Iris lactea var. chinensis Plant Drought Tolerance Depends on Response of Proline Metabolism, Transcription Factors, Transporters and the ROS Scavenging System

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Abstract

Background

Iris lactea var. *chinensis*, a perennial herb, is widely distributed and possesses good drought tolerance traits. However, there is not much information concerning this herb in public databases, so it is difficult to understand its underlying mechanism of drought tolerance.

Results

In this study, we conducted RNA-seq analysis of *I. lactea* var. *chinensis* plants treated under water deficiency condition and rehydration by Illumina sequencing technology to explore potential mechanisms involved in plant drought tolerance. The results showed that *de novo* assembly of the transcriptome generated 126,979 unigenes, of which 44,247 unigenes were successfully annotated. Among these, 1,187 differentially expressed genes (DEGs) were identified from the comparison of water deficiency treatment with the control (T/CK), including 481 up-regulated and 706 down-regulated genes. Additionally, 275 DEGs were identified in the comparison of rehydration plants and water deficiency treatment (R/T). Based on real-time quantitative RT-PCR analysis, the expression levels of eight unigenes arbitrarily selected were consistent with transcriptome data under water deficiency stress and rehydration treatment, as well as the control. According to Gene Ontology annotation and Kyoto Encyclopedia of Genes and Genomes pathway analysis, the results showed that Proline metabolism-related DEGs, including the 'Proline catabolic process', 'Proline metabolic process', and 'arginine and Proline metabolism', possibly play important roles in plant drought tolerance. Additionally, these DEGs were involved in 43 transcription factors, 46 transporters, and 22 reactive oxygen species (ROS) scavenging system-related proteins. Biochemical analysis and histochemical detection showed that Proline and ROS were accumulated under water deficiency treatment, consistent with transcriptomic analysis.

Conclusions

In sum, our transcriptome data revealed that the plant drought tolerance of *I. lactea* var. *chinensis* depends on Proline metabolism, the action of transcription factors and transporters, and a strong ROS scavenging system, which related genes found in this study could be a useful molecular breeding resources to enhance *I. lactea* var. *chinensis* drought tolerance.

Background

Drought is one of the most severe and frequently occurring abiotic stresses threatening plant seed germination, growth, and productivity. However, in the long-term course of the evolution to drought stress, plants develop a dramatically complex and sophisticated process, including diverse physiological, biochemical, metabolic, and defense systems by altering thousands of genes expression patterns [1]. Increasing research has documented that plants have developed sophisticated molecular mechanisms to

adapt and survive during periods of water deficit or drought stress [3-5] and proved that drought is a complex trait which interacts with a number of genes.

The numerous genes which involved in the drought stress-related defenses are divided into three major strategies [6]. The first strategy is the direct protection of essential proteins and membranes, such as osmoprotectants, free radical scavengers, heat shock proteins, etc. Under drought stress, ROS accumulation, including singlet oxygen (O_2^-), superoxide (O_2), hydroxyl (OH^-), and hydrogen peroxide (H_2O_2), may occur in plants, which causes oxidative stress, resulting in plant damage [7-8]. Thereby, plants are subjected to damage by oxidative drought-induced stress, as described by Mano [9]. To avert the excess ROS, plant cells are equipped with antioxidative machinery comprised of both enzymatic and non-enzymatic compounds of a low molecular weight. The antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), etc., play crucial roles in maintaining ROS homeostasis [10-11]. The second strategy is membrane transporters and ion channels, involved in water and ion uptake. The third group consists of regulatory proteins, including kinases and transcription factors that are involved in transcriptional regulation of stress-related genes. Plant transcription factors (TFs) play an important role in plant drought tolerance, directly regulating the gene expression and serving as molecular switches [12]. Recently, a wide range of TF families were characterized and shown to participate in the plant response to drought stress, such as WRKY, MYB, NAC, bZIP, bHLH, etc. [13]. Increasing evidence indicates that many transporters act as major players in plant drought tolerance, e.g., various plant membrane transport systems (ABC transporters, sugar transporters, and so on) play a significant role in adaptation to drought [14-15]. For example, the sulfate transporters (SULTR) may contribute to adjusting the sulfur distribution in plants subjected to drought stress [16].

Iris lactea var. *chinensis*, a perennial herb, is widely distributed in northern China, Siberian regions, eastern Russia, and Mongolia [17]. This plant grows in desert steppe, wasteland, park, etc. with a strong adaptability to the environment, and has been used as a degradation restoration and landscaping plant [18]. *I. lactea* var. *chinensis* possesses higher drought resistance than that of other plants used for roadside ecological restoration, such as *Parthenocissus quinquefolia*, *Opisthopappus taihangensis*, etc., by evaluating comprehensive physiological indexes under various degrees of stress [19]. To date, most research on *I. lactea* has focused on the screening of germplasm resources and the mechanism analysis of salt, heavy metal and PEG-mediated drought tolerance [20-23]. Considering the previous drought stress was simulated by PEG-mediated, the effects of this strategy and directly drought stress (severe dehydration or withholding water) will probably not be the same and the plant generated transcriptomes might exist difference [24]. Moreover, some reports concerning *I. lactea* var. *chinensis*' plant response to drought stress have only shown the morphological characteristics and basic physiological indicators, such as protective enzyme activity and the chlorophyll content [19,25]. Therefore, there is a critical need to explore the underlying molecular mechanisms of *I. lactea* var. *chinensis* of directly water deficiency stress (severe dehydration or withholding water).

In this study, to obtain a comprehensive understanding of the mechanisms of *I. lactea* var. *chinensis*'s plant drought tolerance, we performed a transcriptome analysis of leaf samples from plants treated with

water deficiency stress, rehydration and, the control. The RNA-seq data revealed that *I. lactea* var. *chinensis*'s plant drought tolerance depends on Proline metabolism, the action of transcription factors and transporters, and a strong ROS scavenging system, which may provide new theoretical significance in plant drought tolerance.

Materials And Methods

Plant materials, growth conditions and water deficiency treatments

Seeds of *I. lactea* var. *chinensis* were collected from the Ordos in Inner Mongolia, China, in the early autumn. Seeds were sown in a seedling tray. After 30 days, seedlings that were about 10 cm tall were transferred to plastic pots (10 cm diameter and, 10 cm height) with commercial peat substrate. *I. lactea* var. *chinensis* plants were grown in a greenhouse at the Chinese Academy of Agricultural Sciences with a natural photoperiod, a daily temperature between 32 °C (daytime) and 25 °C (night) and a relative humidity of approximately 60%. Plants were well irrigated from 1^{st} to 30^{th} days (30 days treatment) prior to the start water deficiency stress treatments (Fig. 1).

The seedlings were divided into three groups to be used as the control (CK, normal watering), water deficiency-treated samples (T) and rehydration-treated samples (R). The water deficiency-treated samples were naturally air-dried from 31^{st} to 37^{th} days (7 days treatment) after transplanting them. At this time, about 75 % of the leaves showed wilting. For the rehydration -treated samples (R), the water supply was restored from 38^{th} to 40^{th} day (3 days treatment) after they had been water deficiency-treated (Fig. 1). On the 41^{th} days, the whole plant were collected from CK, T and R groups at the same time to measure the phenotypic characterization, free Proline concentration, ROS accumulation and RNA extraction, respectively.

Phenotypic characterization and the relative water content

The plant height, root length, and root/shoot ratio were measured respectively. The relative water content (RWC) of aerial parts of *I. lactea* var. *chinensis* was measured using the method of Parida et al. [69]. The dry weight resulting from each treatment was obtained by drying at 80 °C for 8 h. RWC was calculated using the following formula: RWC (%) = (FW-DW)/FW×100 (FW: fresh weight and, DW: dry weight).

Determination of Proline concentration and ROS accumulation

Three treatment's fresh leaves Proline concentration and ROS accumulation were quantization respectively. Proline concentration was determined with acid ninhydrin reagent followed by Bates et al. [70] methods and formula: μmoles Proline/g of fresh weight material = [(μg Proline/ml × ml toluene) / 115.5 μg/μmole]/[(g sample)/5]. The accumulation of H_2O_2 and $O_2^{\cdot-}$ production was quantization the ROS activity. H_2O_2 and $O_2^{\cdot-}$ was histochemical detection with diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively followed by Romero-Puertas et al. [71].

RNA isolation, cDNA library construction and sequencing

For each treatment, 30 samples were randomly divided into 3 groups as 3 biological replicates. For each biological replicate (10 individuals), total RNA was extracted from all the overall plant using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). After total RNA extraction, reverse transcription reactions were performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with following DNase I treatment. The poly (A) mRNA was isolated using magnetic beads with Oligo (dT). Then fragmented into short fragments of 200~700 bp after mixing with the fragmentation buffer. Then cDNA was synthesized from mRNA fragments and random primers. Short cDNA fragments were purified and dissolved in TE buffer for end repair and single nucleotide A (adenine) addition. After that, the short fragments were ligated with adapters. The suitable fragments were size fractionated for PCR amplification as templates. The sample libraries were used for the quantification and qualification test by Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System. The library products were sequenced using Illumina HiSeq™ 2000 carried out by the Beijing Genomics Institute (BGI), Shenzhen, China.

