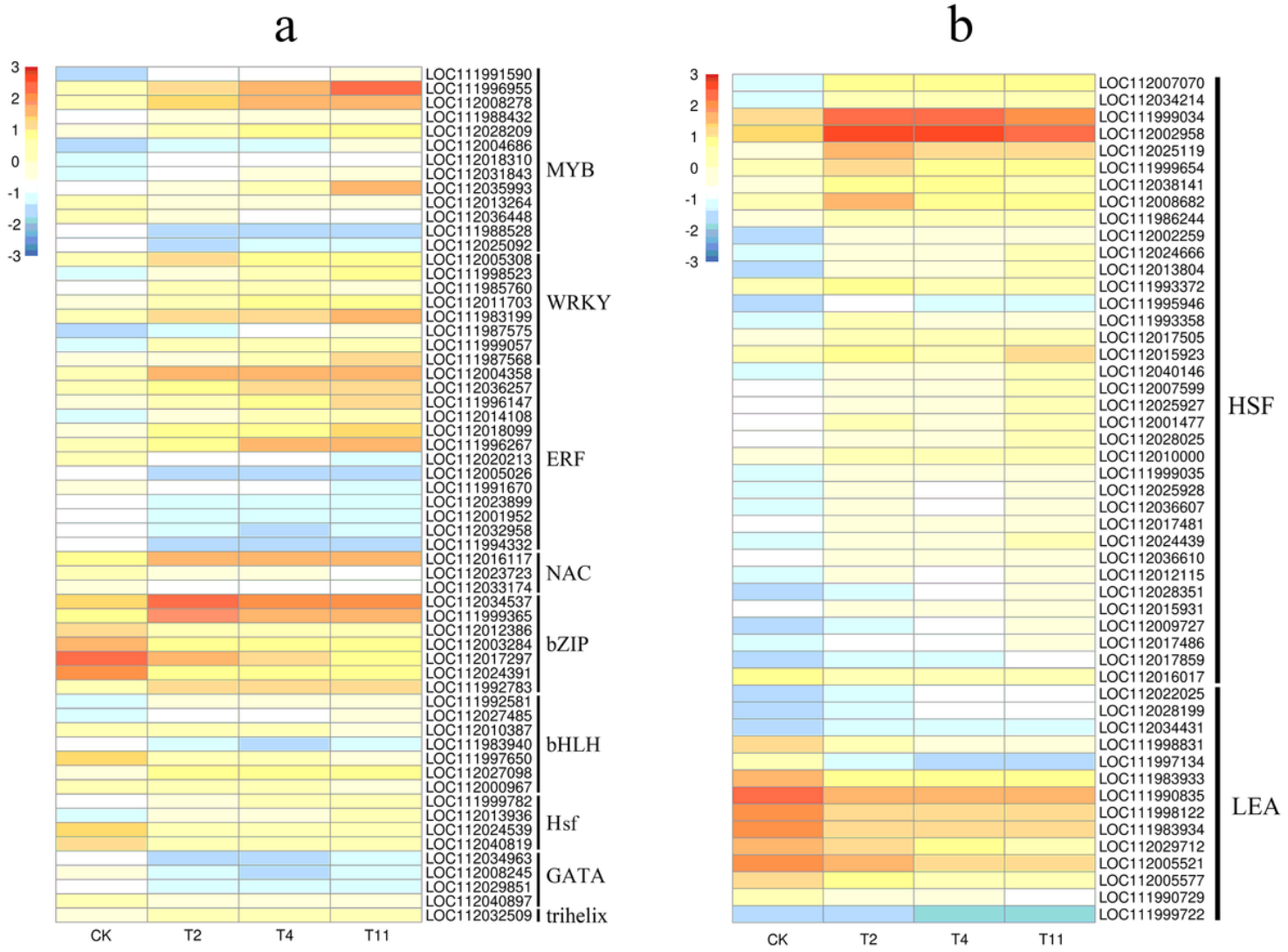

Figure 6

Effects of desiccation on gene expression of transcription factors and protective proteins. Heat maps showed that DEGs related to transcription factor (a) and protective protein (b), respectively.

