



Article

Transcriptomic and Metabolomic Analyses Provide New Insights into the Response of Strawberry (*Fragaria × ananassa* Duch.) to Drought Stress

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Abstract: Strawberry plants have shallow roots and large leaves, which are highly sensitive to variations in water levels. To explore the physicochemical and molecular mechanisms of strawberry response to water stress, and provide new ideas for strawberry scientific irrigation, we measured the transpiration rate, fresh weight, biomass gain, and other indicators of potted “Zhangji” strawberry plants under drought and waterlogging treatments using a Plantarray system. Transcriptomic and metabolomic analyses of strawberry leaves following mild drought, moderate drought, severe drought, and rehydration treatments were performed to identify key genes and metabolites involved in the response to drought stress. Below a certain threshold, the transpiration rate of strawberry plants was significantly lower after the deficit irrigation treatment than the conventional water treatment. Transcriptome analysis revealed that genes involved in oxidoreductase activity and in sulfur and nitrogen metabolism were up-regulated, as well as starch and sucrose. Strawberry plants secrete various endogenous growth hormones to maintain their normal growth under drought stress. The syntheses of salicylic acid (SA) and abscisic acid (ABA) were up-regulated in the mild and moderate drought treatments. However, the syntheses of 1-aminocyclopropanecarboxylic acid (ACC) and indole-3-acetic acid (IAA) were down-regulated in severe drought treatment and up-regulated in rehydration after severe drought treatment.

Keywords: strawberry; drought stress; transcriptomics; metabolomics



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1. Introduction

Drought is a global problem that can lead to major losses in yield. Drought and semi-drought conditions affect approximately 36% of the world's land, and drought-related problems are especially severe in China [1]. Drought stress has various effects on the growth and development of plants [2]. Strawberry, a member of the Rosaceae family, is a shallow-rooted plant that requires loose, water-permeable, ventilated soils. Long-term drought and water shortage can have deleterious effects on the transpiration and photosynthesis of strawberry plants, thereby decreasing the quality and yield of strawberries [3]. The development of drought-resistant varieties is essential for enhancing strawberry yield.

Most previous studies on the drought stress resistance of strawberry have focused on developing drought-resistant varieties and clarifying the physiological response of strawberry plants to drought stress [4]. Ünal et al. (2023) reported that mild drought stress, with half the quantity of water given as the control treatment, resulted in an increase in the content of amino acids in strawberry [5]. Guo et al. (2023) reported the different expression patterns of FvPP2C genes under ABA (abscisic acid), salt, and drought treatments [6]. Han et al. (2023) reported that ICE (inducer of CBF expression)

transcription factors are crucial in the molecular regulation of strawberry confronted by cold and drought stress [7]. And FvICE1 was significantly up-regulated after cold, drought, salt, and heat treatments.

Biotic stress and abiotic stress have been hot research topics in recent years. There is increasing evidence that indicates that phytohormones, in addition to controlling plant growth and development under normal conditions, also mediate various environmental stresses, including salt and drought, and thus regulate plant growth adaptation [8]. Cao et al. (2022) revealed the molecular regulation of drought stress in wild strawberry (*Fragaria nilgerrensis*) via integrated transcriptome and methylome analyses [9]. However, for the cultivated strawberry varieties, there is little information about the physiological and molecular mechanisms of their response to different degrees of drought treatment.

Here, we studied the drought resistance of one of the main strawberry varieties grown in greenhouses ("Zhangji") (*Fragaria × ananassa* Duch.) to clarify the physiological changes in strawberry during drought response. "Zhangji" is the largest cultivated strawberry variety at present, and the cultivated area of "Zhangji" in some areas accounts for more than 80% of the total area. The "Zhangji" fruit has a milky flavor, low acidity, early fruit, and high yield, which are favored by consumers and growers. We used transcriptomic sequencing and mass spectrometry to clarify the molecular mechanism of strawberry stress resistance and the effects of drought on the physiology of strawberry plants. Our findings have important implications for the breeding of drought-resistant strawberry varieties.

2. Materials and Methods

2.1. Experimental Design

Potted "Zhangji" strawberry (*Fragaria × ananassa* Duch.), native to Shizuoka, Japan, was taken as the test material. The "Zhangji" strawberry seedling was made by cutting seedlings at the Jinniushan Base of the Shandong Fruit Research Institute. The cultivation substrate was composed of peat, vermiculite, and perlite with a volume ratio of 2:1:1. The experiment was carried out in an artificial climate room, with temperature (25 ± 2) °C, light cycle 16:8, and relative humidity (60 ± 5)%.

For the strawberry physiological indexes test, three treatments were arranged: conventional water supply (control), deficient water supply (water deficit), and excessive water supply (waterlogging).

For transcriptome and metabolome analyses, drought and conventional water supply treatments were set. Every treatment was repeated 3 times, with 10 pots for each replicate. With the decrease in the substrate water content during the drought treatment, 4 periods were sampled during both the drought and conventional treatments: (1) mild drought (Mild-Dr, 70% of conventional water content), (2) moderate drought (Mod-Dr, 50% of conventional water content), (3) severe drought (Sev-Dr, 20% of conventional water content), and (4) rewatering after drought treatment (RW-Dr).