All raw data were deposited in the National Center for Biotechnology Information (NCBI) and can be accessed in the Short Read Archive (SRA) under the accession number SRP257840.

De novo assembly and annotation

The raw paired end reads were trimmed and quality controlled by SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) with default parameters. Then, clean reads were assembled into contigs using Trinity software (http://trinityrnaseq.sourceforge.net/) [72]. These contigs were subjected to sequence clustering to form longer sequences. Such sequences were defined as unigenes. The Trinity assembly was optimized using TransRate v1.0.3 (http://hibberdlab.com/transrate/) software of the transcriptome assembly sequence filter [73] and CD-HIT v4.6.8 (http://weizhongli-lab.org/cd-hit/) and the sequence alignment Cluster method were used to remove redundancy and similar sequences, and finally obtain the non-redundant sequence [74]. BUSCO (http://busco.ezlab.org) evaluates the assembly integrity of the transcriptome [75]. The sequence assembly quality was evaluated using the number of sequences and bases, GC content, distribution of unigene lengths, average coverage, and N50 statistics [76].

To obtain functional annotation of the assembled unigenes, all of the assembled unigenes were aligned against publicly available databases, including the National Center for Biotechnology Information non-redundant protein (NR, ftp://ftp.ncbi.nlm.nih.gov/blast/db/), the NCBI nucleotide sequence (NT), the Swiss-Prot protein database (Swiss-Prot) [77], Gene Ontology terms (GO) [78], Clusters of Orthologous Groups (COGs) [79], and Kyoto Encyclopedia of Genes and Genomes (KEGG) [80], using the BLASTx algorithm with an E-value threshold of 10^{-5} [81].

Analysis of differentially expressed genes (DEGs)

To identify DEGs between two samples, the expression level of each unigene was calculated according to the transcripts per million reads (TPM) method. RSEM (http://deweylab.biostat.wisc.edu/rsem/) [82] was used to quantify gene and isoform abundances. The R statistical package software DESeq2 package (Empirical analysis of Digital Gene Expression in R, http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html) was utilized for differential expression analysis. Venn Diagrams were generated using the free online platform of Majorbio Cloud Platform (www.majorbio.com).

Thousands of independent statistical hypothesis tests were separately conducted on DEGs. Then, a *P*-value was obtained, which was corrected using the false discovery rate (FDR) method. Parameters for classifying statistically significant DEGs were as follows: at least a two-fold difference in the transcript abundance (|log2FC| >= 1), FC: fold change in expression) and FDR < 0.05(*P*-adjust < 0.05). In addition, functional enrichment analyses, including GO and KEGG database analyses, were performed to identify the GO terms and metabolic pathways in which the DEGs were significantly enriched at a Bonferroni-corrected *P*-value of less than or equal to 0.05 compared with the whole- transcriptome background. GO functional enrichment and KEGG pathway analyses were carried out using Goatools [83] and KOBAS [84].

qRT-PCR Analysis

To validate RNA sequencing's reliability, 8 DEGs were randomly selected from the above detected DEGs for quantitative analysis of gene expression by RT-PCR. The genes sequences used for primers design were generated from assembled unigenes in the present study. Quantitative RT-PCR was performed with ChamQ SYBR Color qPCR Master Mix (2×) (Vazyme Biotech Co., Ltd, Nanjing, China) and BIOER LineGene9600plus Sequence Detector (BIOER, Hangzhou, China), and three replicates were repeated. qRT-PCR was conducted at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and dissociation at 72 °C for 40 s. All primers used for qRT-PCR are listed in Table S17. The relative expression levels were calculated based on the $2^{-\Delta\Delta CT}$ method [85]. The *Actin11* was chosen as a reference gene for normalization and primers were generated from *I. lactea* var. *chinensis* partial CDS sequence of Actin11 (GenBank Accession Number: AB971013).

Results

Phenotypic characterization

I. lactea var. *chinensis* is a drought tolerant plant and can grow in desert steppe and saline lowland meadows. To validate this weed's drought tolerance, we performed a native water deficiency stress and rehydration experiment. The results showed that the wilting rate of *I. lactea* var. *chinensis* plants was approximately 75% after 7 days of native water deficiency stress, and when rehydrated, all wilted plants recovered after 3 days, indicating that *I. lactea* var. *chinensis* possesses a strong phenotypic plasticity of drought stress (Fig. 1). The RWC of water deficiency-treated plants significantly decreased compared to the control (CK). Additionally, the plant height of water deficiency-treated plants was significantly shorter

than the control. When water deficiency-treated plants were rehydrated, RWC and plant height partially recovered to some extent compared to the control (Fig. 2). To further investigate the phenotypes of plants treated and not treated with water deficiency stress, we measured the root and shoot length. The results showed that the root length and root/shoot ratio of the water deficiency stress plants significantly increased compared to the control, similar to the other plants under water deficiency treatment [26].

Primary transcriptome analysis

Three sample groups (CK, T, and R) with three biological repeats were employed to perform RNA-sequencing analysis using the Illumina HiSeq platform. A total of 57.85 Gb of data was obtained, and the three sample groups CK, T, and R, contained 42,979,607, 42,693,515, and 42,884,268 raw reads, respectively. Then, 42,905,695, 42,668,270, and 42,857,576 clean reads were obtained in the CK, T, and R groups after filtering low-quality reads, respectively. Following this, *de novo* transcriptome assembly with Trinity generated 126,979 unigenes, with an N50 of 1,176 bp. These unigenes contained an average length of 810 bp and an average GC content of 42.55% (Table S1), indicating that these unigenes were fine quality and suitable for further annotation. Annotations of the assembled unigenes were carried out according to six public databases including NR, SwissProt, KEGG, KOG, Pfam, and GO. A total of 41,360, 31,799, 18,512, 8,875, 28,920 and 28,385 unigenes were aligned, respectively (Table 1). Finally, 44,247 unigenes (34.85%) were successfully annotated in six databases.

Validity analysis of transcriptome data by qRT-PCR

To validate the reliability of the transcriptome data, eight candidate genes, including *ERF053*, *HSPA1_8*, *ADC1*, *CDPK*, *IAA17*, *APRR5*, *MYBP*, and *ABCC2*, were arbitrarily selected for qRT-PCR analysis. The results showed that the eight candidate genes' expression levels in three samples with water deficiency stress, rehydration or well-watering treatment were similar to the RNA-Seq data (Fig. 3). Together, these data showed that the RNA-seq analysis was reliable in the present study.

Detection of DEGs

To evaluate *I. lactea* var. *chinensis*'s t drought tolerance, differentially expressed genes (DEGs) in three samples were shown in scatter plots (Fig. 4a). A total of 1,187, 275, and 865 DEGs according to $|\log_2 FC| >= 1$ and *P*-adjust 0.05 were detected in comparison groups T/CK, R/T, and R/CK, respectively as shown in Fig. 4a and Table S2~S4. The up-regulated DEG numbers in the three comparison groups of T/CK, R/T, and R/CK, were 481, 185, and 402, respectively, and the corresponding down-regulated DEG numbers were 706, 90, and 463. In the T/CK comparison group, there were more down-regulated DEGs than up-regulated genes, indicating that water deficiency stress globally inhibits gene expression (Fig. 4a, Table S2).

Venn diagrams showing the number of up-regulated DEGs and down-regulated DEGs in each comparison (three ways) is shown in Fig. S1. Interestingly, 73 genes were found to be common between T/CK and R/T, but not R/CK, indicating that these DEGs in rehydration treatment were virtually same as those in the

control group (*P*-adjust > 0.05). Among the 73 DEGs, 29 DEGs were up-regulated in T/CK and down-regulated in R/T (Fig. S1c, Table S5), while 44 DEGs were down-regulated in T/CK and up-regulated in R/T (Fig. S1g, Table S6). These data showed that the 73 DEGs may be have highly plasticity in plant water deficiency stress and rehydration treatment. For example, the gene related to primary-amine oxidase (PAO; TRINITY_DN60247_c0_g2) expression was up-regulated by 32.7 times in response to water deficiency stress and down-regulated to the control level after rehydration. Similarly, the gene related to germacrene D synthase (*GDS*; TRINITY_DN45420_c1_g3) expression increased 655.6 times after water deficiency treatment, then decreased after rewatering. By contrast, two *UGT85A* (TRINITY_DN44873_c0_g4 and TRINITY_DN53280_c0_g1) expression were down-regulated under the water deficiency stress, but up-regulated during the water recovery period. Additionally, two-way Venn diagrams illustrate the overlap between the DEGs identified following well water, drought, and water recovery treatments as shown in Fig. 4b, being similar to the report of Zhang et al. [27].

