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Physiological changes and full-length transcriptome of *Artemisia sphaerocephala* in response to drought stress

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Abstract

Background *Artemisia sphaerocephala* is an important sand-fixing plant in arid sandy areas. To elucidate the physiological and molecular changes of *A. sphaerocephalala* induced by drought stress, this study used a combination of second-generation sequencing and third-generation sequencing technologies to sequence the full-length transcriptome. The sequencing results can identify significant genes and main pathways related to drought resistance of *A. sphaerocephalala*.

Results Changes in physiological characteristics indicated that the relative permeability of plasma (RPP), superoxide dismutase (SOD) activity, peroxidase (POD) activity, catalase (CAT) activity, malondialdehyde (MDA), proline (Pro), soluble sugar (SS), soluble protein (SP) contents increased under drought stress, and all of them decreased sharply after re-watering. The relative water content (RWC) exhibited a declining trend under drought stress, but it increased after re-watering and the chlorophyll content showed a continuous decrease under drought stress and re-watering. Additionally, transcriptome sequencing revealed that important metabolic pathways, such as Plant hormone signal transduction, Starch and sucrose metabolism, Glyoxylate and dicarboxylate metabolism, and Nitrogen metabolism were enriched in *A. sphaerocephala* under severe drought stress. Moreover, the RNA-Seq results of 10 genes were confirmed by real-time PCR, and the results showed that all of them were involved in the process of drought stress adaptation of *A. sphaerocephala*.

Conclusions This is the first reported large-scale sequencing of the full-length transcriptome of *A. sphaerocephala* under drought stress, and these results will provide a better understanding of *A. sphaerocephala* responses to drought stress and lay the groundwork for analysing the expression profile of genes related to drought tolerance in plants.

Keywords *Artemisia sphaerocephala*, Drought stress, Physiological analyses, Transcriptome, qRT-PCR analysis

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Background

Drought, low or high temperatures, and other adverse environments are important factors that affect plant growth. Among them, drought is one of the main factors affecting plant growth. Plant growth and metabolism are inhibited by drought, in severe cases, it can even cause irreversible damage and eventually lead to plant death [1]. This results in a substantial decline in the output of cash crops year by year [2], and the reduction in global crop production due to water shortage in the world exceeds the total reduction in production caused by other factors [3]. When plants suffer from drought stress, it will cause a series of physiological, biochemical, and molecular biological changes, including changes in the structure and composition of biofilms, and many specificities [4], it also promotes protein, sugar, osmotic adjustment substances (betaine, proline, etc.), and accumulate enzyme activities, etc [5].

Artemisia sphaerocephala is a perennial subshrub belonging to the genus *Asteraceae* [6], which is mainly distributed in western China such as Shanxi, Inner Mongolia, Qinghai, Shaanxi, Ningxia, Gansu, and Xinjiang [7]. *A. sphaerocephala* exhibits strong drought resistance and is a dominant species in the barren land. It is not only a pioneer species for restoring vegetation by fixing drifting sand and preserving water and soil [8, 9], but also serves as a geographical substitute species for *Artemisia ordosica* and *Artemisia sieversiana* in arid areas [10]. *A. sphaerocephala* possesses unique anatomical and water physiological characteristics typical of xerophytes. In terms of anatomical structure, the leaf surface has a thick cuticle, which can greatly reduce the water loss caused by transpiration [11]. In addition, the enhanced palisade tissue and the degradation of the spongy tissue affect the area of chloroplasts receiving light and air, thereby improving the rate of light energy utilization [12]. In the field of water relationship, the significantly reduced water potential and transpiration intensity of *A. sphaerocephala* make it able to grow and reproduce faster and better in areas with relatively arid and harsh environments with extreme drought-resistant [13], and it is a very significant pioneer plant in the early successional herbaceous process of sandy areas [14]. At present, research on the drought resistance of *A. sphaerocephala* is mainly focused on a part of specific physiological indices, the comprehensive physiological and molecular mechanisms of drought resistance are still unclear, and there is no report on transcriptome analysis of *A. sphaerocephala.*

First-generation of sanger sequencing is engaged to obtain full-length cDNA sequences to study the structural and functional properties of genomics owing to the advantages such as comprehensive identification of alternative splicing, the discovery of more new genes, effective improvement of genome annotation, and accurate mapping of fusion genes [15–17] before the application of high-throughput sequencing technology. Recently, first-generation sequencing (FGS) has been replaced by second/third-generation sequencing technology (SGS/TGS) because SGS technology improves yield and sequence accuracy while reducing costs [18], and TGS technology can obtain longer read length, more uniform coverage, and construct a complete transcriptome [15]. SGS has been widely used in genomic and transcriptomic studies in the past decades. Nevertheless, the disadvantage of shorter readings of SGS remains unsolved [19], making related bioinformatic analyses difficult and reducing the accuracy of sequence assembly. TGS can effectively overcome problems associated with SGS, whose long read length is useful for the de novo genome and transcriptome assembly of higher organisms [20–22]. The relatively high error rate of TGS might be problematic in bioinformatics analysis and sequence alignment, but it can be improved and corrected by high-precision SGS short readings [23, 24]. Hybrid sequencing approaches combining SGS and TGS are more popular as it can provide more accurate and complete gene assembly in genome and transcriptome studies [25, 26].

Currently, an increasing number of plants such as rice, potato, and poplar have been sequenced and assembled by hybrid sequencing methods, and these studies have identified numerous new genes and alternatively spliced isoforms in different species [27–29]. To clarify the physiological and molecular changes of *A. sphaerocephala* under drought stress, physiological indices were investigated and the drought resistance-related genes were excavated and their expression patterns were further studied under different drought stress in this paper, which can provide a basis for the elucidation of the physiological mechanism of drought resistance of *A. sphaerocephala*, and lay a foundation for clarifying the function of drought resistance genes of *A. sphaerocephala*.

Results

Analysis of physiological changes

In order to study the physiological changes of *A. sphaerocephala* under drought stress, several important physiological properties were analyzed in this study. The overall trend of relative permeability of plasma of *A. sphaerocephala* was increasing with a deepening water stress degree (Fig. 1A). The MDA content increased slowly at 2 days of drought stress (DS1), then increased steadily and reached the maximum at 8 days of drought stress (DS3) (Fig. 1B). Both the relative permeability of plasma and the MDA content decreased rapidly after rehydration but were still relatively high compared with the control group (CK). The relative water content and chlorophyll content showed a continuous decline under drought stress. The RWC increased after re-watering and

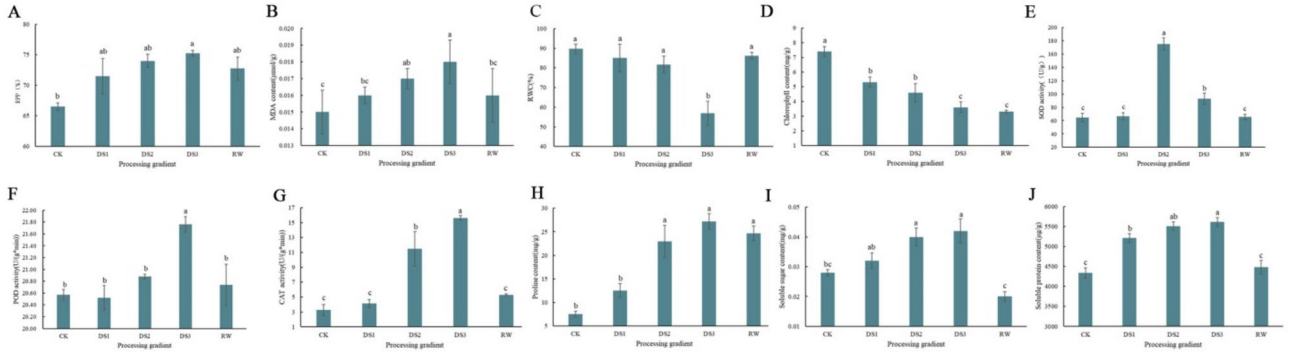

Fig. 1 Physiological indices of *A. sphaerocephala* under drought stress. (**A**) The change of relative permeability of plasma. (**B**) The content of MDA. (**C**) The change of RWC. (**D**) The content of chlorophyll. (**E**) The activity of SOD. (**F**) The activity of POD. (**G**) The activity of CAT. (**H**) The content of Pro. (I) The content of soluble sugar. (**J**) The content of soluble protein. Values are presented as mean ± standard error from three independent biological replicates. All data are presented as the means ± standard errors, while the error bars indicate standard deviation of three replicates

returned to the level of CK. However, the chlorophyll content did not recover and still decreased after rehydration (Fig. 1C-D). In addition, under drought stress, the antioxidant enzyme activity such as SOD, POD, and CAT had no obvious change in the early stress stage, and then increased rapidly and reached a maximum at 4 days of drought stress (DS2) (SOD) or DS3 (POD, CAT) and subsequently decreased rapidly (Fig. 1E-G), and all of them returned to the level of CK after re-watering. The proline content showed a tendency to increase and reached the maximum value at DS3 and decreased after rehydration, but it was still higher than that of CK (Fig. 1H). The content of soluble sugar and soluble protein showed a gradually increasing trend during the drought stress treatment and decreased rapidly after rehydration (Fig. 1I-J).

Transcription group analysis

Quality control of sequencing data

To identify and characterize the *A. sphaerocephala* transcriptomes for control and drought stress treatments, we performed whole transcriptome profiling using a combination of PacBio-SMRT and SGS technologies. In total, Illumina sequencing yielded over 720 million clean reads. The analysis result is referred to as 'Illumina'. SMRT sequencing yielding 512,434 reads of inserts, of which 344,868 were full-length non-chimeric reads (containing 5′ primer, 3′ primer, and the poly(A) tail) and 54,052 were non-full-length non-chimeric reads. The average length of the full-length non-chimeric read was 2701 bp.