The 4th functional leaves were sampled. After the surface was washed with deionized water and sucked up with filter paper, the leaves were freeze-dried in liquid nitrogen and stored at -80 °C for subsequence analysis.

2.2. Measurement of Physiological Indexes

The plant fresh weight, soil temperature, soil volume water content, daily transpiration, transpiration rate, and biomass gain (estimated using the leaf dry weight method) of "Zhangji" strawberry plants under conventional water supply (control), deficient water supply (water deficit), and excessive water supply (waterlogging) were measured using a Plantarray system, soil temperature tester (FOCHOU ST-02, Guangdong, China), constant temperature drying box (REALE RVO-0B (6020), Guangdong, China), and other instruments and equipment [10].

Daily transpiration: the daily transpiration of plants was measured using the water balance method [11]. The strawberry leaves were placed in a closed environment with a set relative humidity gradient; the daily transpiration of the leaves was calculated by

measuring the change in the relative humidity in the environment before and after the leaves were placed in this environment for 24 h.

Transpiration rate: leafy branches with normal growth were used for measurements of the transpiration rate. Immediately after cutting, the weights of the branches were measured and recorded. The detached leafy branches were then returned to their original mother plant; after 3–5 min under the original environmental conditions, they were weighed again. The transpiration loss within 3–5 min was then calculated. The leaf area was measured using the paper-cut weighing method. White paper with the same thickness was cut into 10 cm × 10 cm pieces (1 dm²) and weighed (mg); the tested leaves were spread on the same white paper, and the leaf shape (without petiole and branches) was traced with a pencil; the paper leaves were then cut and weighed (mg).

$$\text{Leaf area (dm}^2\text{)} = \text{Leaf weight (mg)} / \text{Paper weight (mg)}$$

$$\text{Transpiration rate} = \text{Transpiration water loss} / \text{unit leaf area} \times \text{time}$$

2.3. Transcriptome Sequencing and Analysis

2.3.1. Extraction and Detection of RNA Samples

The total RNA from the strawberry leaf samples was extracted using the Trizol method (Invitrogen, Carlsbad, CA, USA) [12]; the RNA was then stored at −80 °C. The integrity of the extracted total RNA was detected using 1.2% agarose gel electrophoresis, and the 28S rRNA/18 rRNA ratio of high-quality RNA was 2.0. A Nanodrop 2000 ultraviolet spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the concentration and purity of the RNA. RNA was considered high quality if A260/A230 = A260/A280 and >1.8 and if RNA integrity (RIN value) > 9.0, as determined by an Agilent 2100 bioanalyzer (2100 Bioanalyzer, AGILENT, Waldbronn, Germany).

2.3.2. RNA Library Construction

rRNA was removed from the total RNA samples (5 µg) using the rRNA Removal Kit (Ribo-zero™ rRNA Removal Kit, EpiCentre, Manalapan Township, NJ, USA). cDNA libraries were constructed using the Truseq™ RNA sample prep Kit (Illumina, San Diego, CA, USA). dUTP was used instead of dTTP for second-strand synthesis; after the synthesis of double-stranded cDNA, short, spliced fragments were connected, and the UNG enzyme was added to induce the degradation of the second-strand cDNA. The cDNA was amplified via 15 PCR cycles, and the cDNA library was obtained using 2% Low Range Ultra Agarose (Bio-Rad, Hercules, CA, USA). Quantification by TBS380 (Picogreen dsDNA Assay, XYbscience, Yueyang, China) was performed according to the data ratio. The bridge PCR amplification was performed on cBot (cBot Truseq PE Cluster Kit v3 murcBotcopyright HSL, Illumina, San Diego, CA, USA) to generate clusters. The results were sent to the HiSeq sequencing platform for 2 × 150 bp sequencing [12].

2.3.3. Analysis of Differentially Expressed Genes (DEGs)

DEGs were analyzed using DEseq software (Bioconductor, <https://www.bioconductor.org/>) [13]. The overall distribution of DEGs in samples was visualized using a volcano map. Genes were considered differentially expressed if padj ≤ 0.05. The GO software (Bioconductor, <https://www.bioconductor.org/>) was used to enrich the selected DEGs (gene ontology), and significant enrichment was defined as *p* ≤ 0.05. The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway of differentially expressed genes were enriched by KOBAS, with a significant threshold of *Q* ≤ 0.05.

GO analysis of the DEGs was performed using Blast2GO software (<https://www.blast2go.com/>) [14], and GO terms were considered significant if the corrected *p*-value < 0.05. KEGG pathway enrichment analysis of DEGs was performed using BlastKOALA, and KEGG pathways were considered significant if the *Q* value ≤ 0.05.