GO functional and pathway enrichment analysis of DEGs

To elucidate the potential mechanism of *I. lactea* var. *chinensis*'s t drought tolerance, DEGs were mapped against the GO database and subjected to enrichment analysis, which was classified into three major functional categories based on the criteria of *P*-adjust<0.05. In the comparison of water deficiency treatment with and the control, 42 GO terms were significantly enriched in biological process (BP) category. For example, the DEGs involved in the amino acid biosynthetic and metabolic process included the 'glutamine family amino acid catabolic process', 'Proline catabolic process', 'amide biosynthetic process', and 'cellular amide metabolic process'; those associated with recognition included 'cell recognition' and the 'recognition of pollen'; those concerning the organic substance metabolic process included the 'nitrogen compound metabolic process', 'cellular aromatic compound metabolic process', and 'heterocyclic metabolic process'; and the DEGs related to the biosynthetic and metabolic process of nucleic acids and proteins included the 'DNA metabolic process', 'RNA biosynthetic process', 'peptide biosynthetic process', and 'cellular protein metabolic process'. Additionally, the expected DEGs related to the response to various other types of abiotic stresses were detected in the 'cellular response to osmotic stress', 'cellular response to salt stress', and 'cellular response to blue light' (Fig. 5; Table S7~S9). For the cellular component (CC) category, only 15 GO terms were significantly enriched in the comparison of drought stress and the control, which were mainly related to the components of membrane cytoplasmic part and organelle part (Fig. 5). For the molecular function (MF) category, lots of proteinase activity types, including Proline dehydrogenase activity, protein kinase activity and transferase activity were highly enriched (Fig. 5). We found that only four and eight GO terms were significantly enriched in the R/T (Table S8) and R/CK group (Table S9), respectively.

To reveal the underlying mechanism of *I. lactea* var. *chinensis*'s tolerance to drought stress, pathway enrichment analyses of the DEGs based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were performed. In the T/CK group, 64 DEGs were significantly enriched in six pathways (*P*-adjust < 0.05), including 'Plant-pathogen interaction', 'alpha-Linolenic acid metabolism', 'Circadian rhythm - plant', 'ABC transporters', 'Arginine and Proline metabolism', and so on (Table 2). Some of the 64 DEGs

play an important role in plant drought tolerance [28-30]. For example, receptor-like kinases encoding genes *BAK1/SERK1* (TRINITY_DN60727_c4_g2), hydroperoxide dehydratase encoding genes *AOS* (TRINITY_DN51813_c4_g1), and pseudo-response regulator encoding genes *PRR5* (TRINITY_DN56996_c1_g3, TRINITY_DN62580_c5_g2, TRINITY_DN51806_c4_g2, and TRINITY_DN56996_c1_g1) were up-regulated in T/CK comparison group. The whole report for the DEGs in each comparison group, respectively, is shown in Table S10~S12.

TFs, transporter proteins (TPs), and ROS scavenging systems affect the plant response to drought stress

TFs In our database, at least 43 DEGs encoding TFs were found (Table S13), and were included in the heatmap (Fig. 6). The concerning TFs were divided into 14 subfamilies, including APETALA2/Ethylene-responsive transcription factor (AP2/ ERF), MYB, WRKY, Zinc finger proteins, NAC, growth-regulating factors (GRF), etc. As shown in Fig. 6, most DEGs coding TF unigenes exhibited a down-regulated expression in plants treated with water deficiency stress compared to the control, consistent with other DEGs aforementioned (Fig. 4 and Fig. 6). However, some DEGs coding TFs exhibited up-regulated expression in water deficiency treatment, e.g. two related genes encoding AP2/ERF (TRINITY_DN59365_c4_g7, TRINITY_DN48541_c0_g3), six related genes encoding MYB (TRINITY_DN58225_c1_g1, TRINITY_DN63921_c6_g1, TRINITY_DN56464_c2_g2, TRINITY_DN57679_c2_g2, TRINITY_DN43447_c3_g3, TRINITY_DN44333_c4_g3) and four related genes encoding Zinc finger (TRINITY_DN59156_c3_g4, TRINITY_DN64408_c4_g1, TRINITY_DN44819_c3_g3, TRINITY_DN57150_c6_g1) were up-regulated expression significantly in water deficiency treatment. In the R/CK group, most genes' expression levels were similar to those in the T/CK group, suggesting that rehydration might affect a small amount of gene expression, which was validated by the gene expression level in R/T (Fig. 6).

TPs Forty-six DEGs encoding transporters were detected in the RNA-seq data, including ABC, Nitrate, Hexose, Polyamine, and so on (Fig. 7; Table S14). In T/CK and R/CK groups, most DEGs were down-regulated, similar to the DEGs related to TFs. While there were some up-regulated expression DEGs under water deficiency stress e.g. two genes related bidirectional sugar transporter (TRINITY_DN50598_c1_g2, TRINITY_DN44794_c7_g1), one gene related Potassium transporter (TRINITY_DN45507_c4_g1), one gene related Metal-nicotianamine transporter (TRINITY_DN46504_c5_g1), one gene related Polyol transporter (TRINITY_DN52400_c0_g1), etc. However, rehydration can affect the remarkable change of these DEGs in terms of their gene expression, as shown in R/T (Fig. 7).

ROS scavenging system In only the T/CK comparison group, there were 22 DEGs encoding ROS scavenging enzymes, categorized as the glutathione peroxidase (GPX) pathway, catalase (CAT) pathway, Peroxidase (POD) pathway, NADPH oxidoreductase pathway and water-water cycle (Fig. 8; Table S15). Among them, the GPX pathway contains the most DEGs in the ROS scavenging system, including glutathione S-transferases (*GSTs*) and glutaredoxin (*GRX*). Under water deficiency stress, one *GRX* (TRINITY_DN47416_c1_g1) and four *GSTs* (TRINITY_DN53475_c0_g3, TRINITY_DN52312_c2_g1, TRINITY_DN62418_c3_g3, and TRINITY_DN53475_c0_g2) had a significantly down-regulated expression,

only two *GSTs* (TRINITY_DN67517_c0_g1 and TRINITY_DN47288_c0_g1) were significantly up-regulated. In the NADPH oxidoreductase pathway, most DEGs coding three key enzymes, viz., thioredoxin (TRX), NAD(P)H-quinine oxidoreductase, and peroxiredoxin (PrxR), were down-regulated (Fig. 8). Additionally, two *SODs* (TRINITY_DN58115_c0_g1, TRINITY_DN57370_c0_g2) were significantly down-regulated expression under water deficiency stress. In addition, these DEGs were associated with two *POD*s, two *PEX*s, one *CAT*, and one *APX*, which possibly participated in plant response to drought stress (Fig. 8). After rehydration, the expression level of 22 DEGs recovered to a certain extent compared to the control. To verify *in situ* the accumulation of H_2O_2 and $O_2^{\cdot-}$ under water deficiency treatment, histochemical with diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) to quantization the ROS activity was performed. After water deficiency-treated, dark blue spots (stained with DAB, Fig. 9a) and brown spots (stained with NBT, Fig. 9b) were deposited the *I. lactea* var. *chinensis* leaves. And after rehydration-treated, the deposited of dark blue spots (stained with DAB, Fig. 9a) and brown spots (stained with NBT, Fig. 9b) turned to lighten. The histochemical experiment further demonstrate that ROS accumulation was involved in drought stress of *I. lactea* var. *Chinensis*.

Proline metabolism Proline dehydrogenase (ProDH), a mitochondrial enzyme, is a key enzyme in the first step of the Proline metabolic process (Fig. 10a). Under water deficiency stress, *ProDH* in *I. lactea* var. *chinensis* plants had a down-regulated expression. When rehydrated, the *ProDH* expression level increased compared to water deficiency treatment (Fig. 10a; Table S16). The genes located downstream of the Proline metabolism process, including *ADC*, *SpeE,* and *APX*, exhibited a down-regulated expression under drought stress (Fig. 10a; Table S16). The free Proline concentration determination result was as consistent as the transcriptome analysis. After water deficiency-treated, The *I. lactea* var. *chinensis* plants' free Proline concentration sharply increased and would decrease after rehydration-treated (Fig. 10b). These data suggested that Proline can be accumulated under drought stress, consistent with previous reports of water deficiency-treated plants.

Discussion

Drought is one of the key factors threating plant growth and limiting the distribution of plant. Drought environments have spread to many regions because of ongoing global climate change. Plants evolve many elaborate mechanisms to adapt to drought stress, but the underlying mechanisms have yet to be investigated. *I. lactea* var. *chinensis* is an ecologically important herb, and possesses strong phenotypic plasticity, leading to growing in many types of habitats, even in the desert. Previous studies on *I. lactea* var. *chinensis* have mainly focused on its phytoremediation capability, salt, heavy metal ion-resistant and PEG-mediated drought traits [23,27-28,31]. As for PEG-mediated drought stress, Ni et al. [23] found osmotic stress signal transduction, ABA signal transduction pathway, TFs, and ROS were mediated *I. lactea* var. chinensis resistance. The underlying mechanism of *I. lactea* var. *chinensis* tolerance to abiotic stresses, especially directly water deficiency stress (severe dehydration or withholding water), needs to be explored. In the present study, RNA-seq technology was applied for *I. lactea* var. *chinensis* transcriptome

profiling under water deficiency stress in order to characterize the molecular mechanism of plant drought tolerance.