SMRT sequencing has the advantage of long read lengths, but the technique has a high single-base error rate. To reduce the high incidence of subread errors, all 512,434 SMRT readings were corrected using approximately 720 million Illumina clean reads as input. A total of 90,730 non-redundant transcripts were generated using the CD-HIT v4.6.8 procedure (https://github.com/weizhongli/cdhit, Parameters: c0.95-T6-G0-aL0 .90-AL100-aS0.99-AS30) to correct errors and remove redundant transcripts, each represented a unique full-length transcript with an average length of 2991 bp and N50 of 3521 bp. For simplicity, the results are referred to as "SMRT".

The length distribution of SGS and TGS transcripts showed that approximately 38.64% of the Illumina reads were less than 500 bases, while only 2.78% of the SMRT transcripts were less than 500 bases. Of the SMRT transcripts assembled, 71.64% were greater than 2000 bases, while only 8.09% of the assembled transcripts from Illumina were greater than 2000 bases. In addition, the average length of genes detected by SMRT was longer than Illumina. Our results suggest that SMRT sequencing produced more full-length, high-quality transcripts and that correcting low-quality SMRT reads using SGS data improved the accuracy of PacBio long-read.

Functional annotation and classification of unigenes

To obtain the most comprehensive annotation, all SMRT full-length transcripts were aligned to public databases such as the NCBI non-redundant protein (NR) database, NCBI non-redundant nucleotide database(NT), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Protein family (Pfam), EuKaryotic Ortholog Groups (KOG) and Gene Ontology (GO) by BLASTX ($E\text{-}value < 1e^{-10}$). A total of 81,864 (90.23%), 57,794 (63.70%), 81,135 (89.42%), 70,966 (78.22%), 55,743 (61.44%), 55,743 (61.44%) and 53,359 (58.81%) unigenes had significant hit rates ($E\text{-}value < 1e^{-10}$) in NR, NT, KEGG, SwissProt, PFAM, GO and KOG, respectively (Fig. 2). Among the 90,730 high-quality unique sequences, 84,114 (92.71%) unigenes were annotated to at least one of the 7 databases, and 28,372 (31.27%) unigenes were annotated to proteins in all 7 databases.

Five major public databases (NT, NR, KOG, GO, and PFAM) were selected from seven databases to draw the Venn map (Fig. 3) to identify the number of unigenes

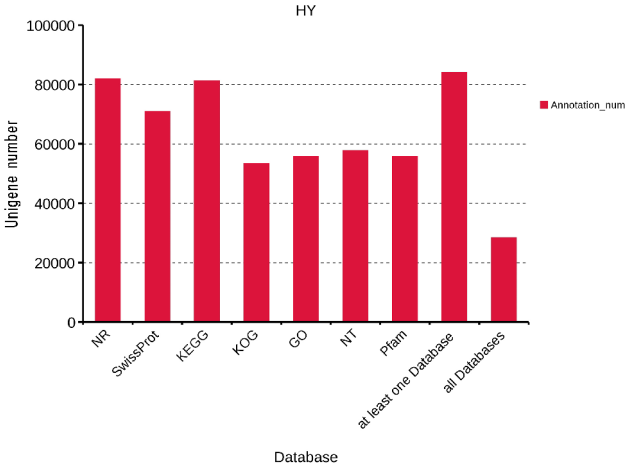

Fig. 2 Unigenes matched in seven databases

with important motifs (*E-value* < $1e^{-10}$) at each intersection of the Venn diagram, and there were 28,690 unigenes matched in all five databases. According to the BLASTx matching results in the GO protein database, a total of 55,743 unigenes were classified by BLAST GO (*E-value* < $1e^{-10}$), and at least one GO item was assigned to them. Single-gene belongs to three main GO categories and 56 subcategories, including biological processes (BP), of which there are 25 major subcategories, cellular compartment (CC), with 19 subcategories, and molecular function (MF), including a total of 11 main subcategories (Fig. 4).

Within the unigenes of *A. sphaerocephala*, 53,359 unigenes were categorized in 26 KOG clusters (*E-value* < $1e^{-10}$) (Fig. 5). The five largest categories were general function prediction only (12,313 unigenes), signal transduction mechanisms (8,963 unigenes), posttranslational modification (5,265 unigenes), transcription (3,012 unigenes), intracellular trafficking, secretion, and vesicular transport (2,856 unigenes).

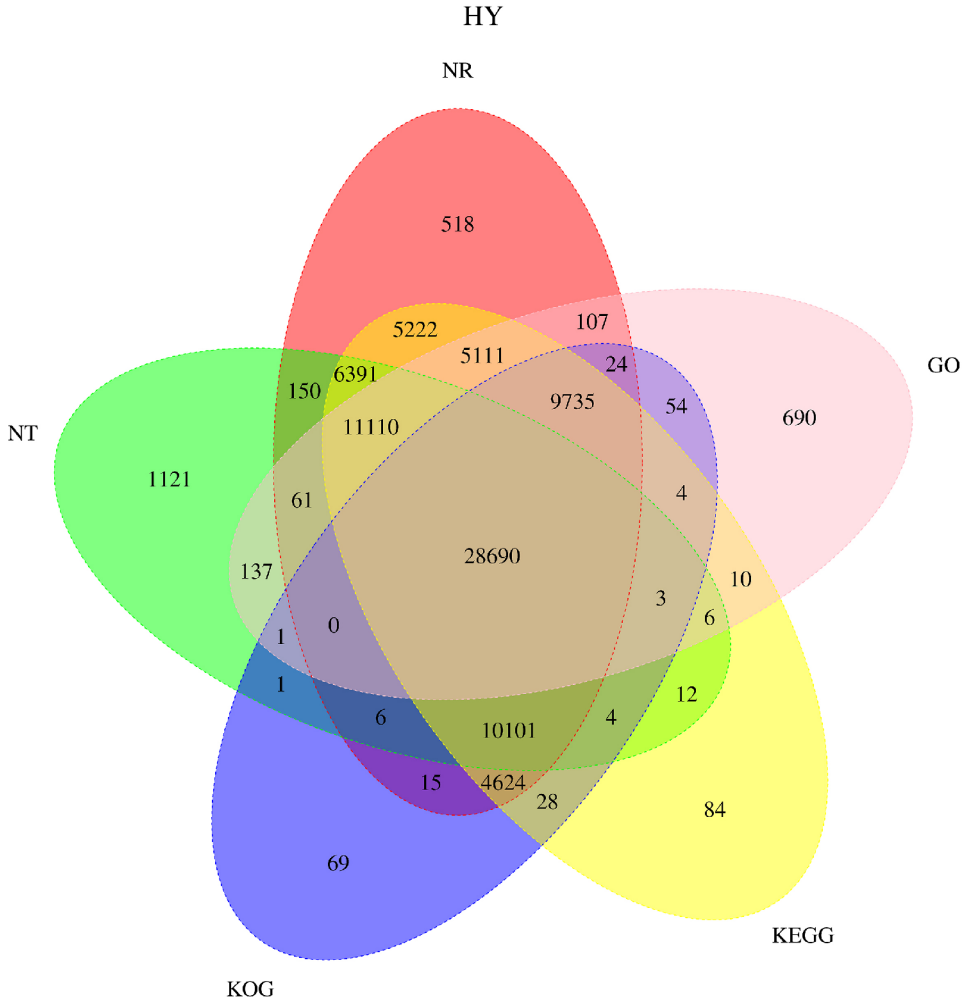

Fig. 3 Venn map of differential databases

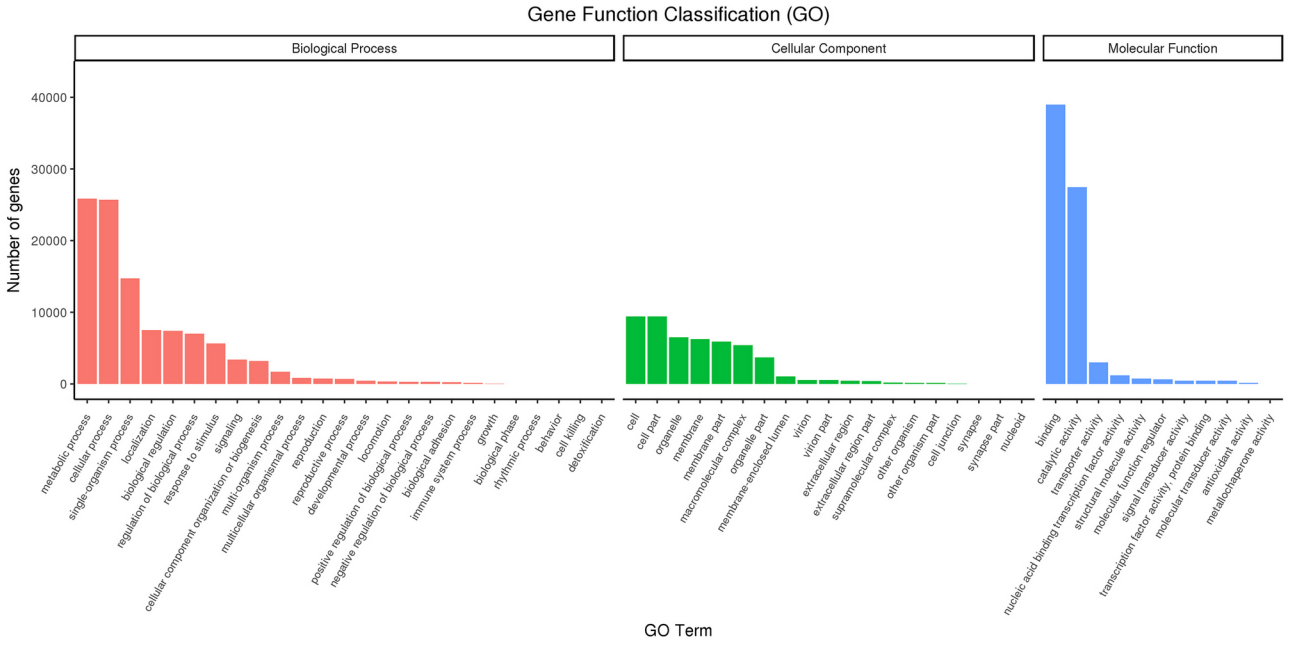

Fig. 4 Functional classification of GO terms of *A. sphaerocephala* transcripts. The number of genes in a specific subcategory within the main category is shown on the y-axis, and the name of the subcategory is shown on the x-axis