2.4. Targeted Metabolomic Analysis

2.4.1. Preparation of the Metabolomic Standards

A targeted metabolomic analysis of plant hormones was used to study the metabolism of strawberry leaves in different treatments. The 24 plant hormones examined (24 plant hormone standards and 7 stable isotope labeling standards were purchased from Shanghai Zhenjun Biotechnology Co., Ltd., Shanghai, China) included Indole-3-acetic acid (IAA), 3-indolebutyric acid (IBA), indole-3-carboxylic acid (ICA), methyl indole-3-acetate (ME-IAA), indole-3-carboxaldehyde (ICA), N6-isopentenyladenine (IP), isopentenyl adenosine (IPA), *trans*-zeatin-riboside (tZR), *trans*-zeatin (tZ), dihydrozeatin (Dh-Z), kinetin (K), methylsalicylate (MESA), brassinolide (BL), methyl jasmonate (MeJA), dihydrojasmonic acid (H₂JA), N-Jasmonic acid-isoleucine (JA-Ile), (±)-jasmonic acid (JA), Salicylic acid (SA), abscisic acid (ABA), gibberellin A1 (GA₁), Gibberellin A3 (GA₃), Gibberellin A4 (GA₄), Gibberellin A7 (GA₇), and 1-aminocyclopropanecarboxylic acid (ACC). The 24 hormone standard products were weighed, mixed, and used to prepare a mother liquor.

The stock solution of individual phytohormones was mixed and prepared in a phytohormone-free matrix to obtain a series of phytohormone calibrators. Certain concentrations of IAA-D4, JA-D5, N6-isopentenyladenine-D6, dihydrozeatin-D3, gibberellin A1-D4, SA-D4, and ABA-D6 were mixed to make internal standard solutions. The stock solution of these phytohormones and the working solution were stored at −20 °C.

LC-MS was used to determine the concentrations of a series of standard solutions. The linearity of the standard solutions was evaluated using concentrations of the standards (abscissa) and the ratio of the internal standard peak area (ordinate).

2.4.2. Metabolite Extraction

After grinding with liquid nitrogen, 100 mg samples containing mixed internal standard were homogenized with 400 µL of acetonitrile (50%) and extracted for 30 min at 4 °C, then centrifuged at 12,000 rpm for 10 min. The supernatant passed through the HLB sorbent, which was subsequently eluted with 500 µL of acetonitrile (30%). With two fractions combined into the same centrifuge tube and mixed well, these solutions were injected into the LC-MS/MS system for analysis.

2.4.3. Chromatographic and Mass Spectrometry Detection

Metabolic extracts [15] were used for liquid chromatography–mass spectrometry (LC-MS, Thermo-Fisher, Waltham, MA, USA). An ultra-high performance liquid chromatography coupled with a tandem mass spectrometry (UHPLC-MS/MS) system (ExionLC™ AD UHPLC-QTRAP 6500+, AB SCIEX Corp., Boston, MA, USA) was used to determine the concentrations of phytohormones at Novogene Co., Ltd. (Beijing, China). Separation was performed on a Waters XSelect HSS T3 column (2.1 × 150 mm, 2.5 µm), which was maintained at 45 °C. The mobile phase, consisting of 0.01% formic acid in water (solvent A) and 0.01% formic acid in acetonitrile (solvent B) (LC-MS, Thermo-Fisher, Waltham, MA, USA), was delivered at a flow rate of 0.30 mL/min. The solvent gradient was as follows: 10% B, 1 min; 10–50% B, 3 min; 50–65% B, 4 min; 65–70% B, 6 min; 70–100% B, 7 min; 100–10% B, 9.1 min; and 10% B, 12 min.

The mass spectrometer was operated in multiple reaction mode. The parameters were as follows: ion spray voltage (negative mode: −4500 V, positive mode: 4500 V), curtain gas (35 psi), ion source temp (550 °C), and ion source gases of 1 and 2 (60 psi).

2.4.4. Metabolic Data Analysis

MetaboAnalysis 6.0 [16] was used to analyze the targeted metabolic data. Differential metabolites were identified using the following criteria: VIP ≥ 1, according to the orthogonal partial least squares–discriminant analysis model and *p* < 0.05, according to a Student's *t*-test. A bubble map of differential metabolite enrichment was made using the ggplot2 package in R software (<https://www.r-project.org/>).

3. Results

3.1. Effects of Drought on the Physiology of Strawberry

Recent studies have shown that stress exposure induces a series of physiological changes in plants, and many plants have evolved strategies that allow them to tolerate drought. The daily transpiration, transpiration rate, fresh weight, and biomass gain of “Zhangji” strawberry plants under a regular water supply (control), deficient water supply (water deficit), and excessive water supply (waterlogging) were measured using a Plantarray system. We found that the transpiration rate in the deficit irrigation treatment was significantly lower than that of the control (Figure 1).