TFs play important roles in plant responses to abiotic stresses through the activation or inhibition of target genes' expression [32]. In PEG-mediated drought stress, 85 DEGs were mapped to TFs pathway, including 71 upregulated DEGs: 1 *ABF*, 17 *WRKY*s, 3/6 *bHlH*s, 23 *NAC*s, 6/11 *MYB*s, 12/18 *AP2/ERF*s and 9 *GRAS*s [23]. In the present study, 43 DEGs were related to TFs in *I. lactea* var. *chinensis* plant in a comparison of water deficiency stress and the control, which were divided into 14 subfamilies (Table S13, Fig. 6). Among them, 15 up-regulated and 28 down-regulated DEGs were shown, we detected 2/8 *AP2/ERF*s, 6/7 *MYB*s, 1/4 *NAC* DEGs were upregulated, besides, 4/5 Zinc finger, 1 *BES1* and 1/3 *GRF* DEGs were also upregulated drought-mediated DEGs (Table S13, Fig. 6). An increasing number of reports have documented that TFs play important roles in regulating plant responses to drought stress. For example, DREB2 is an *AP2/ERF* transcription factor, and transgenic *Arabidopsis* plants overexpressing OsDREB2B showed an enhanced expression of DREB2A target genes and improved drought stress tolerance [33]. In *Arabidopsis*, *AtMYB104* is up-regulated under drought under drought stress, while *AtMYB81* is up-regulated [34]. Plant *WRKYs* respond to drought stress and abscisic acid (ABA) signaling in plants [35-36]. Maize *NAC111* was reported to be highly responsive to drought stress, and the over-expression of *ZmNAC111* resulted in increased drought tolerance [37]. Plant zinc finger TFs are positive regulators in plant responses to drought stress [38-39]. In this study, the AP2/ERFs were the largest TF subfamilies, there were two related genes expressed up-regulation in response to the drought conditions. And the expressions of *MYB* were differentially expressed in response to drought conditions (Fig. 6, Table S13). The expression of the *WRKYs* was down-regulated during the drought and water recovery periods. Two genes encoding NAC expression were up-regulated during the drought and water recovery periods (Fig. 6, Table S13). Together, these DEGs coding TFs possibly play important roles in *I. lactea* var. *chinensis*'s drought tolerance.

In this study, 46 DEGs associated with transporters including ABC, phosphate, potassium, and ammonium transporters, participated in *I. lactea* var. *chinensis*'s drought tolerance. In our data, four phosphate transporter DEGs were identified (Fig. 7), which were reported to determine the plant's drought tolerance. Several studies have shown that phosphate transporters are involved in drought responses in poplar [40] and *Arabidopsis thaliana* [41]. In our research, there were five DEGs involved in nitrogen-related transporters including two nitrate transporter genes, and two polyamine transporter genes, and two ammonium transporter genes, which participate in nitrogen uptake, reduction and metabolism (Fig. 7). Recently, two ammonium transporter genes, AMT1;2 and AMT1;6, were demonstrated to regulate plant responses to drought stress by participating in NH^{4+} uptake in *Populus simonii* [42]. It's reported that bidirectional sugar transporter can mediates both low-affinity uptake and efflux of sugar across the plasma membrane with waterlogging stress [43]. The metal-nicotianamine transporter acts probably as a transporter of iron- and metal-nicotianamine chelates [44]. Increased transport of polyols, both in the phloem and the xylem occurs frequently as a result of drought stress [45]. In this study, some genes encoding Bidirectional sugar transporter, metal-nicotianamine transporter, and Polyol transporter were up-

regulated after drought which indicated *I. lactea* var. *chinensis* plant drought tolerance was related to sugar, potassium transport, and iron transport, etc. Thereby, these results showed that *I. lactea* var. *chinensis*'s drought tolerance possibly depends on transporter genes' expression changes.

In general, abiotic stresses cause ROS accumulation, leading to the inhibition of plant growth [46]. Thereby, it is important to scavenge ROS to protect cells from oxidative damage through antioxidants and antioxidant enzymes. In PEG-mediated drought stress, Ni et al. [23] found 23 *POD*, 10 *CAT* and 13 *GST* were related to non-enzymatic antioxidants. And in the present study, there were 22 DEGs in a comparison of water deficiency treatment and the control, which were enriched in the GO category 'cellular response to osmotic stress' (GO: 0071470) (Table S5), we also found mediated DEGs in NADPH oxidoreductase pathway (*Trx* and *NADPH*) and water-water cycle (*SOD* and *APX*). Among them, *six GST*s were associated with *I. lactea* var. *chinensis* plant drought tolerance (Fig. 8; Fig. 10; Table S15). Interestingly, a recent report showed that 16 *GST*s likely participated in *I. lactea* var. *chinensis*'s salt tolerance [22]. These results suggested that GSTs participating in drought and salt tolerance resulted from their function in ROS scavenging. Additionally, two *SOD*s were up-regulated in response to drought stress in transcriptome data of *I. lactea* var. *chinensis* (Table S15), which catalyze the disproportionation of O_2 into H_2O_2 and O_2 that converts the H_2O_2 back into H_2O in the water-water cycle catalyzed by ascorbate peroxidase (APX) [47]. There have been many reports demonstrating that SODs participate in plant responses to drought stress, such as *Ipomoea batatas* [48], *Oryza sativa* [49], and *Lens culinaris* [50]. According to RNA-seq analysis, the ROS scavenging system is important to *I. lactea* var. *chinensis*'s drought tolerance.

Proline is considered as an important nonenzymatic antioxidants to scavenge the negative effect of ROS [51] and inhibit ROS-mediated apoptosis in response to cellular stress [52]. Proline accumulation is a common physiological response to drought stresses in many plants [53-55]. In this study, there were several GO terms and KEGG pathways involved in Proline metabolism in a comparison of drought stress and the control. The GO terms mainly included the 'glutamine family amino acid catabolic process' (GO: 0009065), 'Proline catabolic process' (GO: 0006562) and 'Proline metabolic process' (GO: 0006560) (Table S7~S9), and the KEGG pathways contained 'Arginine and Proline metabolism' (ko00330) and 'Glutathione metabolism' (ko00480) (Table 2), which affected Proline accumulation in plants under drought stress. In plants, a major Proline synthesis pathway is synthesized by glutamate. The precursor substance glutamate could be catalyzed by two enzyme, pyrroline-5-carboxylate synthase (*P5CS*) and pyrroline-5-carboxylate reductase (P5CR) to synthesize Proline [56]. Su and Wu [57] reported that *Oryza sativa* could tolerant higher salt and water deficiency stress by induced increasing expression of *P5CS* to accumulate Proline concentration. In the present study, we also observed an increased expression of *P5CS* under drought stress to promote Proline accumulation. On the other hand, the Proline degradation is a reversible reaction which catalyzed by Pro dehydrogenase (*ProDH*) and P5C dehydrogenase (*P5CDH*) [58]. In the present study, the *I. lactea* var. *chinensis* plant's *ProDH* had a significantly down-regulated expression under drought stress in our study, indicating that Proline metabolism is inhibited to lead to its accumulation (Fig. 10). In line with the results, the *ProDH* expression of *Arabidopsis* in is down-regulated by dehydration and up-regulated after rehydration [59]. Thereby, the change of Proline

metabolism could be beneficial for Proline accumulation in *I. lactea* var. *chinensis* for drought tolerance by two main strategies: the first strategy is to promote the synthesis of Proline by increased expression of *P5CS*, the second strategy is to inhibit the degradation of Proline by decreased expression of *ProDH*.

Though establishing novel homeostasis during rehydration after drought stress is vital for plant growth and development, rarely researches involved in and the rehydration mechanism is uncharacteristic so far. The extent of recovery from rehydration may depend on pre-drought intensity and duration and species/varieties [60]. Talame et al. [61] compared the expression changes in leaves of barley plants during drought-stress and rehydration treatments, and found that a very low overlap differentially regulated transcripts (unknown function). The rehydration-inducible genes identified may involve in ABA degradation [62], recovery process from dehydration-induced damage (protein degradation, ROS scavenging enzymes, and regulatory proteins), suggested that regulation during the recovery from drought may involve regulation at both transcriptional and post-transcriptional levels. Ford et al. [63] tested three drought tolerant wheat cultivars responses during rewatering after water deficiency treatment. The most drought tolerant cultivar's glycolysis enzymes abundance increased sharply, indicated the need for energy during recovery phase. In this study, 73 DEGs were found to be common between T/CK and R/T but not R/CK (Fig. S1, Table S2~S4). Their expression of exhibited a reversible trend during water deficiency and the rehydration period. However, these genes were functionally unknown which was similar to Talame et al. [61]. Moreover, several genes may be focused on the future research. For example, one primary-amine oxidase (*PAO*) related gene was more highly expressed in the drought-stressed seedlings than in the control following the water recovery. It has been reported that *MmPAO2* gene was highly responsive to drought stress [64]. Sesquiterpenoids show involvement in many biological properties as a component of plant secondary metabolic pathways [65], which participate in response to drought stress in *Salvia dolomitica* [66]. The germacrene D synthase (*GDS*) coding gene, a main enzyme in the biosynthesis of sesquiterpenoids, was up-regulated in response to drought stress and down-regulated in rehydration treatment of *I. lactea* var. *chinensis*. In plants, UGTs (UDP-glycosyltransferases) glycosylate various phytohormones and metabolites in response to abiotic stress. For example, the expression levels of *UGT85A1* and *UGT85A2* were shown to be downregulated under drought stress in an *Arabidopsis* mutant [67]. In line with the result, our RNA-seq data revealed that two genes related to *UGT85A* expression were down-regulated during the drought and up-regulated during the water recovery period. Thereby, the results imply that over-expressing or knocking down these DEGs through genetic manipulations may increase the drought tolerance in *I. lactea* var. *chinensis*.