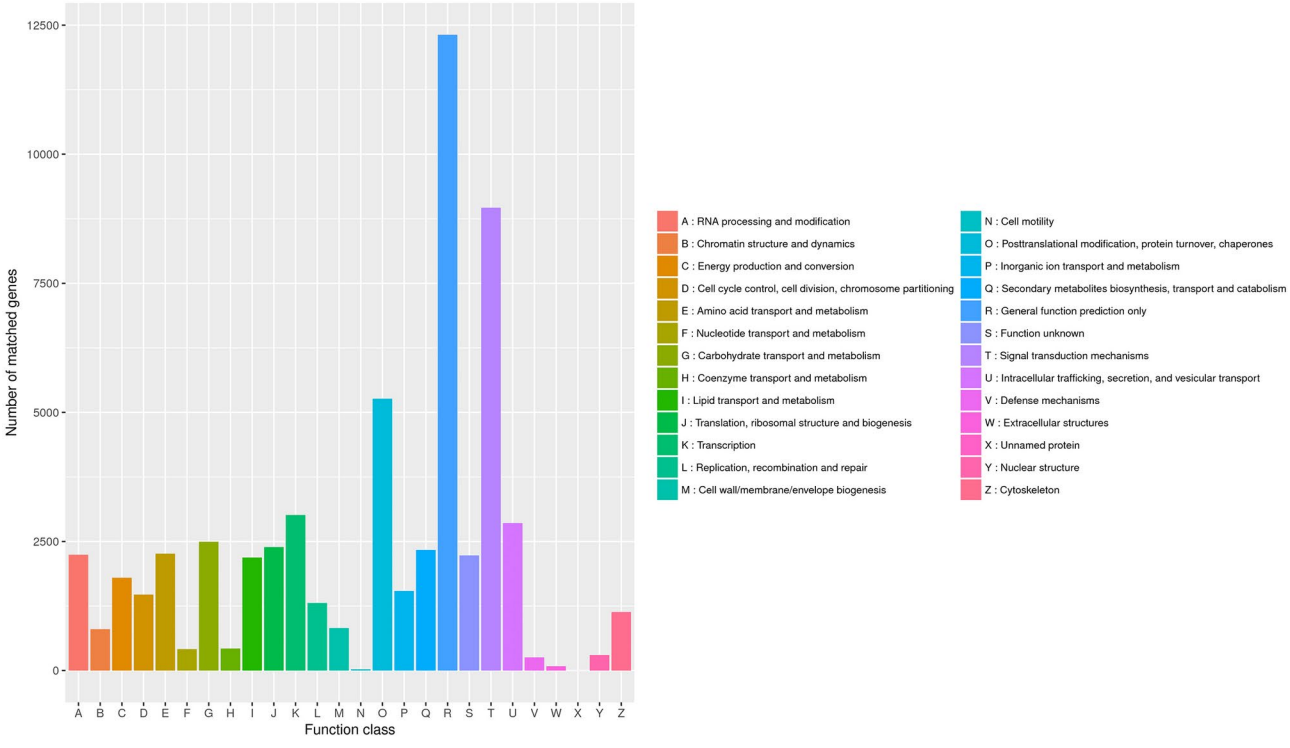

Fig. 5 KOG functional classification of the *A. sphaerocephala* transcriptome. Capital letters on the x-axis indicate KOG categories on the right side of the histogram, and the y-axis indicates the number of unigenes

A total of 81,135 unigenes were annotated in the KEGG database and assigned to 45 KEGG pathways which can be divided into 6 groups (Fig. 6). There were 4,125 unigenes in the cellular processes group, 4,561 in the environmental information processing group, 6,693 in the genetic information processing group, 10,835 in the human diseases group, 15,840 in the metabolism group, and 7,864 in the organismal systems group.

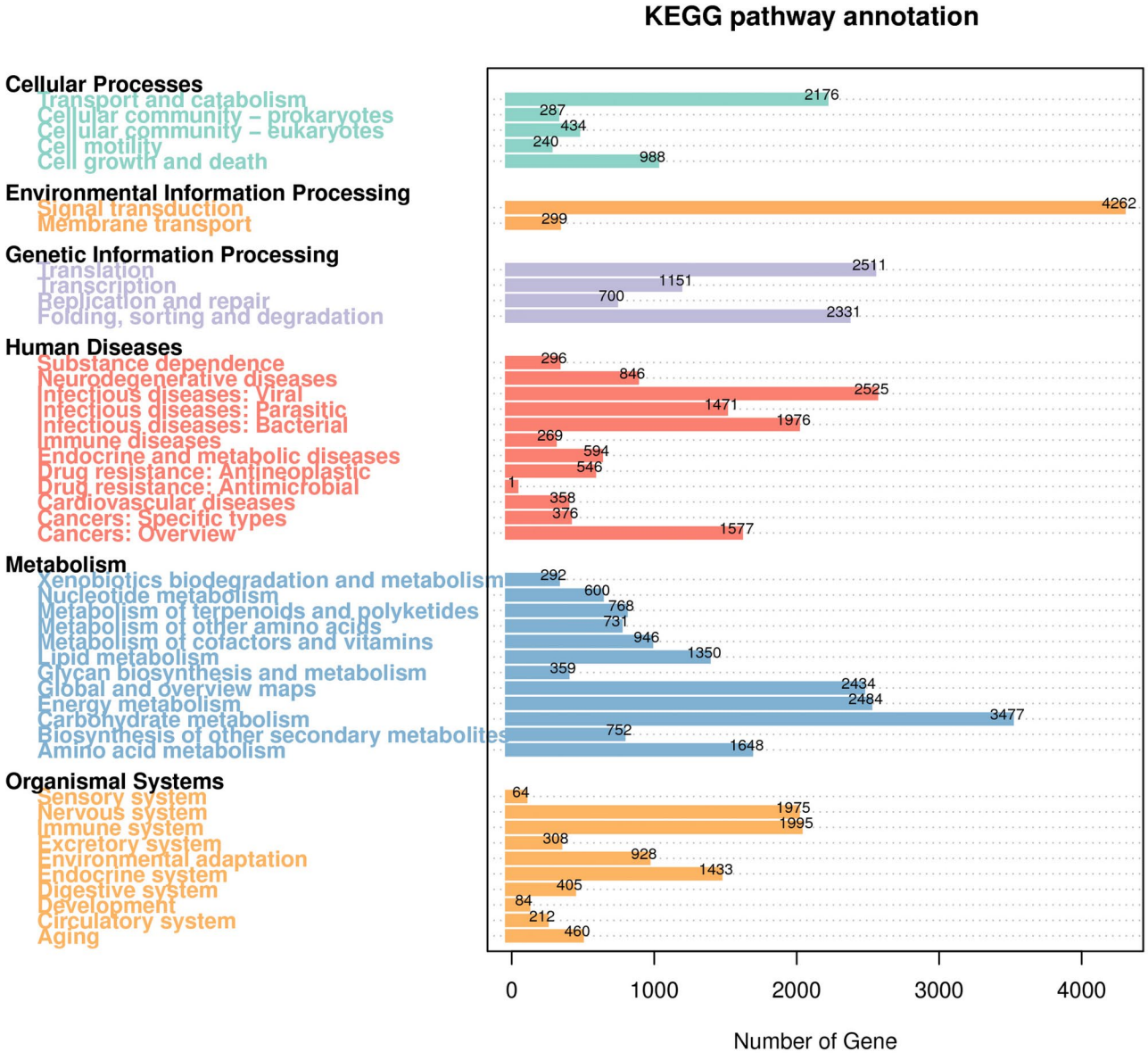

Fig. 6 KEGG pathway annotation of *A. sphaerocephala*. The number of unigenes in each pathway is shown on the x-axis, and the pathway categories are shown on the y-axis

Distribution of differentially expressed genes

The gene expression levels (FPKM) of *A. sphaerocephala* between different treatments were compared in pairs to find the differentially expressed genes based on the standard for significant differences as|log2(FoldChange)|>0, qvalue < 0.05. In this experiment, 1,000 genes of *A. sphaerocephala* were determined in all samples, and 18 differential genes were shared by all samples after comparison with CK(Fig. 7A), a total of 164 DEGs were obtained between CK and DS1, of which 82 were significantly up-regulated and 82 significantly down-regulated (Fig. 7B). 562 DEGs were obtained between CK and DS2, of which 307 were significantly up-regulated and 255 down-regulated (Fig. 7C). 274 DEGs were obtained between CK and DS3, among them146 were significantly up-regulated and 128 DEGs were significantly down-regulated(Fig. 7D).