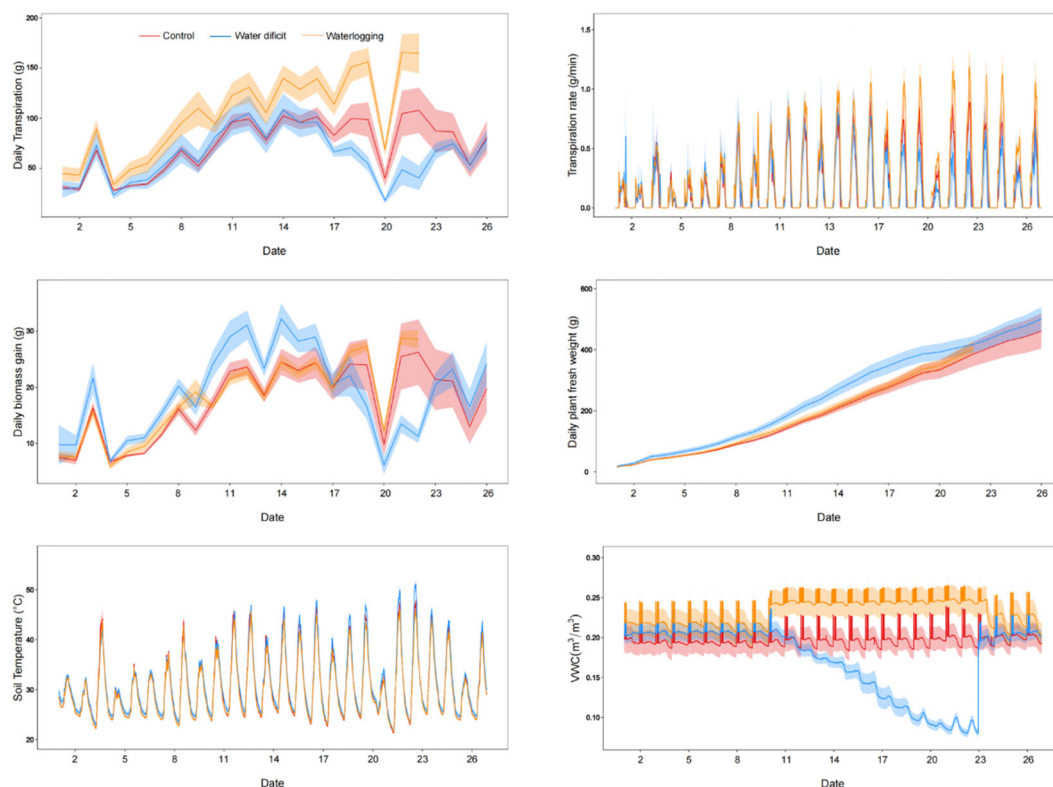


Figure 1. Effects of drought on the physiological indexes of strawberry plants.

The daily transpiration rate of strawberry was consistently lower during the deficit water treatment than the control. The biomass of plants increased continuously, and the rate of increase was initially rapid and then slow. The increase in the biomass of plants was higher in the early stage of the water deficit treatment than in the conventional water treatment, but it was lower in the late stage of the water deficit treatment than in the conventional water treatment. The fresh weight of plants increased gradually over time, and the increase in the fresh weight of plants was significantly higher in the water deficit treatment than in the control. The soil temperature fluctuated over time in the three treatments, and the variation in soil temperature was greater in the water deficit treatment than under control treatment. In the early stage of the experiment, the soil water content was stable and lower in the water deficit treatment than in the conventional water treatment. From day 11 to 23, the soil volume water content gradually decreased in the water deficit treatment; in the excessive water treatment, the soil volume water content gradually increased and was higher than the conventional water treatment at the end of this period.

3.2. Transcriptome Analysis

3.2.1. Transcriptome Sequencing and Identification of DEGs

Differences in the physiological responses of strawberry plants were observed in the Mild-Dr, Mod-Dr, Sev-Dr, RW-Dr, and CK groups, and DEGs in each of these groups were identified.

A total of 428 (209 up-regulated and 219 down-regulated), 5666 (3014 up-regulated and 2652 down-regulated), 3693 (2172 up-regulated and 1521 down-regulated), and 2573 (1079 up-regulated and 1494 down-regulated) DEGs were identified in the Mild-Dr vs. CK, Mod-Dr vs. CK, Sev-Dr vs. CK, and RW-Dr vs. CK comparisons, respectively (Figure 2).