Plants live in constantly changing environments that are often suffered abiotic stress, including drought, heat, cold, nutrient deficiency, salt or toxic metals in the soil. Plant often suffers multiple abiotic stresses simultaneously that overlapping signals and pathways may contribute to the plant stress resistance [68]. So far, the mechanisms of *I. lactea* var. *chinensis* abiotic stress to toxic heavy metal elements (cadmium and lead) [21], salt [22-23], and PEG-mediated drought [23] were investigated in succession. Transcription factors (TF) and reactive oxygen species (ROS) detoxification system were found to mediate toxic heavy metal, salt and drought stress. However, they may have unique signal transduction and response mechanism against different types of abiotic stress. Ni et al. [23] compared the resistance response

under NaCl or PEG stress from transcriptome data, found that transporter activity, transmembrane transporter activity, cell wall, cell periphery, and membrane were co-regulatory under the two stresses. While the above enriched pathways had different regulatory strategy, suggesting the same genes play different roles facing different stress. Combined with Ni et al. [23] PEG-mediated study, the drought tolerance mechanism involves in several metabolic pathways associated with repair or reduction of damage due to stress, including osmotic stress signal transduction, ABA signal transduction, transcription factors, transporters, ROS scavenging system, Proline metabolism etc.

Conclusion

In conclusion, we employed the RNA-seq technique to obtain global information on the gene expression of the *I. lactea* var. *chinensis* plant in response to water deficiency stress as well as rehydration. Based on the assembled *de novo* transcriptome, 1187, 275, and 865 DEGs were identified from the comparison of T/CK, R/T, and R/CK, respectively. GO and KEGG pathway analysis uncovered that the DEGs were enriched in several important terms and pathways related to Proline metabolism, such as the 'Proline catabolic process', 'Proline metabolic process' and 'Arginine and Proline metabolism'. Moreover, lots of DEGs involved in transcription factors, transporters, and the ROS scavenging system were shown. The expression of these 73 DEGs displayed a reversible trend during drought and the rehydration period, such as the genes encoding PAO, GDS, UGT85A, etc., which may need to be focused on future research. Taken together, our findings showed that *I. lactea* var. *chinensis*'s drought tolerance might depend on Proline accumulation, the action of transcription factors and transporters, and a strong ROS scavenging system.

Declarations

Ethics approval and consent to participate

We declarated that we have the permission to collected the seeds of *I. lactea* var. *chinensis* from the Ordos in Inner Mongolia. The experimental research and field studies on plants (either cultivated or wild), including the collection of plant material, comply with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not Applicable.

Availability of data and materials

The datasets generated and analysed during the current study are available in the NCBI repository, with the Accession Number SRP257840.

Competing interests

The authors declare that they have no conflict of interests.

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Authors' contributions

J.W. and G.Z. conceived and designed the experiments; R.Z., Y.Z., Z.S., and H.W. performed the bioinformatics analysis; J.G., L.Y. and G.H. performed the RNA extraction and qRT-PCR experiments; W.F. and Z.W. contributed reagents/materials/analysis tools. R.Z. and Y.Z. wrote the paper; All authors read and approved the final manuscript.

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Tables

Table 1. Number of functional annotations for all unigenes

Values	Total	NR	SwissProt	KEGG	COG	Pfam	GO	Overall
Number	126,979	41,360	31,799	18,512	8,875	28,920	28,385	44,247
Percentage	100%	32.57%	25.04%	14.58%	6.99%	22.78%	22.35%	34.85%

Table 2. Expression profiling of DEGs in *Iris lactea* var*. chinensis* significantly enriched pathways under drought stress (*P*-adjust<0.05)

Pathway ID	Description	*P* value	*P*-adjust
ko04626	Plant-pathogen interaction	1.19805E-06	8.39E-05
ko00592	alpha-Linolenic acid metabolism	1.13685E-05	0.00039
ko04712	Circadian rhythm - plant	0.00053171	0.01240
ko02010	ABC transporters	0.00122627	0.02146
ko00330	Arginine and Proline metabolism	0.00382203	0.04459
ko00480	Glutathione metabolism	0.00356642	0.04993
ko04075	Plant hormone signal transduction	0.0079252	0.07925
ko04146	Peroxisome	0.01387324	0.12139
ko04016	MAPK signaling pathway - plant	0.03042858	0.23666
ko04141	Protein processing in endoplasmic reticulum	0.03785114	0.26495

Figures

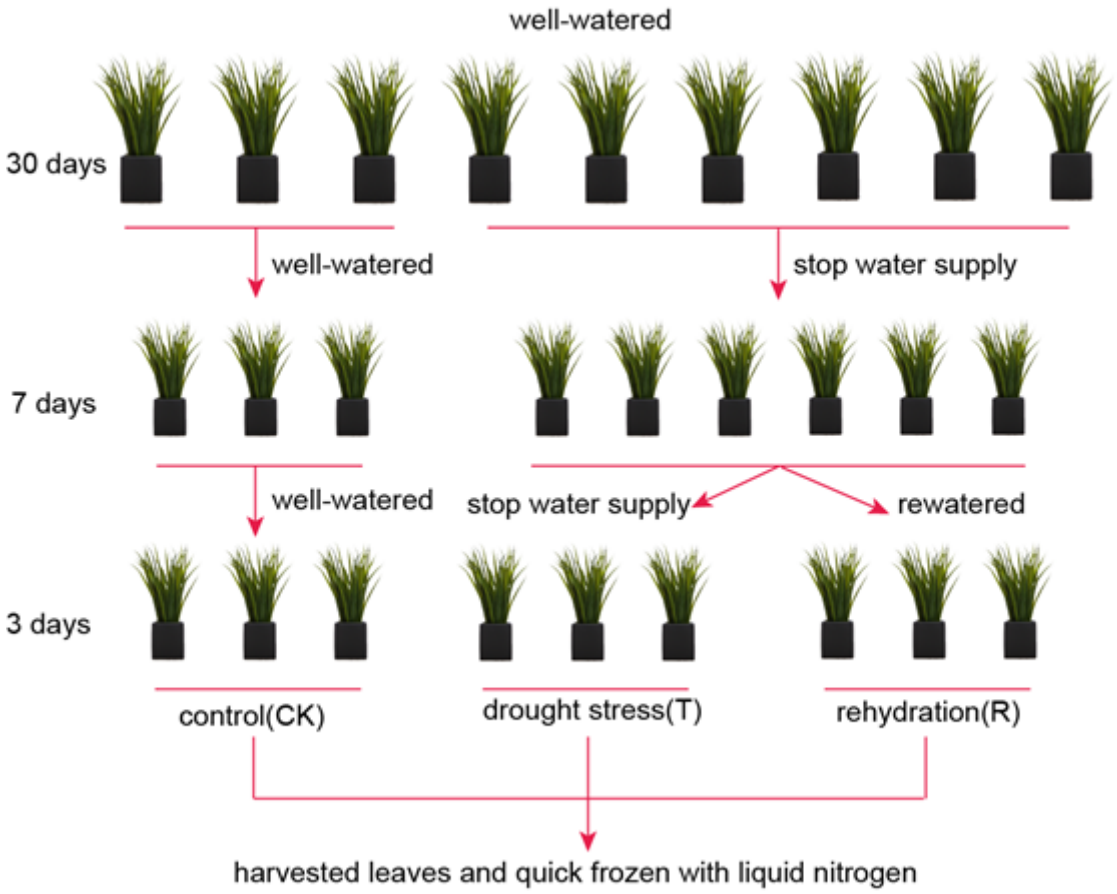

Figure 1

A scheme representing the experimental design.