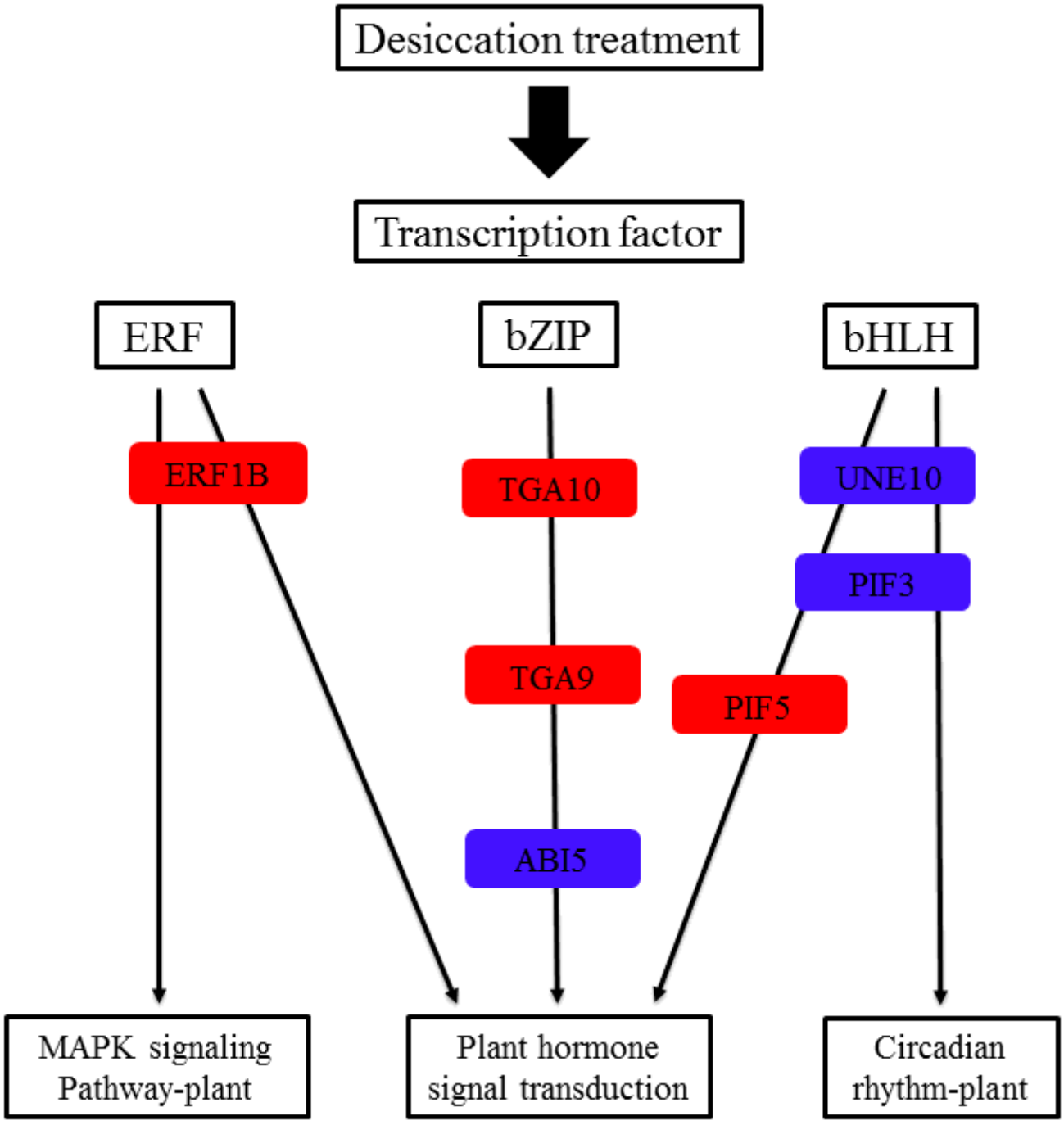

Figure 7

A proposed working model for desiccation transcription factor signaling network in Q. variabilis seeds.