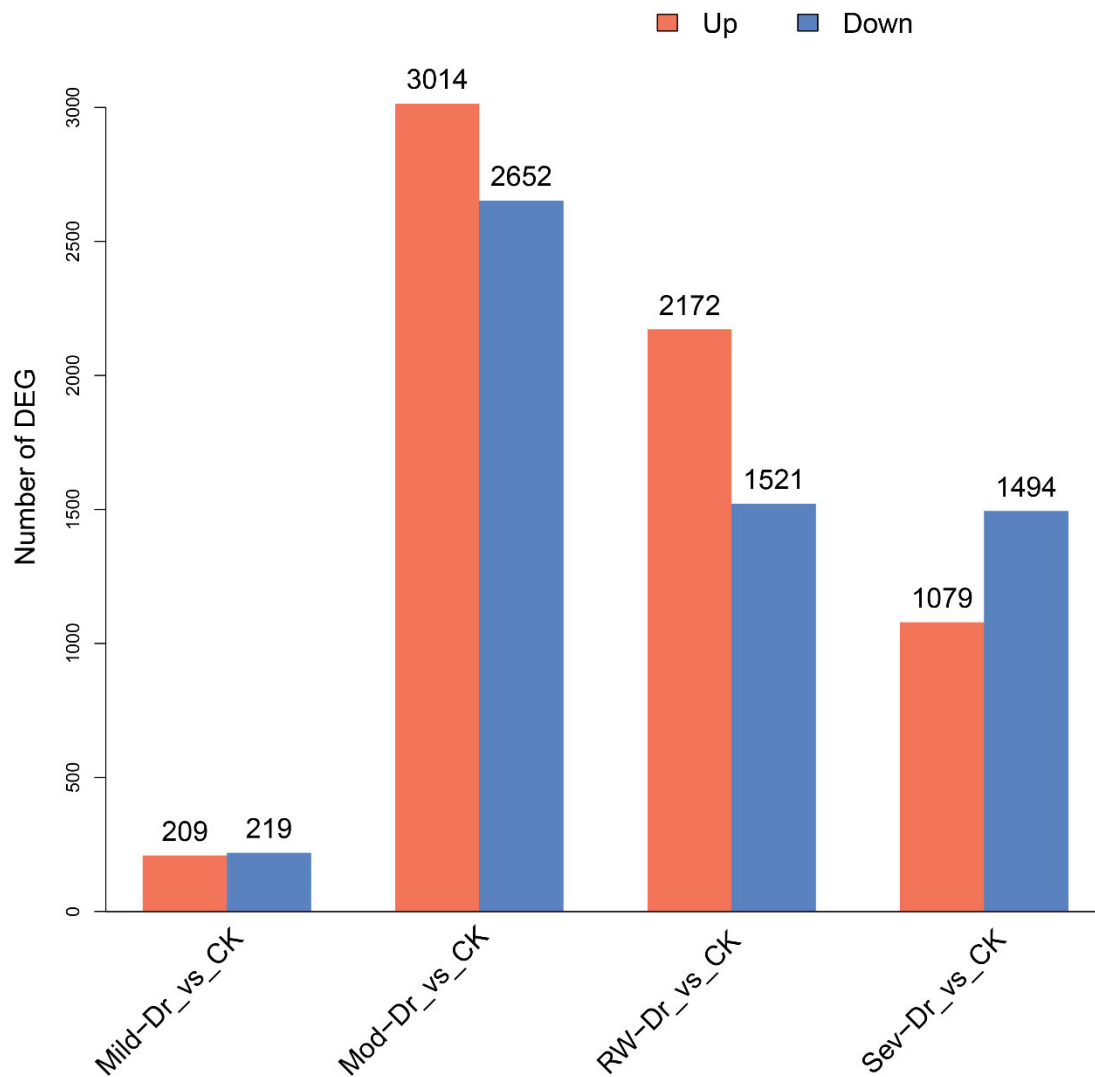


Figure 2. Up-regulated and down-regulated DEGs in four comparison groups.

3.2.2. GO and KEGG Pathway Enrichment Analyses

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted on DEGs under normal conditions and drought treatment to clarify their biological functions. GO terms from three different categories—molecular function (MF), cell component (CC), and biological process (BP)—were determined for DEGs in the comparison of different drought (mild, mod, severe and rewatering after drought) degrees with CK; the differential DEGs were all mainly enriched in binding, catalytic activity, metabolic process, and cellular process (Figure 3).