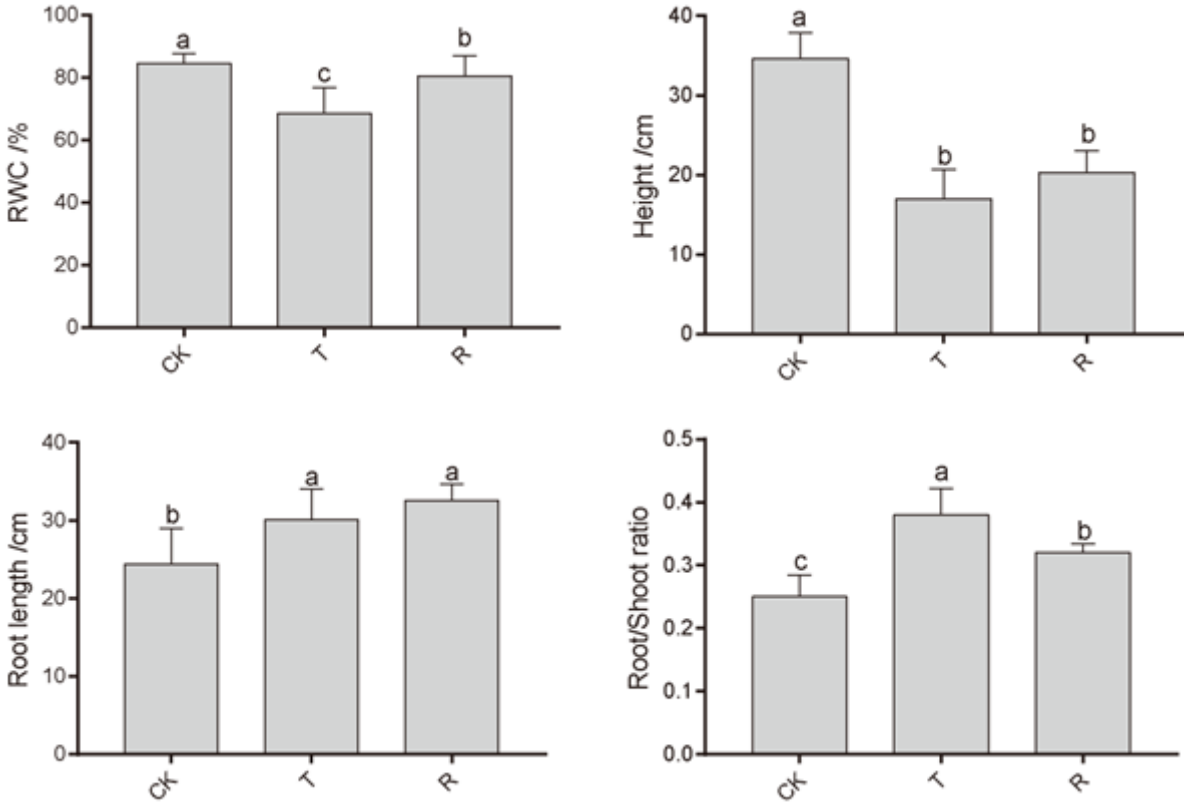

Figure 2

The phenotypic characterization of *Iris lactea* var. *chinensis* following different treatments.

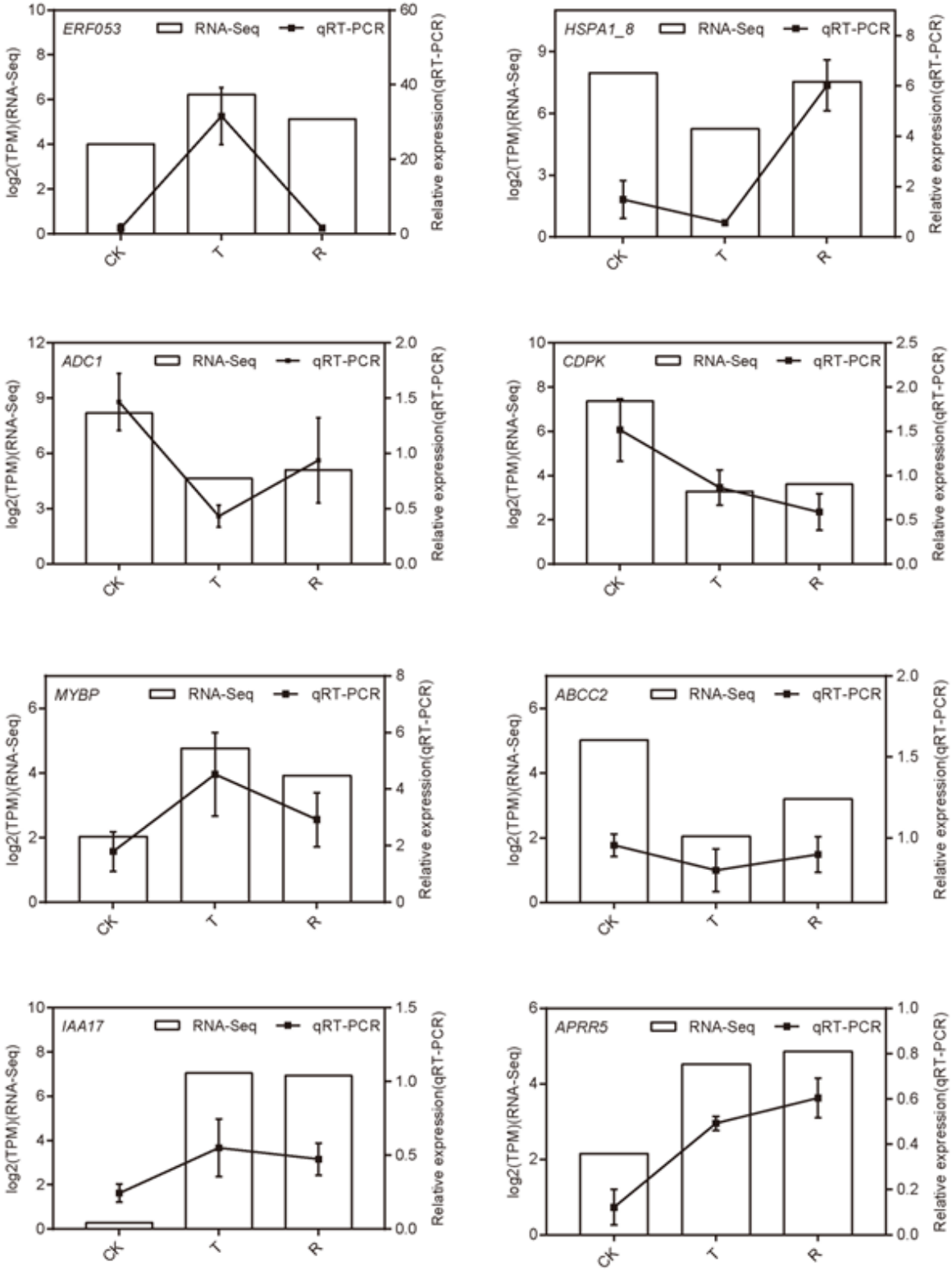

Figure 3

RT- qPCR confirmation of the relative expression levels of transcripts. Relative expression levels in transcript abundance obtained by both qRT- PCR and RNA-Seq are presented for eight different genes. The signal intensity of each transcript was normalized using actin1. The y-axis shows the normalized expression level of the transcript.

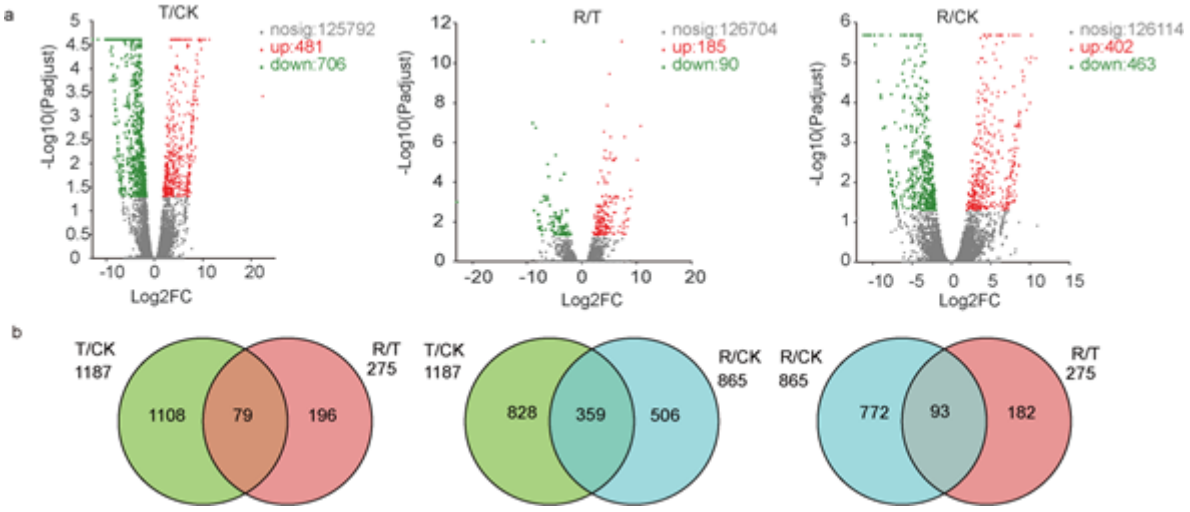

Figure 4

Expression profiling of differentially expressed genes (DEGs) in *Iris lactea* var. *chinensis* for T (water deficiency), CK (control), and R (rehydration). (a) Scatter plot showing DEGs in the comparison groups. (b) Venn diagrams showing the total number of DEGs and the total number for each comparison is given.