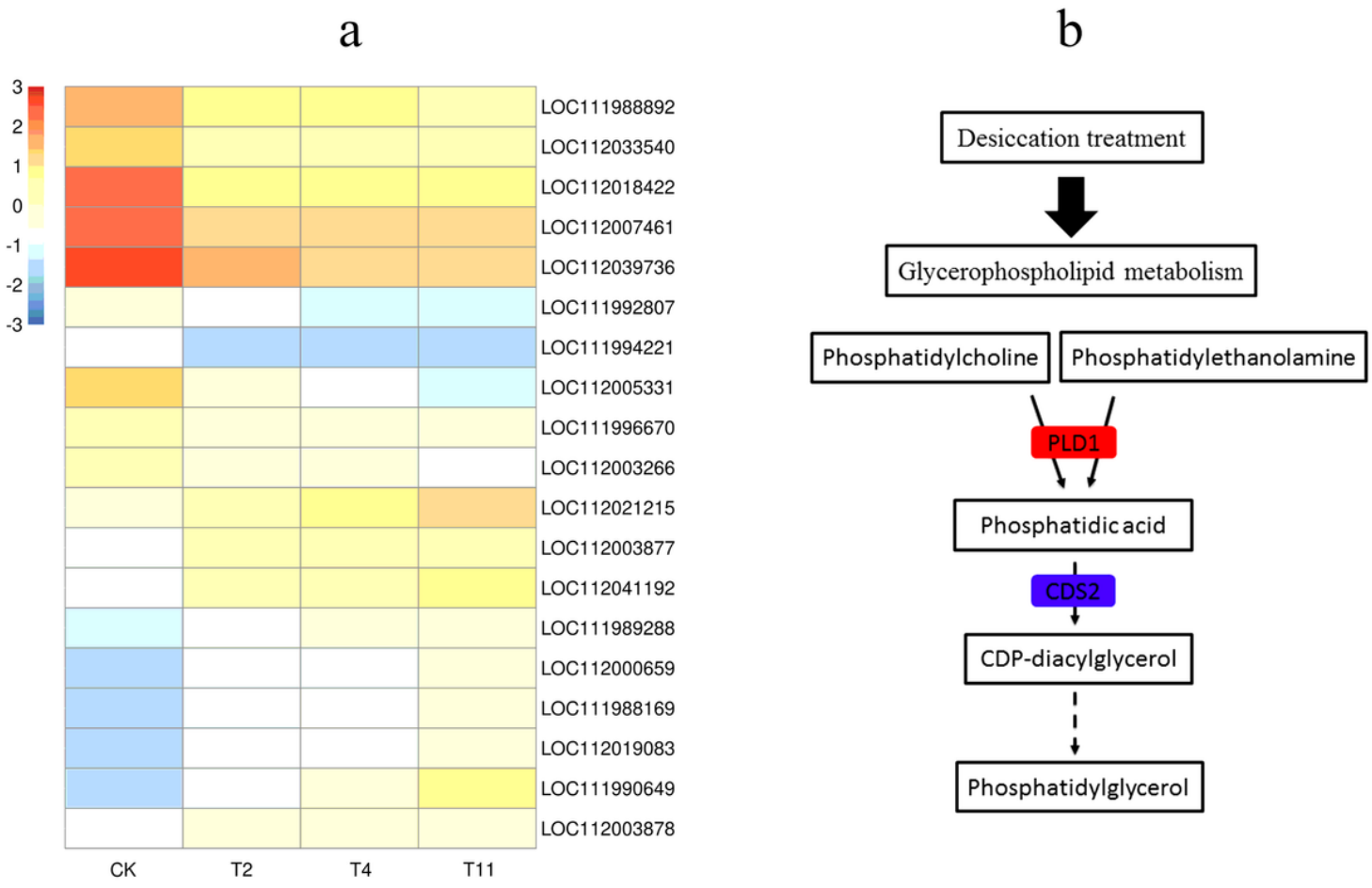

Figure 8

Gene expression level in glycerophospholipid metabolism pathway after treatment by desiccation. (a) Heat map illustrates that DEGs related to glycerophospholipid metabolism pathway, (b) Partial pathway of glycerophospholipid metabolism. Red part indicates the expression of the up-regulated gene, while blue indicates that of the down-regulated one.