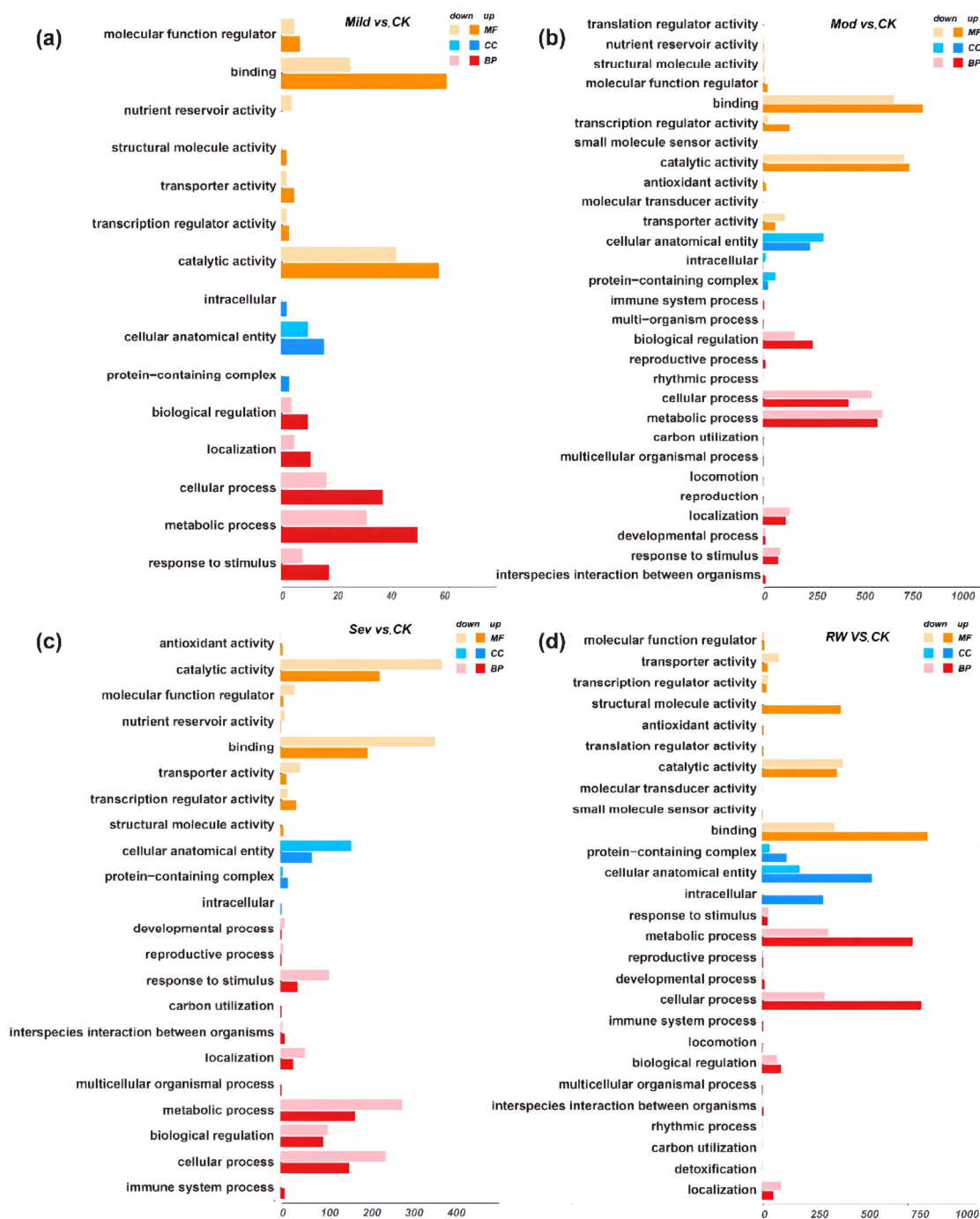


Figure 3. Results of the GO enrichment analysis of DEGs in different comparison groups.

Bubble maps were used to visualize the results of the KEGG pathway enrichment analysis for all DEGs and clarify the effects of drought stress on the enriched pathways (Figure 4). DEGs in the mild-Dr vs. The CK comparison group was highly enriched in “pentose and glucose interconversions” and “sulfur metabolism”. DEGs in the Mod-Dr vs. CK comparison group were highly enriched in “biosynthesis of secondary metabolites” and “nitrogen metabolism”. DEGs in the Sev-Dr vs. CK comparison group were highly enriched in the “galactose metabolism” and “fatty acid elongation” pathways. DEGs in the RW-Dr vs. CK comparison group were highly enriched in “ribosome”, “ribosome biogenesis in eukaryotes”, and other pathways. During the moderate drought treatment, the normal growth and development of plants were

enhanced through the regulation of sugar, nitrogen, and sulfur metabolism. During the excessive drought treatment, drought resistance was mainly achieved through alterations of the synthesis and metabolism of amino acids and fatty acids. Normal development and growth were restored during the rehydration treatment after drought through changes in the nucleotide metabolism pathway.