The Euclid distance method related to complete linkage was used to cluster all 1000 DEGs [30], and the clustering patterns of DEGs under different experimental treatments were determined (Fig. 8A). It showed that a group of genes was rapidly activated in DS2, other genes were activated in other stress periods, and some of them were continuously and highly expressed during drought stress. All of the identified 1000 DEGs were divided into six subclusters with different time expression patterns (Fig. 8B). The pattern of genes up-regulation in cluster 4 was the most pronounced, and genes of DS2 were the

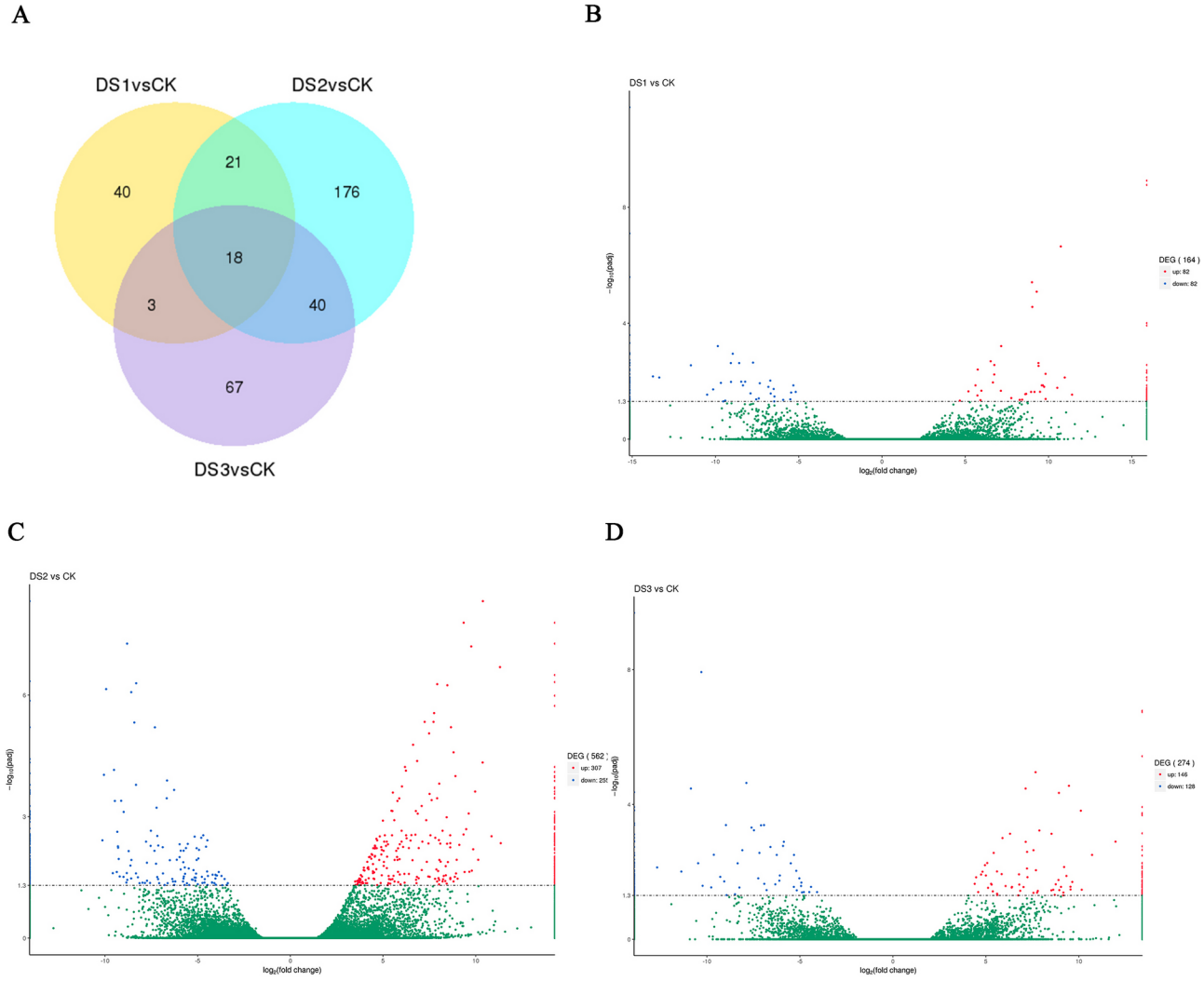

Fig. 7 The numbers of specific expressed and overlapped unigenes. (**A**) The numbers of specifically expressed and overlapped unigenes in CK and DS1, DS2, and DS3 are shown in the Venn diagram; (**B**) DEGs of DS1 vs. CK; (**C**) DEGs of DS2 vs. CK; (**D**) DEGs of DS3 vs. CK

most strongly expressed in cluster 4. The analysis of the gene of DS2 in cluster 4 by GO analysis showed that most genes were related to catalytic activity, metabolic process, and protein binding, indicating that these transcripts play a vital role in the drought tolerance of *A. sphaerocephala*.

In the current study, some unreported genes (genes that have not been annotated in various databases) such as Cluster-15733.79385, Cluster-15733.24638, Cluster-15733.10883, Cluster-15733.79657, and Cluster-15733.30554 were significantly up-regulated, Cluster-15733.55557, Cluster-15733.40997, Cluster-15733.61526, Cluster-15733.47110, Cluster-15733.18705 were significantly down-regulated in *A. sphaerocephala* under drought stress, prompt thta they may play important roles in response to drought stress. In order to clarify the molecular mechanism of drought resistance in *A. sphaerocephala*, it is necessary to further study the drought resistance function of the above-mentioned genes in the future, and the research will also provide new effective genes for transgenic cultivation of drought resistant plants.

GO enrichment analysis of differential expressed genes

Based on the differential genes screened, studying the distribution of specific genes in Gene Ontology, which will help clarify the differences in the samples in the experiment in terms of gene function [31]. In GO enrichment analysis, biological process (BP) and molecular function(MF) are two main categories. Compared with CK, in DS1 there were no genes that reached a significant difference, and the differential genes in DS2 in the biological process were mainly enriched in the cellular amino acid metabolic process (GO:0006520), carboxylic acid metabolic process (GO:0019752), oxoacid metabolic process (GO:0043436), organic acid metabolic process (GO:0006082), single-organism metabolic

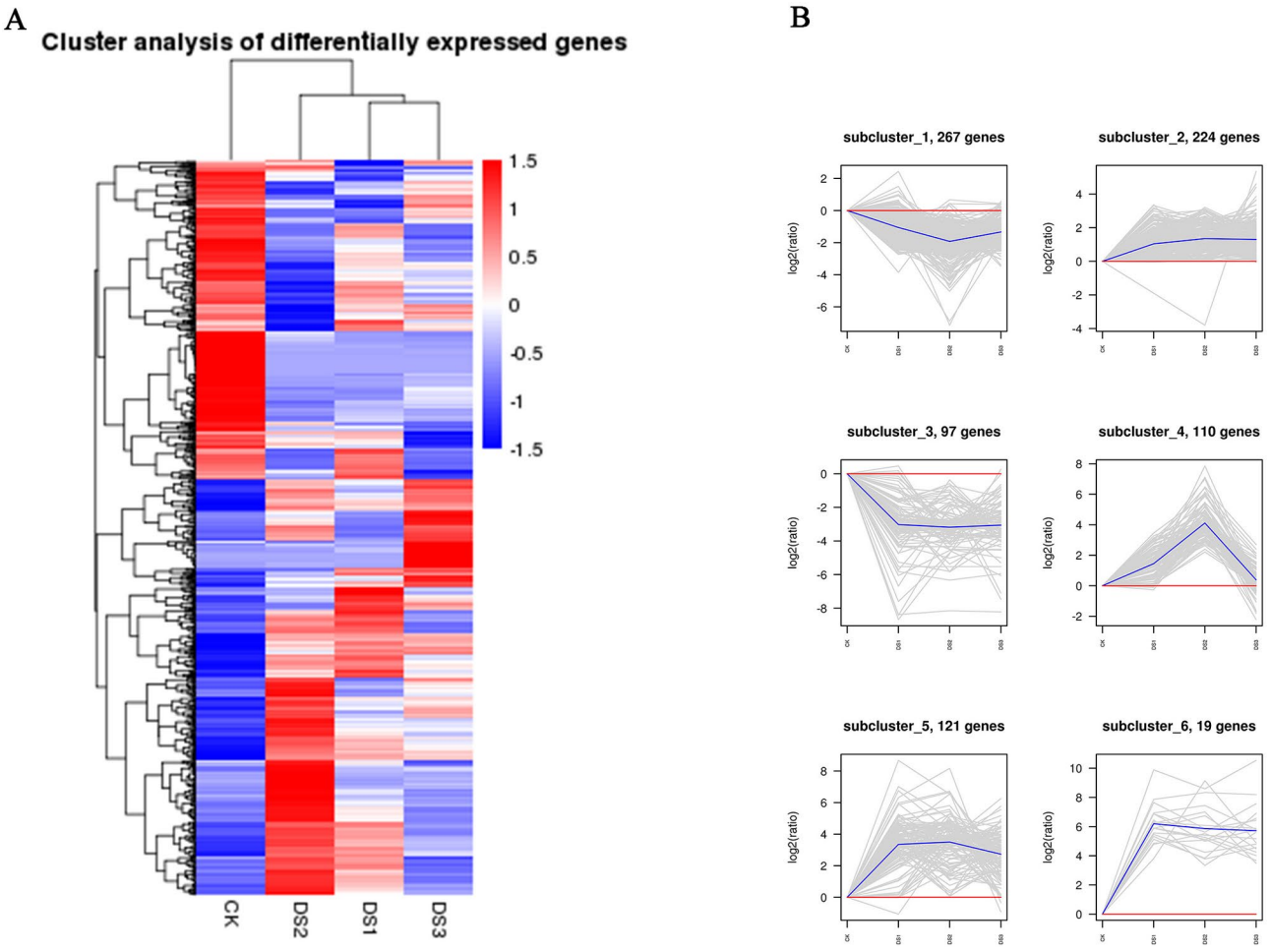

Fig. 8 Clustering analysis of the differentially expressed genes (DEGs). (**A**) Heatmap showing the clustering analysis of drought-responsive genes; (**B**) The 1000 DEGs were clustered into six subclusters. The number of genes in each cluster is shown at the top of each cluster. Blue lines show the average values for relative expression levels in each subcluster, and gray lines represent the relative expression levels of each gene in each cluster

process (GO:0044710), small molecule metabolic process (GO:0044281), transmembrane transport (GO:0055085), single-organism process (GO:0044699), nitrogen compound metabolic process (GO:0006807), and organonitrogen compound metabolic process (GO:1901564). In molecular function (MF), differential genes were mainly enriched in transmembrane transporter activity (GO:0022857) (Fig. 9A). In DS3, in the biological process, the differential genes were mainly enriched in the cellular amino acid metabolic process (GO:0006520), carboxylic acid metabolic process (GO:0019752), oxoacid metabolic process (GO:0043436), organic acid metabolic process (GO:0006082), single-organism cellular process (GO:0044763), and in the molecular function (MF), the differential genes were mainly enriched in the primary active transmembrane transporter activity (GO:0015399), and P-P-bond-hydrolysis-driven transmembrane transporter activity (GO:0015405) (Fig. 9B).