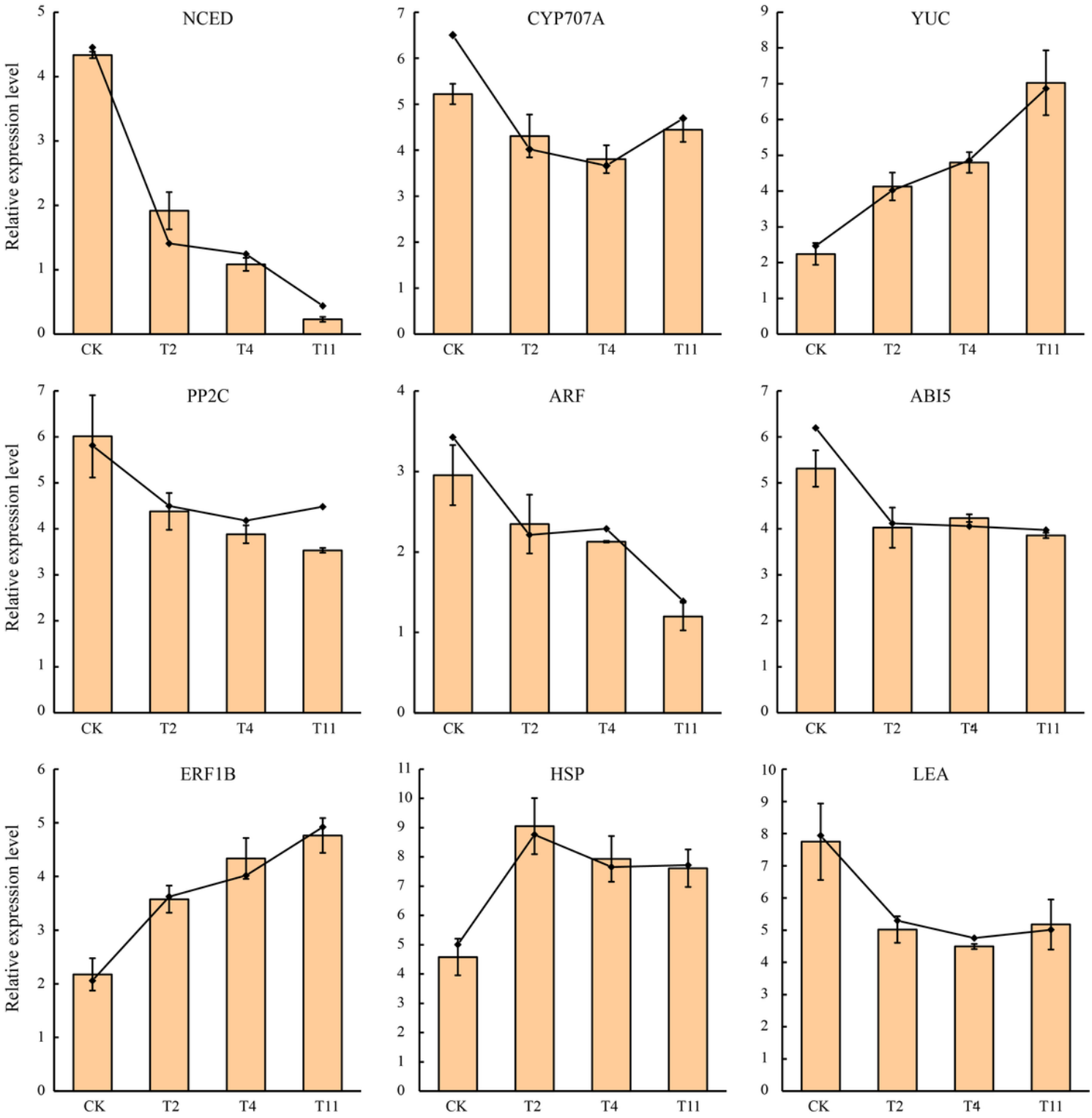

Figure 9

Expression patterns of nine selected DEGs in Q. variabilis seeds according to qRT-PCR and RNA-seq. The broken line indicates RNA-Seq data, while histogram indicates qRT-PCR data. Vertical lines represent ± SE of the means.