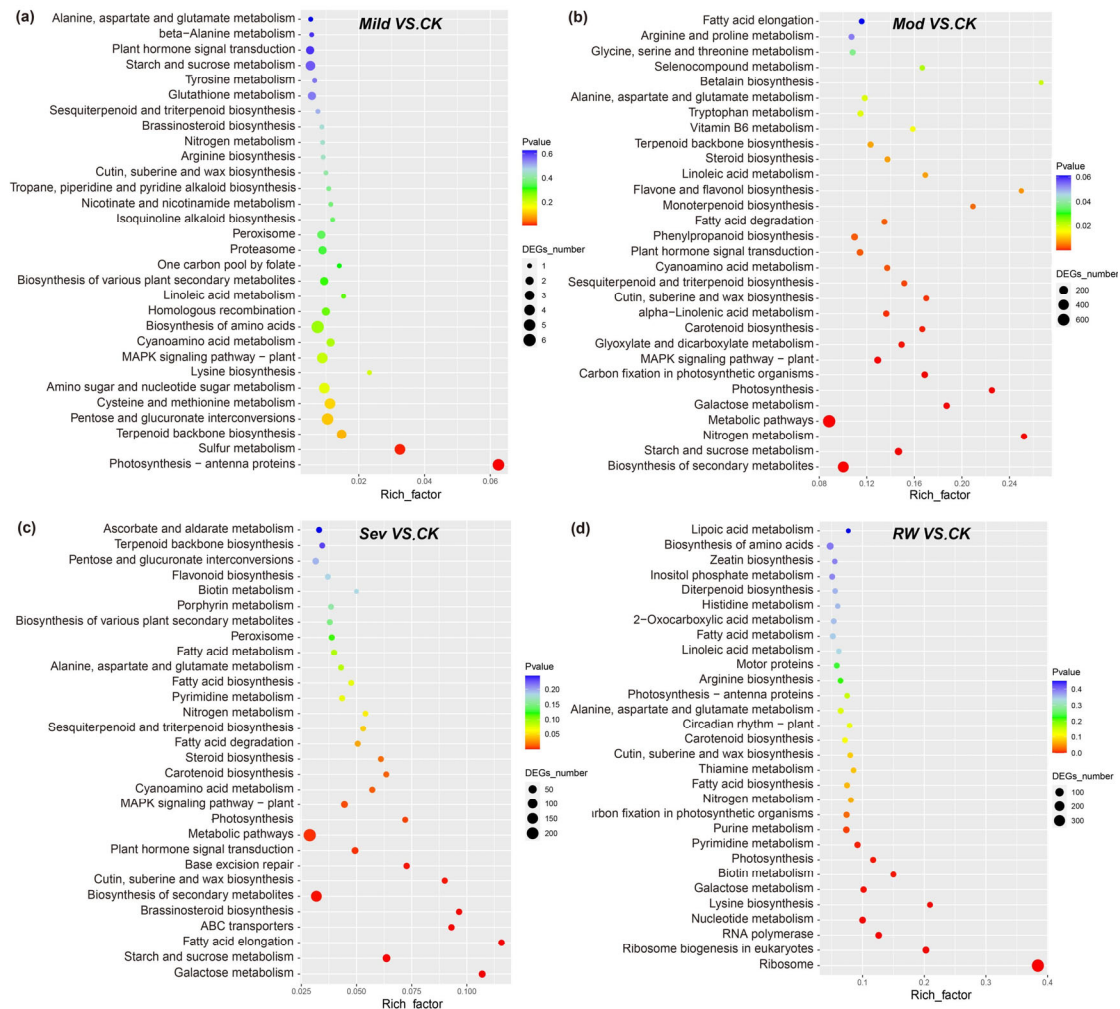


Figure 4. KEGG enrichment analysis of DEGs in different comparison groups.

3.3. Metabolomic Analysis

A targeted metabolomic analysis of “Zhangji” strawberry plants was conducted to identify the metabolites related to drought stress and clarify the mechanism by which “Zhangji” strawberry plants respond to drought stress. As shown in Figure 5, changes in 12 hormones were detected in the mild-Dr, Mod-Dr, Sev-Dr, and RW-Dr treatments compared with CK. Compared with the control, the syntheses of 3-indolebutyric acid (IBA), salicylic acid (SA), and abscisic acid (ABA) were up-regulated in the mild drought treatment. The syntheses of indole-3-carboxyl acid (ICA), salicylic acid (SA), abscisic acid (ABA), gibberellin A1 (GA1), and jasmonic acid (JA) were up-regulated in the moderate drought treatment. The syntheses of salicylic acid (SA), 1-aminocyclopropanecarboxylic acid (ACC), and indole-3-acetic acid (IAA) were down-regulated in severe drought treatment. Although 3-indolebutyric acid (IBA) was down-regulated, 1-aminocyclopropanecarboxylic acid (ACC), indole-3-acetic acid (IAA), indole-3-carboxaldehyde (ICA), and isopentenyl adenosine (IPA) were up-regulated in the rehydration after severe drought treatment compared with the other treatments.