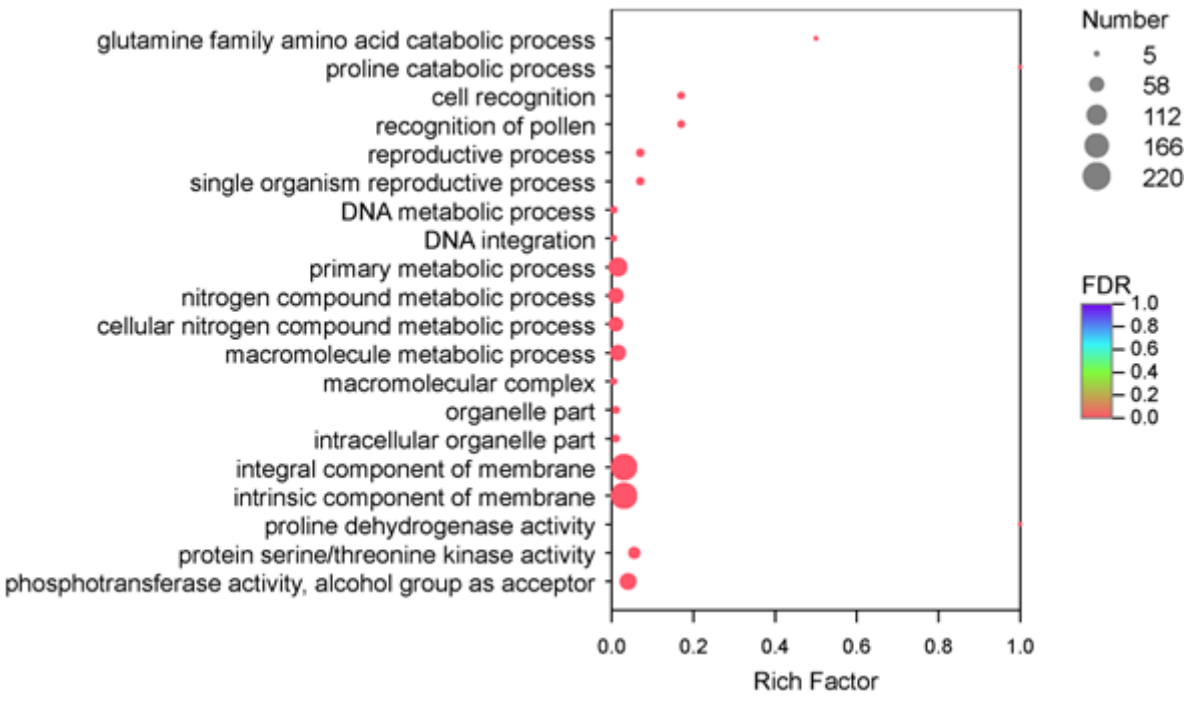

Figure 5

Gene ontology (GO) of DEGs in *Iris lactea* var. *chinensis* under drought stress.

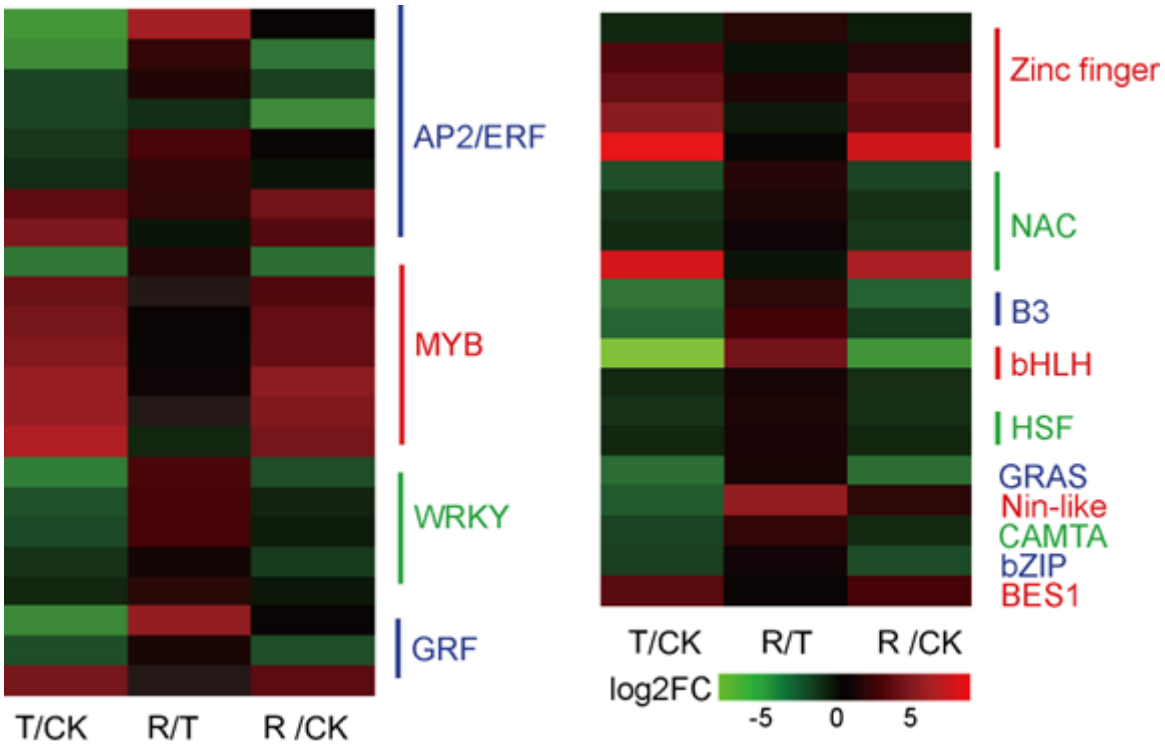

Figure 6

Expression patterns of drought-regulated differentially expressed genes (DEGs) of transcription factors (TF) genes.

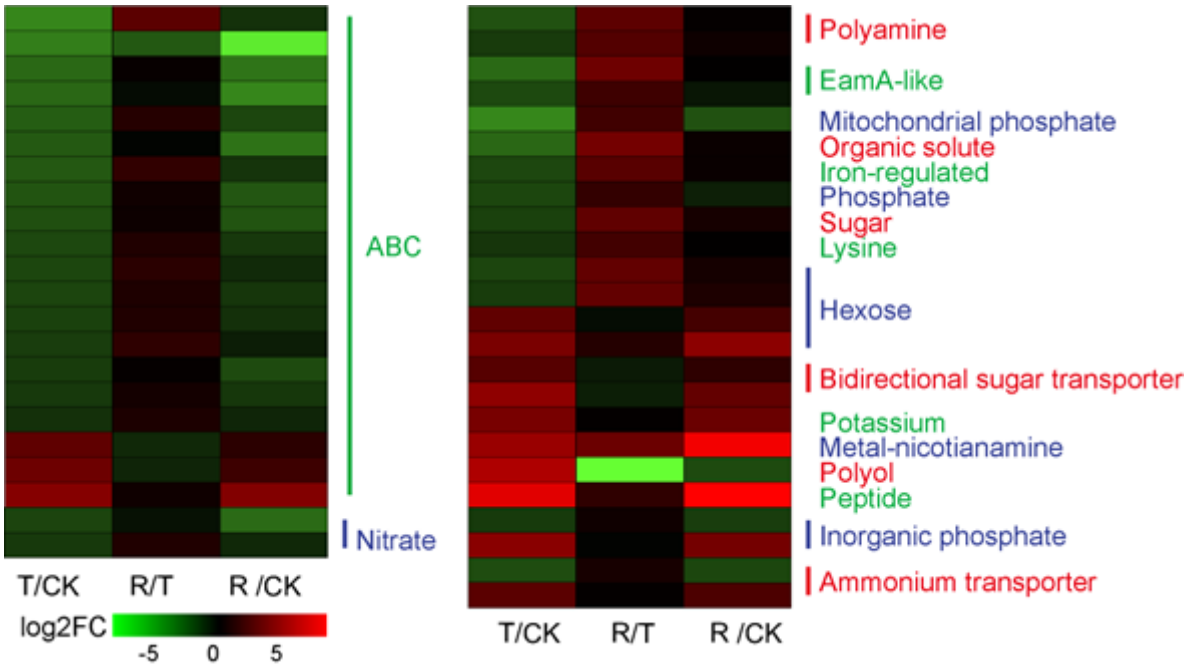

Figure 7

Expression patterns of drought-regulated differentially expressed genes of transporter genes.