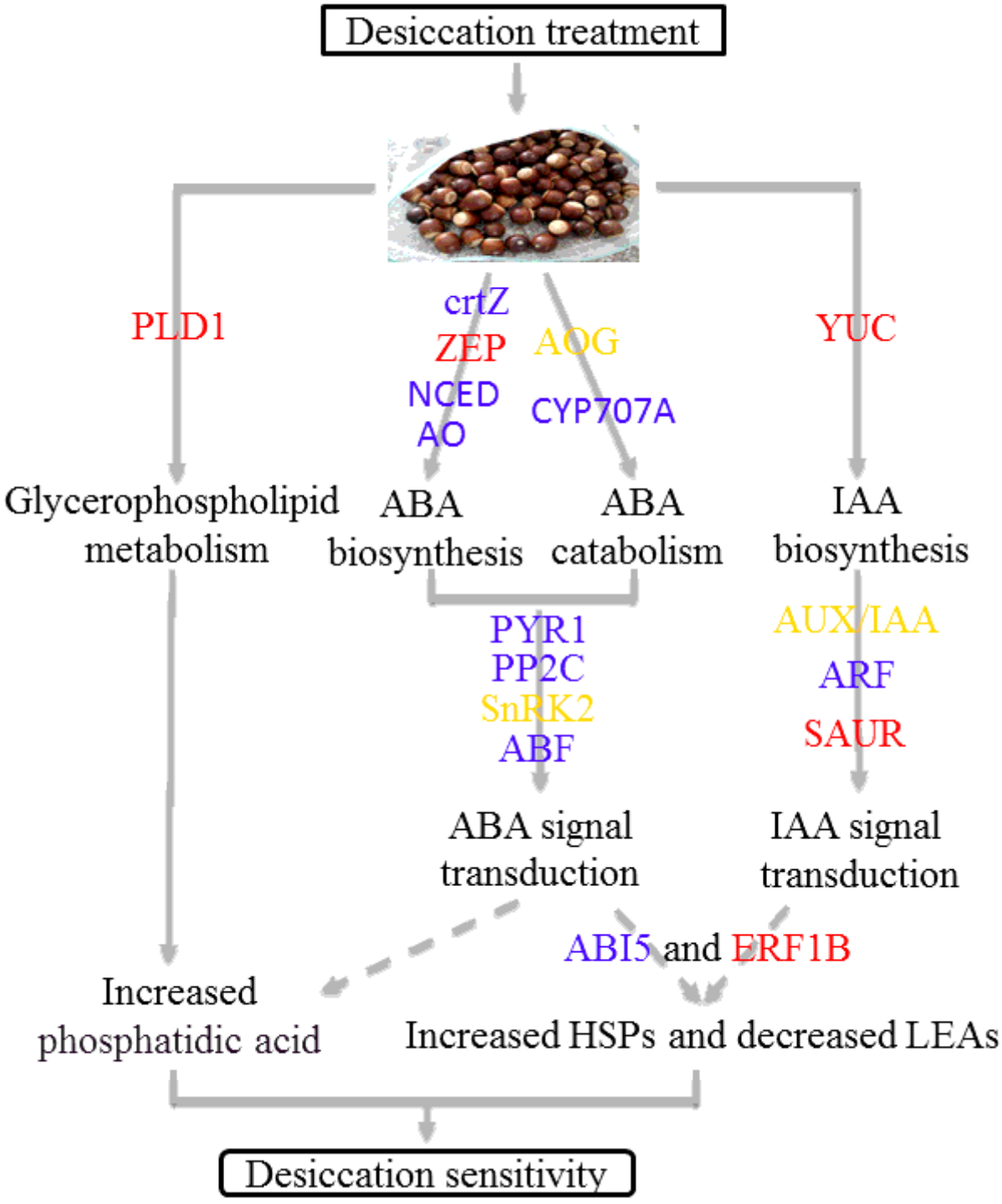

Figure 10

A model for mechanisms underlying desiccation sensitivity of Q. variabilis seeds. Solid lines indicate activation, and dashed lines indicate postulated regulation. Red indicates that the gene expression is up-regulated, blue indicates that the gene expression is down-regulated, while orange indicates that there are both up-regulated genes and down-regulated genes in the gene family.

Supplementary Files

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