KEGG analysis of metabolic pathways of differential gene

In organisms, different genes coordinate with each other to perform biological functions [32]. The significant enrichment through the KEGG pathway can determine the most important biochemical metabolic pathways and signal transduction pathways involved in specific genes. Based on the KEGG pathway analysis, the differential genes in DS1 of *A. sphaerocephala* were mainly enriched in Glyoxylate and dicarboxylate metabolism (4 unigenes), Nitrogen metabolism (3 unigenes), Starch and sucrose metabolism (3 unigenes) (Fig. 10A), the differential genes in DS2 were mainly enriched in plant hormone signal transduction (10 unigenes), glyoxylate and dicarboxylate metabolism (8unigenes), starch and sucrose metabolism (7 unigenes), and aminoacyl-tRNA biosynthesis (7 unigenes) (Fig. 10B), while in DS3 were mainly enriched in glyoxylate and dicarboxylate metabolism (5unigenes), nitrogen metabolism (4 unigenes) (Fig. 10C).

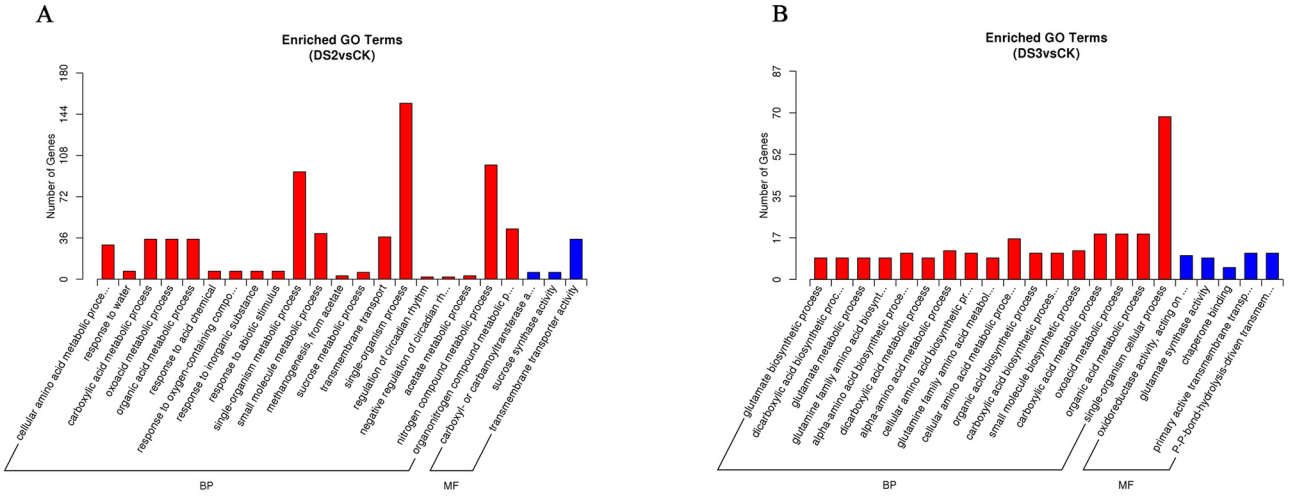

Fig. 9 GO-enriched items. (**A**)GO-enriched items of DS2 VS CK; (**B**) GO-enriched items of DS3 VS CK

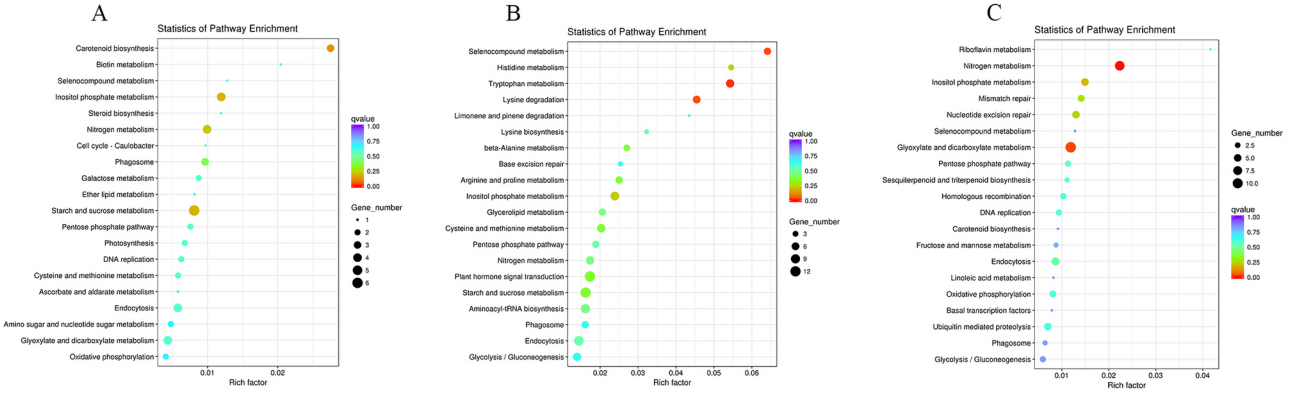

Fig. 10 KEGG pathway enrichment of DEGs. The graph shows only the top 20 enriched pathways, different colors denote different Q-Values, and the size of the bubble represents the number of DEGs. **A**: The enriched pathways comparing DS1 with CK; **B**: The enriched pathways comparing DS2 with CK; **C**: The enriched pathways comparing DS3 with CK

Analysis of drought-responsive transcription factors

TFs play crucial roles in plant responses to abiotic stress and can activate or inhibit gene expression at the transcriptional level, and helping plants preserve normal physiological activities under stress. In this paper, based on iTAK, we found a total of 2472 TFs in A. *sphaerocephala*, belonging to 77 families (Supplementary S1, Data comes from second-generation sequencing technology). Differential analysis showed 1006 TFs that were differentially expressed comparing DS with CK, of which 465 TFs were up-regulated and 541 TFs were down-regulated. The most abundant TF family was the MYB (55) family, followed by the AP2 (32), NAC (30), C2H2 (26), WRKY (23), Orphans (21), bZIP (20), HB (19) families, and the dynamic changes in the expression of genes associated with these TFs may indicate their important function in the drought stress in A. *sphaerocephala*.

Confirmation of the transcriptome data by qRT-PCR

To validate the RNA-Seq gene expression results, quantitative reverse transcription-PCR (qRT-PCR) was performed to assess the expression levels of 10 genes related to drought resistance of *A. sphaerocephala* under control and drought stress conditions (Fig. 11). The results showed that expression patterns obtained by qRT-PCR were moderately correlated with those obtained by transcriptome data, with a correlation coefficient of 0.7044(Fig. 12, Supplementary S2). However, RNA-Seq gene expression results of SnRK2 and pip genes was not fully consistent with qRT-PCR results. The principles and methods of RNA-Seq and qRT-PCR are inconsistent, and there may be a tendency of inconsistent expression between qRT-PCR and RNA-Seq results, which can be caused by a number of reasons, including the filtering of raw data, amplification efficiency, and annotation of the reference transcriptome [33]. In addition, RNA-Seq covers a wider range and is usually used for large-scale screening of gene, reflecting the overall gene expression

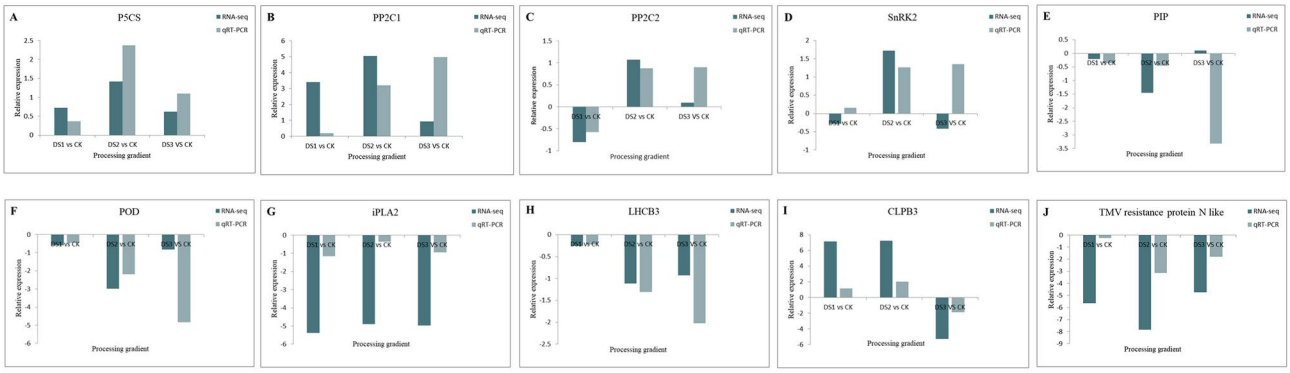

Fig. 11 qRT-PCR Analysis of 10 Gene in *A. sphaerocephala* leaves. (**A**)Delta-1-pyrroline-5-carboxylate synthetase(P5CS); (**B**)Protein phosphatase 2 C-1 (PP2C1); (**C**) Protein phosphatase 2 C-2(PP2C2); (**D**) sucrose non-fermenting1-related protein kinase 2; (**E**) Plasma membrane intrinsic protein(PIP); (**F**) Peroxidase(POD); (**G**) Calcium-independent Phospholipase A2(iPLA2); (**H**) Light-harvesting chlorophyll a-PSII binding protein 3 (LHCB3); (**I**) Caseinolytic protease B3 (CLPB3); (**J**) TMV resistance protein N-like