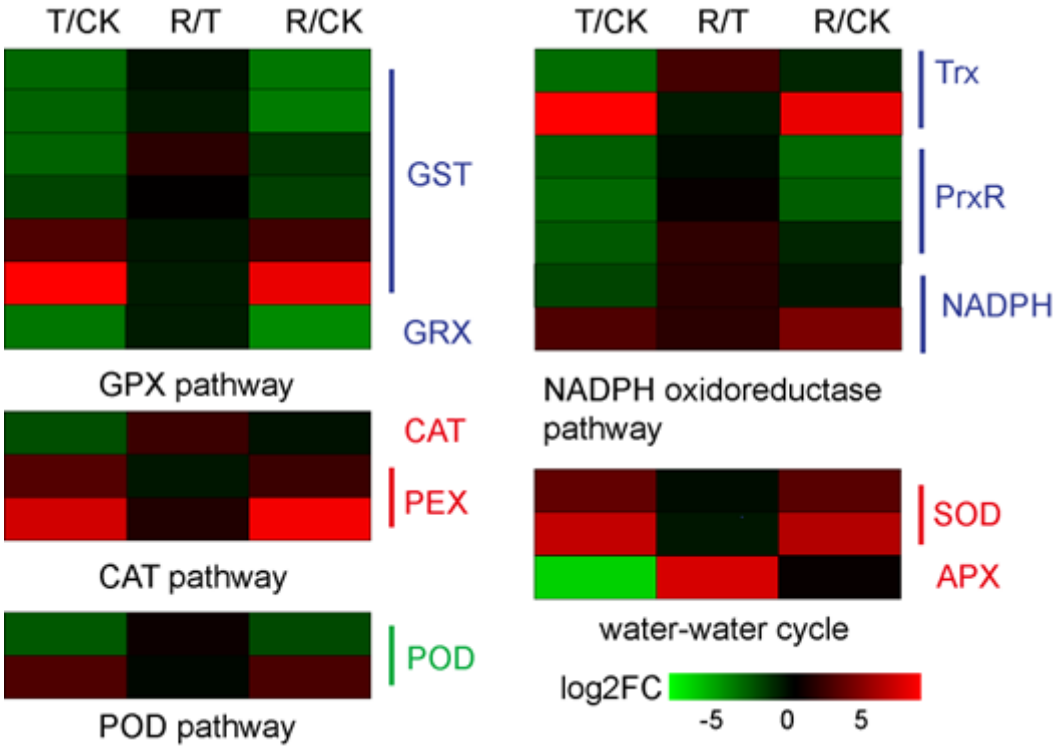

Figure 8

Expression patterns of drought-regulated differentially expressed genes encoding enzymes related to the reactive oxygen species (ROS) scavenging system.

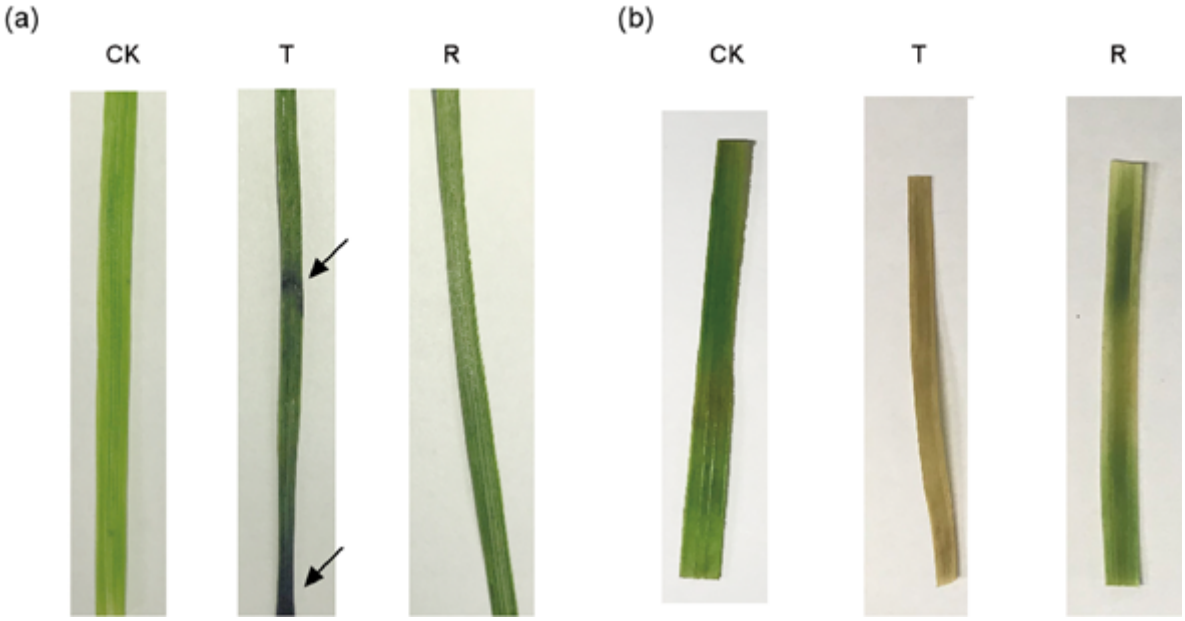

Figure 9

Comparison of $O_2^{\cdot-}$ and H_2O_2 accumulation in *Iris lactea* var. *chinensis* leaves under normal watering (CK), water deficiency-treated (T), and rehydration -treated (R) condition. (a) NBT staining, (b) DAB staining. Arrow indicate the location of $O_2^{\cdot-}$ and H_2O_2 accumulation.

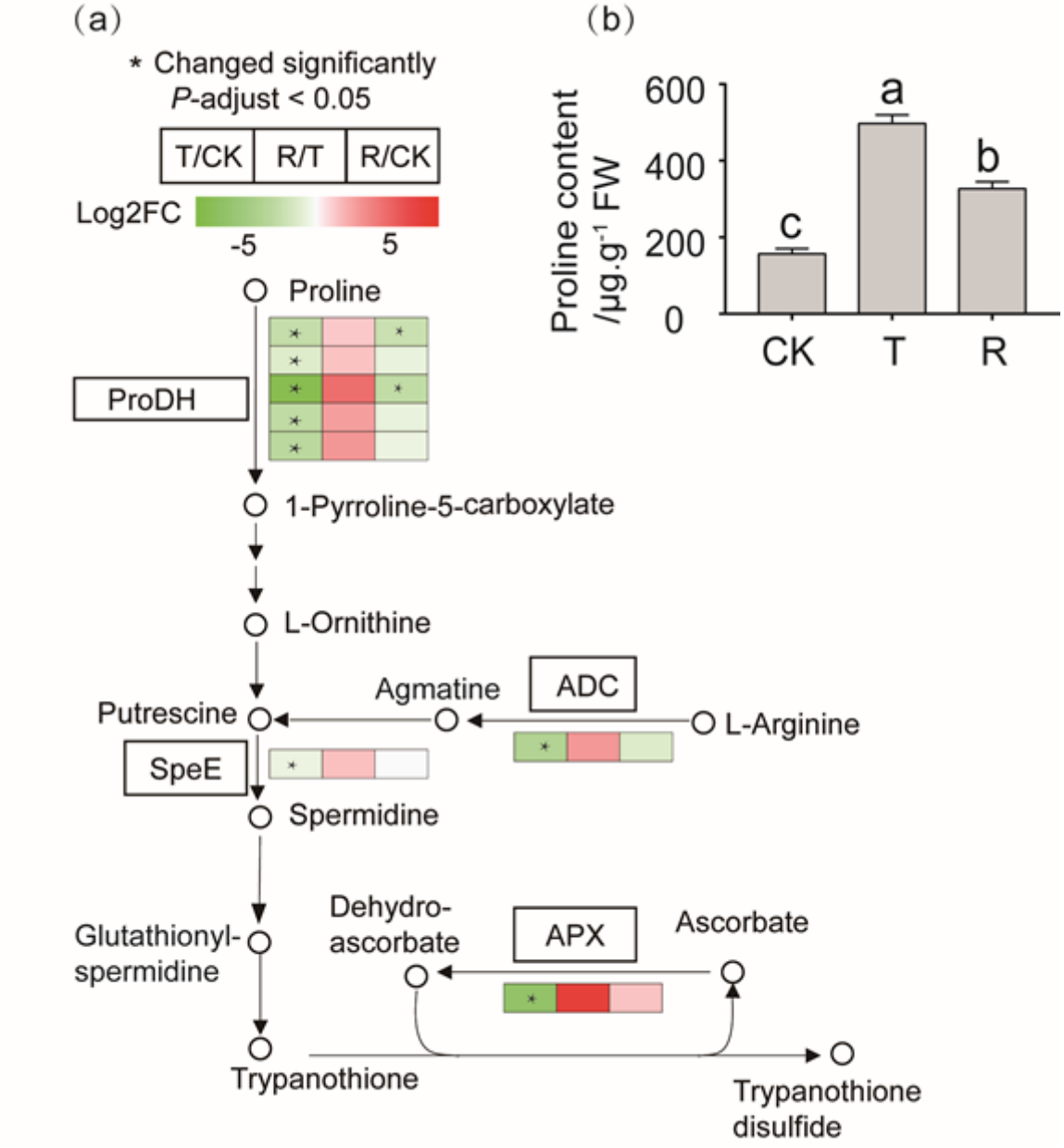

Figure 10

(a) Gene expression of the Proline and glutathione metabolism pathway and the response in *Iris lactea* var. *chinensis* under water deficiency treatment and rehydration. Metabolites detected shown in bold, solid lines represent one-step reactions, and dashed lines represent multi-step reactions. The abbreviations in boxes indicate enzymes catalyzing the reactions. The expression patterns of unigenes encoding these enzymes under drought stress and rehydration compared to the controlsare shown in the color boxes. The small boxes in the left, middle and right sides of each colored box indicate altered levels between T/CK, R/T and R/CK, respectively. Significantly altered expression (*P*-adjust < 0.05) is indicated by an asterisk in the boxes. Abbreviations for enzymes: ProDH: Proline dehydrogenase; speE: spermidine synthase; APX: L-ascorbate peroxidase; ADC: Arginine decarboxylase. (b) Comparison of Proline concentration in *Iris lactea* var. *chinensis* under normal watering (CK), water deficiency-treated (T), and rehydration -treated (R) condition.

Supplementary Files

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