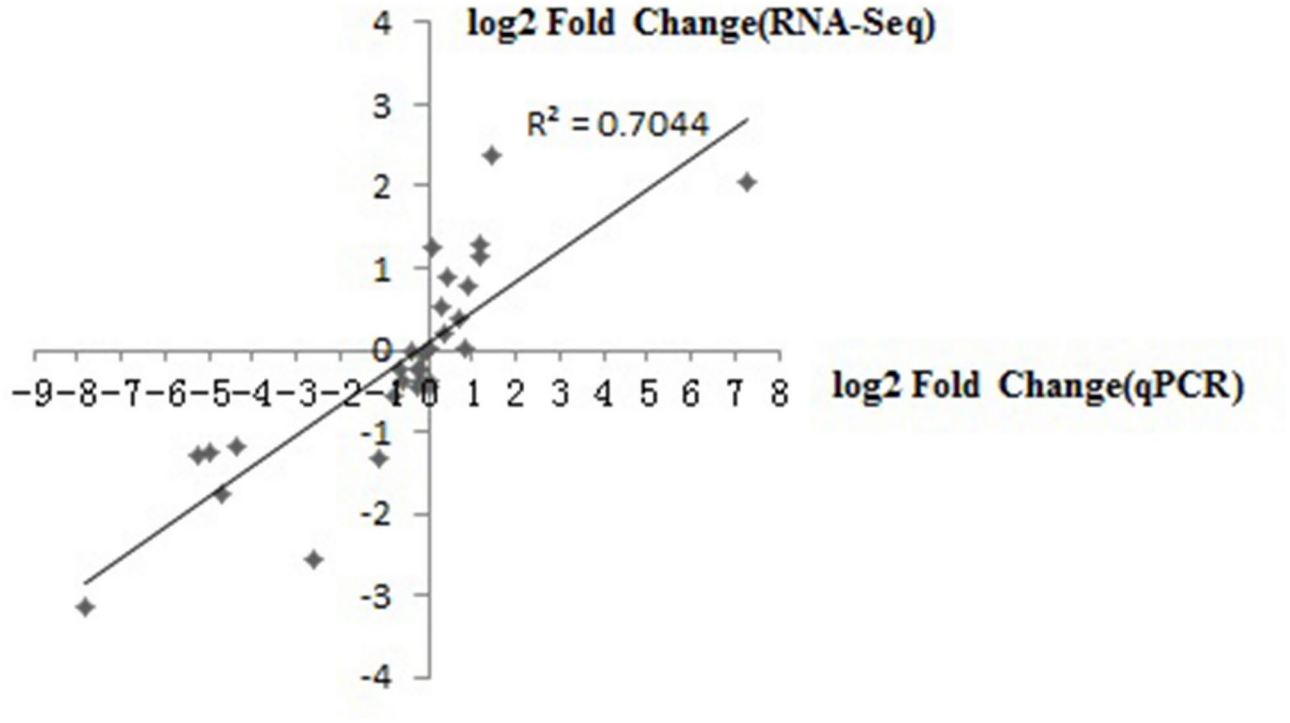

Fig. 12 Analysis of the correlation between transcriptome sequencing and qRT-PCR

pattern of the sample. Studies benchmarking RNA-seq processing workflows typically target RT-qPCR data for only a few hundred genes, and the expression measurement results of qRT-PCR and RNA-Seq are inconsistent when certain genes were typically lower expressed, smaller and had fewer exons [34]. In addition, The consistent results for other genes indicate that our transcriptome results were reliable.

Discussion

SGS technology has greatly accelerated transcriptome research in recent decades, and SGS generates highly accurate short read segments. Nevertheless, short read lengths reduce the accuracy of sequence assembly and complicate bioinformatics analysis [15]. TGS technology can obtain longer read length, more uniform coverage, and construct a complete transcriptome [18]. Although the relatively high error rate of TGS may be problematic in bioinformatics analyses and sequence alignment, it can be improved and corrected reads by high-precision SGS short readings. Thus, a hybrid sequencing approach

combining SGS and TGS could provide more accurate and complete gene assembly in genome and transcriptome studies. In this study, we combined SGS and SMRT sequencing to generate a more complete *A. sphaerocephala*, and our SMRT data were of high quality. In addition, the use of Illumina short reading to correct SMRT long reading resulted in high-quality full-length transcripts with reduced misassembly, which plays an important role in the study of functionally important genes in plants.

Physiological changes under drought stress

Drought stress can lead to changes in plant molecular, physiological, and morphological responses [35] and plants adopt a series of complicated regulatory mechanisms to prevent, reduce, or repair damage to maintain a normal physiological state when experiencing water deficit [36], including an increase in osmoregulation substances and the synthesis of antioxidants. The cell membrane is the boundary membrane for material and information exchange between inside and outside of the cell and it is selectively permeable [37]. The membrane damage caused by drought will increase the membrane permeability, and also increase the content of MDA which is a product of membrane lipid peroxidation. Thus, MDA content and cell membrane permeability are significant indicators of the membrane lipid peroxidation rate and cell damage under stress [38]. The cell membrane controls the transport of water between the cell and its environment, resulting in changes in the RWC which can be used to determine the level of cell dehydration and assess the degree of drought by plants. The photosynthetic mechanism of plants is very sensitive to stress and the synthesis and degradation of chlorophyll which is an important substance in photosynthesis also affected and reduced under drought stress [39]. Drought stress can aggravate the production of reactive oxygen species ROS(e.g., H_20_2, -OH, O^{2-}, etc.) [40] in desert plant cells. ROS can be used as a signal to induce plants to produce defense mechanisms against adversity stress, but too much ROS can damage membrane lipids, nucleic acids, proteins, and other biological macromolecules, causing secondary stress [41]. Superoxide dismutase (SOD), peroxidase (POD) and catalase(CAT) as an important antioxidants, can minimize cellular damage by scavenging and detoxifying ROS-generated H_20_2 [42]. SOD, POD and CAT contents are induced rapidly to deal with oxidative damage during the period of drought stress. Furthermore, various osmoregulation substances such as pralines, soluble sugars, and soluble proteins rapidly accumulate under drought stress [37]. Thus, the content of these molecules and the activity of these enzymes are widely used as parameters to assess the characteristics of plants subjected to drought [38].

In this study, ten traits including RPP, MDA content, RWC, chlorophyll content, the activity of SOD, POD, and CAT, and the content of proline, soluble sugar, and soluble protein were investigated in *A. sphaerocephala* leaves under different drought stresses. Similar to previous studies conducted in other species [43, 44], these 10 examined characteristics revealed a rapid induction in response to drought stress in *A. sphaerocephala*. From the test results, it can be seen that the changes in RPP and MDA showed an overall increasing trend, RWC, and the total chlorophyll content gradually decreased. These results may suggest that the cell membrane of *A. sphaerocephala* suffered from drought stress is damaged, which leads to the cell membrane lipid being released and the membrane structure being damaged. The expected reductions in the multiple physiological parameters collectively indicated that *A. sphaerocephala* suffered physiological damage under severe drought stress. In addition, the content of antioxidative enzymes (SOD, POD, CAT) and osmoregulation substances (proline, soluble sugar, soluble protein) increased under stress. Delta-1-pyrroline-5-carboxylate synthetase (P5CS) is considered to be the key enzyme for proline biosynthesis in the plant, and based on the result of RNA-Seq of *A. sphaerocephala*, the expression of the P5CS(i2_LQ_HY_c52210) gene was elevated under drought stress conditions, which is consistent with the change in proline content. In addition, the expression levels of POD(i1_LQ_HY_c18002/f1p0/1725), and CAT(i2_HQ_HY_c3014/f6p24/2239, i2_LQ_HY_c14397/f1p40/2102)genes increased under drought stress which was also consistent with the trend of enzyme activity in physiological results. These results confirmed that *A. sphaerocephala* can accumulate antioxidant enzymes and osmoregula-tions (proline, soluble sugars, soluble proteins) when subjected to drought treatment. These behaviors can maintain a relatively stable membrane structure to mitigate damage caused by stress and reduce intramembrane osmotic pressure to ensure normal water sup-ply under stress conditions. Therefore, A. *sphaerocephala* is highly resistant to drought.

Plant hormone signal transduction under drought stress

A series of changes in plant endogenous hormones under drought stress reflect the flexibility of plants to the adverse stress, and drought resistance in plants is achieved by the complex and coordinated actions of multiple hormones, rather than by the action of a single hormone [45]. In our study, it was established that genes related to three plant hormone signaling pathways were found to be differentially expressed in *A. sphaerocephala*, including the ABA signaling pathway, auxin signaling pathway, and JA signaling pathway [46–49].

The plant hormone ABA plays a key role in many aspects of plant response to various stress signals [50].

Under drought stress, ABA levels in plants increase, leading to stomata closing and preventing water loss through stomatal transpiration [51]. The core components of ABA signaling include Pyracbactin Resistance/Pyracbacin Resistance like (PYR/PYL), protein phosphatase 2Cs (PP2Cs), and SNF1-related kinase 2 (SnRK2s) [52]. Under adversity stress, ABA binds to PYR/PYL and interacts with PP2Cs to inhibit the activity of PP2Cs, which relieves the repression of PP2Cs to SnRK2s, the activation of SnRK2s will lead to phosphorylated of the downstream transcription factors or membrane proteins, then turn on the ABA signaling response [53]. Transcription factors of the ABRE binding factor (ABF) subfamily which are activated by SnRK2 can be induced by drought and the ABRE/ABFSnRK2 pathway as a positive regulator of ABA-dependent gene expression. In this study, we found that 1 gene encoding SnRK2(i1_LQ_HY_c4134/f1p20/1339) and 1 gene encoding ABF (i2_LQ_HY_c53103/f1p2/2595) were up-regulated (Supplementary S1), 1 gene encoding PP2C (i4_HQ_HY_c15887/f4p0/4625) was down-regulated. As negative regulators of ABA signaling, PP2Cs play a critical role in regulating stomatal movement [54], a decrease in the expression of PP2C would reduce the inhibition of ABA levels, and the increase of the expression of ABF and SNRK would elevate the ABA levels in the cells, which induced stomatal closure, reduced transpiration, and maintained water balance in A. *sphaerocephal* under drought stress.

Under drought stress, auxin can positively modulate root biomass especially the number of lateral root and leaf water uptake [55] and the accumulations of compatible solutes especially multiple sugars and sugar alcohols [56]. In addition, auxin can positively regulate enzymatic antioxidant enzymes such as SOD, CAT, POD, and GR, thus providing potent ROS detoxification [57]. The auxin-triggered defense responses mentioned above improved drought resistance. Auxin is transported into the cell through the AUX1 vector and binds to the TIR1 receptor, which then interacts with AUX/IAA ubiquitination, and the ubiquitination of AUX/IAA releases auxin response factors (ARFs) from the inhibitory control of AUX, thereby accelerating the transfer of auxin signal to affect the plant [58]. Auxin-responsive protein (IAA and SAUR), and indole-3-acetic acid-amido synthetase gene (GH3) are reported to be involved in the auxin signaling pathway and play a crucial role in drought tolerance [59]. In the current study, the expression level of the gene encoding GH3 (i2_LQ_HY_c51988/f1p12/2444), and IAA (i2_HQ_HY_c11908/f2p0/2710) in *A. sphaerocephala* were obviously up-regulated (Supplementary S4), indicating that auxin was closely related to the drought resistance of *A. sphaerocephala*.

The JA signaling pathway is conducive to the reduction of drought stress by activating antioxidant systems (SOD, POD, CAT) [60], accumulating amino acids, soluble sugars, and soluble proteins [61], and regulating stomatal opening and closing [62] to improve drought tolerance in plants. Jasmonate ZIM-domain proteins (JAZ) are regulators and typically repressors in the JA signaling pathway. Fu et al. [63] showed that OsJAZ1 plays a negative regulatory role in rice resistance to drought. Gretchen Hagen 3 (GH3) family is an important gene family involved in the regulation of jasmonic acid signaling which mainly converts jasmonic acid into biologically active compound jasmonoyl-isoleucine (JA-Ile) to induce changes in the expression of response genes, indicating that auxin was closely related to drought resistance. In the current study, the expression level of the gene encoding JAZ (i2_LQ_HY_c26066/f1p1/2735) was obviously down-regulated, and IAA (i2_HQ_HY_c11908/f2p0/2710) was up-regulated (Supplementary S4), indicating that JA was closely related to the drought resistance of *A. sphaerocephala*.

Drought stress can cause changes in the contents of various endogenous plant hormones. The regulation of plant growth and development by various hormones forms a very delicate and complex network, and the synergistic action of hormones is an important method of regulating drought tolerance [64]. The above results showed that plant hormones signal transduction pathways play an important role in drought resistance in *A. sphaerocephala*.

Sugar metabolism under drought stress

Glucose and sucrose are significant soluble sugars for maintaining the osmotic potential of cells, and their accumulation can enhance the drought tolerance of plants [65]. By analyzing the changes in genes related to sugar metabolism in *A. sphaerocephala* under drought stress, we found that significant changes in carbohydrate metabolism pathways such as starch and sucrose metabolism and glycolysis/gluconeogenesis pathways were activated, and there are a large number of significantly up-regulated genes involved in the metabolism of sucrose and starch have been identified like sucrose synthase (i2_LQ_HY_c71228/f1p3/2809,i2_LQ_HY_c51736/f1p5/2656,i5_LQ_HY_c26342/f1p10/5102) which can hydrolyze sucrose into glucose and fructose, and beta-amylase (i1_LQ_HY_c5624/f1p0/1849, i2_HQ_HY_c6838/f3p3/2364, i2_LQ_HY_c76022/f1p4/2274), which can degrade starch to glucose and maltose (Supplementary S4). These results suggest that genes involved in regulating starch and sucrose metabolism may play a significant role in drought stress in *A. sphaerocephala* as it tries to utilize more carbohydrates to cope with the hostile environment under drought stress.

The glycolysis/gluconeogenesis pathway mainly influenced the supply of ATP in response to abiotic stress [66]. In this research, we found that glyceraldehyde-3-phosphate dehydrogenase (GAPD) (i1_LQ_HY_c3582/f1p3/1953) and fructose-1, 6-bisphosphatase (i2_LQ_HY_c14983/f1p8/1888) were increased in *A. sphaerocephala*, resulting in the plentiful production of ATP and NADPH (Supplementary S5). GAPD plays an important role in glycolysis and gluconeogenesis which is an essential enzyme for glycolysis and gluconeogenesis, it catalyzes the formation of ATP from 1,3-diphosphoglycerate, which is the first reaction to produce ATP in glycolysis [67]. Fructose-6-phosphate kinase 1 (PFK1) is one of the most important rate-limiting enzymes in glycolysis, it catalyzes fructose-6-phosphate to form fructose-1,6-diphosphate in the glycolysis pathway, which is the second phosphorylation reaction in the glycolysis pathway [68]. As an intermediate product of glycolysis, fructose-1,6-diphosphate plays an important role in glycolysis [69]. Therefore, the regulation of the glycolysis/gluconeogenesis pathway might contribute to drought tolerance in *A. sphaerocephala* under severe drought stress.

Glutamate related to drought stress

Nitrogen metabolism mainly participates in plant stress resistance from nitrogen absorption, nitrogen assimilation, and amino acid metabolism, which can affect the ability of plants to resist abiotic stress through physiological mechanisms such as regulating the absorption and transport of ions, stabilizing cell morphology and protein structure, maintaining hormone balance and cellular metabolism level, and reducing the production of reactive oxygen species (ROS) in the body [70]. Nitrogen assimilation in plants can be divided into nitrate (NO_3^-) assimilation and ammonium ($NH4^+$) assimilation [71]. The NO_3^- absorbed by plants from the soil is reduced to $NH4^+$ in the cytoplasm by nitrate reductase (NR) and nitrite reductase (NiR) which is called NO_3^- assimilation. NO_2^- and $NH4^+$ produced by NO_3^- assimilation are toxic to plants, so the resulting $NH4^+$ rapidly enters the $NH4^+$ assimilation pathway [72]. $NH4^+$ assimilation is primarily the conversion of $NH4^+$ to amino acids via the GS/GOGAT pathway [71]. $NH4^+$ and glutamate(Glu) are first converted to glutamine(Gln) under the catalysis of glutamine synthetase(GS). Then Gln and α-ketoglutarate are catalyzed by glutamate synthase (GOGAT) to form two molecules of Glu [73]. Among them, one molecule of Glu can be directly used by the plant as a nitrogen compound, and the other molecule re-enters the GS/GOGAT cycle as a raw material [74]. From the above, it is clear that NR, NiR, GS, GOGAT, and GDH are key enzymes involved in the process of plant nitrogen metabolism, and their activities reflect to some extent the nutritional status and the level of nitrogen assimilation in plants. Zhong et al. [75, 76] demonstrated through physiological experiments that higher nitrogen levels enhanced the adaptability of rice to drought stress, which was manifested by increased antioxidant enzyme activity and proline content in the leaves. The current study showed up-regulation of genes encoding GOGAT (i3_LQ_HY_c11050/f1p0/3840,i5_LQ_HY_c11823/f1p0/5520,i5_LQ_HY_c10058/f1p18/5567,i5_LQ_HY_c33431/f1p0/5474, i4_LQ_HY_c11548/f1p0/4954, i5_LQ_HY_c27783/f1p0/5412, i5_LQ_HY_c23367/f1p0/5280) and GS(i1_LQ_HY_c8059/f1p5/1368, i2_HQ_HY_c18051/f2p5/2783) which were the key enzyme of nitrogen metabolism, indicating that the activity of GOGAT and GS increased in *A. sphaerocephala* under drought stress (Supplementary S6). It supported the assimilation of nitrate and ammonium, synthesized proline and other nitrogenous compounds as osmotic substances, and retained water balance, and the increased activity of GOGAT also increased antioxidant enzyme activities and reduced oxidative damage to cells.

Glyoxylate and dicarboxylate metabolism is a key pathway related to abiotic stress that can balance metabolic disorders to improve tolerance [77] and mainly affects ATP supply in response to abiotic stress [78]. Glu is converted to 2-oxoglutarate which is an intermediate product of glyoxylate and dicarboxylate metabolism [79]. In the present study, the expression level of genes encoding GOGAT(i4_LQ_HY_c11548/f1p0/4954,i5_LQ_HY_c27783/f1p0/5412,i5_LQ_HY_c23367/f1p0/5280,i5_HQ_HY_c2502/f2p9/5365,i5_LQ_HY_c35060/f1p6/5513,i5_LQ_HY_c33431/f1p0/5474,i3_LQ_HY_c11050/f1p0/3840,i5_LQ_HY_c11823/f1p0/5520,i5_LQ_HY_c10058/f1p18/5567,i5_LQ_HY_c33431/f1p0/5474) were up-regulation (Supplementary S7), indicating that *A. sphaerocephala* improved the drought resistance by increasing ATP levels in vivo.

Differential expression of transcription factors(TFs) under drought stress

During drought stress, when plants receive stress signals, transcription factors (TFs) are activated through a series of signaling cascades [80], and the activated TF specifically binds to the cis-acting element of their respective target genes to initiate transcription of the downstream specific response gene [81]. The activity of transcription factors usually depends on developmental stages, the presence of co-regulatory proteins, and exogenous stimuli [82]. Different transcription factors are used to regulate the expression of different genes, allowing plants to respond to environmental changes in a highly specific and flexible manner [83]. Different TF subfamilies or even members of the same subfamily may exhibit disparate transcriptional regulations under various stress conditions [84]. It has been confirmed that members of the transcription factor family such as AP2-EREBP, NAC,

WRKY, C2H2, bHLH, HB, and MYB are key regulators in signal transduction pathways, and play a vital role in the response to drought stress and water stimulation [80, 85].

MYB proteins are shown to be involved in the regulation of numerous stress-related genes directly or indirectly in response to abiotic stresses [86]. GmMYB118 confers drought tolerance by reducing the contents of ROS and MDA [87]. MYB TFs play a significant role in hormone signaling and ScMYB2 can participate in the ABA-mediated leaf senescence signaling pathway and play an active role in drought-induced senescence [88]. The APETALA2/ethylene-responsive element binding protein (AP2/EREBP) can be divided into three subfamilies: AP2 (apetala 2), RAV (related to ABI3 / VP1) and ERF (ethylene responsive factor). These proteins play a crucial role in plant growth and response to drought stress, in which they also respond to hormones with improved plant survival during stress conditions [89]. Interestingly, overexpression lines of ERF1 show greater tolerance to drought and salt stress, suggesting that ERF1 contributes to plant tolerance to various drought stresses [82]. NAC TFs also play a key role in plant responses to abiotic stress and most NAC TFs respond to drought stress [90]. At present, many NAC TFs have been confirmed to participate in ABA-mediated drought signal transduction, which is an important component of this pathway. C2H2 TFs also play crucial roles in plant responses to drought stress. Overexpression of OsWRKY45 and WRKY57 increased drought tolerance [91]. In the sequencing results of this experiment, the number of genes encoding transcription factors such as MYB, AP2-EREBP, NAC, C2H2 and WRKY, accounted for the main proportion, among which MYB(55), AP2(32), NAC(30), C2H2(26), WRKY(23), indicating that these types of transcription factors play a very significant role in regulating the response to drought stress in *A. sphaerocePhala.*

Materials and methods

Experimental materials and treatments

The seeds of *A. sphaerocephala* were collected from the Qaidam Basin (36°01'23.4"N, 97°40'27.2"E, altitude 2700 m) and planted in pots (peat: garden soil: Perlite = 40:30:30 (% by volume). All of the seedlings were cultivated in the greenhouse (20 ± 25℃,60 ± 70% rH) and watered every three days (100 mL/pot) and the RWC was maintained at approximately 80% of the field capacity (field capacity was 36%). The seedlings were watered with Hoagland nutrient solution every two weeks. After 4 months of cultivation, the seedlings with well growth and consistency were selected to start drought stress treatment. The roots, stems, and leaves of *A. sphaerocephala* were collected at 8:00–9:30 AM after 0(CK), 2(DS1), 4(DS2) and 8(DS3) days of drought stress treatments. After 8 days of stress, when the seedlings were severely wilted, the stress group was re-watered, and after one day of rehydration, the roots, stems, and leaves were harvested(RW) and stored. The materials were divided into two parts, one of them was quickly frozen in liquid nitrogen and then stored at -80℃ for transcriptome sequencing and gene expression analysis, and the other part was placed in the ice box and brought back to the laboratory for physiological indices determination immediately, and each was repeated three times.

Experimental method

Measurement of physiological indices

The relative permeability of plasma was measured by the oven drying method [92], the content of MDA was determined by the thiobarbituric acid [93], and the activity of SOD was measured by nitro blue tetrazolium (NBT) [94], the activity of POD was measured by guaiacol, the activity of CAT was determined by UV absorption [94], the chlorophyll content was determined by the acetone method [95], the relative water content was determined by gravimetric method [96], the soluble sugar content was determined by the anthrone method [95], the soluble protein content was determined by the Coomassie brilliant blue G-250 method [97], and the proline content was determined by the sulfosalicylic acid method [98]. Each measurement was repeated three times.

RNA extraction and quality control

Total RNA was extracted from the leaves of CK, DS1, DS2, and DS3 in *A. sphaerocephala* using the RNA38 (Aidlab Biotech, China) according to the manufacturer's instructions. RNA degradation and contamination were monitored through 1% agarose gel. The quality and purity of RNA were evaluated by NanoDrop One Spectrophotometer (Thermo Scientific, USA). RNA was used only if OD260/280 was greater than 1.8 and less than 2.2. The total RNA was stored at -80 °C for future use.

Total RNA was extracted from the leaves of CK, DS1, DS2, and DS3 in *A. sphaerocephala* using the RNA38 (Aidlab Biotech, China) according to the manufacturer's instructions. The total RNA of each sample was quantified and qualified via Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), NanoDrop One Spectrophotometer (Thermo Scientific, USA), and 1% agarose gel analyses. RNA was used only if OD260/280 was greater than 1.8 and less than 2.2. The total RNA was stored at -80 °C for future use.

Illumina transcriptome library Preparation and sequencing

The input material for our sample preparation was 1.5 μg RNA. The NEBNext1 Ultra™ RNA library preparation kit from Illumina1 (NEB, USA) was used. TruSeq PE Cluster Kit v3-cBot-HS (Illumina) clusters index-coded samples

on the cBot cluster generation system. After cluster generation, the library preparations were placed on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) for sequencing (completed by Novogene) to generate paired-end reads. The raw reading of the fastq format was first processed by SMRT-Link v6.0(https://www.pacb.com/support/software-downloads). Clean readings were obtained by removing readings containing adapters or ploy-N and discarding low-quality readings from the original readings. The contents of Q20, Q30, GC, and clean read sequence repetition levels were calculated. Subsequently, all clean reads were assembled with Trinity software using default parameters.

PacBio iso-seq library Preparation and sequencing

The Iso-Seq library was prepared according to the Isoform Sequencing protocol (Iso-Seq) using the Clontech SMARTer PCR cDNA Synthesis Kit and the BluePippin Size Selection System protocol as described in Pacific Biosciences (PN 100-092-800-03). Sequence data were processed using SMRTlink 6.0 software and circular consensus sequences (CCS) were generated from subread BAM files. The output BAM files were read by pbclassity.py, ignore polyA false, minSeqLength 200 classified as full-length and non-full-length reads. The generated full-length and non-full-length fasta files were then fed into the cluster step for isoform-level clustering (ICE) and finally for Arrow polishing. Additional nucleotide errors in consensus reads were corrected using the Illumina RNA-seq data with LoRDEC v0.7 (http://atgc.lirmm.fr/lordec). Eventually, the redundancies were transferred using CD-HIT v4.6.8 (https://github.com/weizhongli/cdhit) to obtain final transcripts for subsequent ones.

Gene functional annotation

The online BLAST program of NCBI was used to compare the spliced UNigene with the protein database includes the nonredundant protein database, NR, and Swissprot protein database. Blast 2Go and we go software were used to classify and count all unigenes. Unigenes were analyzed by the cluster of homologous groups (KOG) functional classification and signal pathway analysis (KEGG).

Differential gene expression analysis

On the RSEM v1.3.0 program (http://deweylab.github.io/RSEM/), the expression level of each gene is calculated based on the number of fragments per kilobase in the transcribed sequence per million base pairs (FPKM). Differentially expressed genes (DEGs) between CK and DS were identified using the DESeq-R 1.10.1 package (http://www.bioconductor.org/packages/release/bioc/html/DESeq.html). The genes with adjusted P-value (padj) < 0.05 are considered as DEGs. GO seq-R package was used to perform GO enrichment analysis on DEGs and KOBAS was used to test the statistical enrichment degree of DEGs in the KEGG pathway.

Quantitative PCR analysis

To verify the reliability of the RNA-Seq data, 10 unigenes were selected for qRT-PCR analyses using Power SYBR Premix Ex Taq TM II Kit (Perfect Real-Time, Takara, China) with LightCycler96 Real-Time PCR system (Roche, Switzerland). β-actin was selected as a reference gene with 3 replicates. The relative expression was calculated by the delta-delta CT and expressed as the fold change from expression in the null controls (expression = 1).

Conclusions

In this study, physiological and transcriptomic analyses were performed in *A. sphaerocephala* under drought stress. The changes in various physiological indicators of *A. sphaerocephala* under drought stress showed that it had a strong ability to resist drought. Differential gene expression analysis with RNA-Seq of *A. sphaerocephala* under drought stress identified a large number of differentially expressed genes. These DEGs can either be positively (up-regulated) or negatively (down-regulated) in response to drought. In addition, the up-regulated DEGs were mostly enriched in the pathways involved in plant hormone signal transduction, starch and sucrose metabolism, nitrogen metabolism, glyoxylate and dicarboxylate metabolism, which regulate the expression of drought-resistance genes thus allowing plants to avoid the adverse effects of drought stress. The results of this experiment provide candidate genes for promoting drought resistance in other plants, through qRT-PCR analysis of related drought resistance genes, it lays a foundation for the follow-up study of the molecular mechanism of drought resistance-related genes in *A. sphaerocephala*.

Abbreviations

RW	Restore-water
SMRT	Single-molecule real-time sequencing
NR	NCBI non-redundant proteins
NT	NCBI non-redundant nucleotide database
KEGG	Kyoto Encyclopedia of Genes and Genomes
PFAM	Protein family
GO	Gene ontology
KOG	EuKaryotic Ortholog Groups
FPKM	Fragments per Kilobase of transcript per Million mapped reads
CC	Cellular component
MF	Molecular function
BP	Biological process
qRT-PCR	Quantitative RT-PCR
ABA	Abscisic acid
IAA	Indole-3-acetic acid
JA	Jasmonate
ROS	Reactive oxygen species
Glu	Glutamate
Gln	Glutamine
GS	Glutamine synthetase

GOGAT	Glutamate synthase
AQP	Aquaporin

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

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Author contributions
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Data availability
The data was accessible in NCBI Sequence Read Archive (SRA) with the direct link and login number: https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA961293, PRJNA961293.

Declarations

Ethics approval and consent to participate
The experiments did not involve endangered or protected species. The data collection of plants was carried out with permission of related institutions, and complied with national or international guidelines and legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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