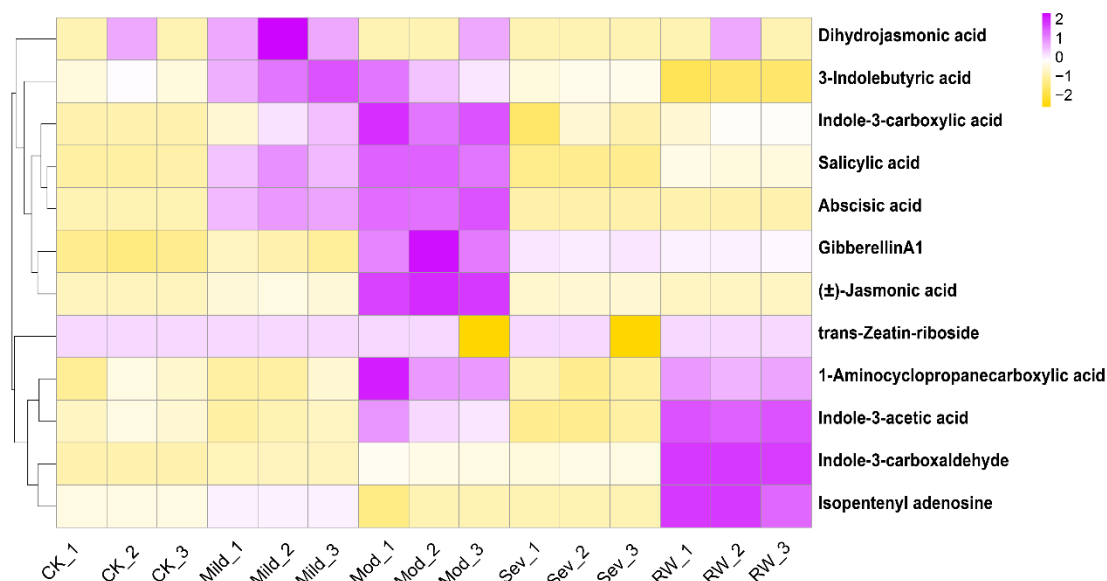


Figure 5. Comparison of heat maps of phytohormones in different treatment groups.

4. Discussion

Strawberry is one of the most well-received fruits because of its sweet smell and rich nutrients [17]. In terms of physiological structure, cultivated strawberry has a large leaf surface and a shallow root system, making it more sensitive to drought [18]. And drought is the most considerable environmental stressor [19], caused by a decrease in water content in plant tissue and resulting in a decrease in somatic osmotic pressure and abnormal metabolism, which has a certain impact on the growth and development of strawberry [20].

The response of strawberry plants to stress is a complex process. Recent studies have shown that plant physiological changes are the primary response mechanism to stress, and many plants have evolved a response mechanism to drought [1]. In this study, the water content of “Zhangji” strawberry plants can be maintained under drought conditions by reducing the transpiration rate and daily transpiration. Under mild drought stress, the physiological processes and hydraulic signals of strawberry plants are altered to mediate adaptation to stress. However, as the stress exposure increases, the expression of genes involved in stress resistance increases, affecting the phenotypic features of plants.

Transcriptomic analyses can be used to characterize quantitative changes in the expression of genes at different times and under different states [21]. In this study, we identified genes that play a key role in the drought tolerance of “Zhangji” strawberries to clarify the mechanism underlying the response to drought. The expression of genes involved in pathways such as “oxidoreductase activity”, “cell metabolism”, “sulfur metabolism”, “sugar metabolism”, “pentose and glucose transformation”, and “nitrogen metabolism” was up-regulated in the four treatments (mild water deficiency, moderate water deficiency, severe water deficiency, and rehydration after drought), which indicated that strawberry plants could resist the damage induced by drought stress through the accumulation of sugars in leaves. Soluble sugars can reduce the membrane osmotic potential by combining with membrane lipid bilayers, which alleviates changes in osmotic pressure and promotes cell expansion; this might explain why the increase in soluble sugars, such as glucose and sucrose, can enhance the resistance of plants to abiotic stress [22]. In addition, sugars can be used as small molecular signaling substances to mediate resistance to abiotic stress. Previous studies have shown that soluble sugars in roots act as energy storage substances and osmotic regulators in plants, but they also play key roles as signal substances under drought and heavy metal stress [23]. Nitrogen metabolism and carbon metabolism are closely related via a complex network of metabolites, and this has implications for plant growth. The energy and carbon sources needed for nitrogen metabolism are provided

by carbon metabolism [24]. The activity of nitrogen metabolism enzymes and the soluble protein content were enhanced in strawberry leaves in the moderate water deficiency treatment. The above results indicate that moderate drought treatment can promote nitrogen metabolism and the conversion of inorganic nitrogen and sulfur into amino acids, which can then be used to assemble proteins. The expression of genes involved in endogenous auxin such as “pyrimidine metabolism”, “lysine biosynthesis”, “biotin metabolism”, and “eukaryotic nucleic acid generation” was significantly up-regulated in the rehydration treatment after drought, which indicates that the growth and development of the plants were restored after drought stress.

Endogenous hormones are important growth regulators in plants. The relative abundance of endogenous hormones play an important role in regulating the resistance of plants to drought stress, and their responses to drought stress are complex and dynamic [25]. Previous studies have shown that, under drought stress, the synthesis of hormones such as IAA, GA3, and zeatin nucleoside is often suppressed [26], and ABA can be used as a signaling molecule to induce stomatal closure and enhance drought resistance [27]. In this study, under mild water shortage, the concentrations of IBA, SA, and ABA increased. Increases in GA1, ($\pm$)-JA, and ICA were observed in the moderate water deficit treatment. Levels of SA, ACC, and IAA during the severe water deficit treatment were lower than those during other treatments. Increases in ACC, IAA, ICA, and IPA were observed in the rewatering after drought treatment. The above results indicate that drought stress induces an endogenous response in “Zhangji” strawberry plants. The secretion of various endogenous growth hormones maintains the normal growth of plants under drought stress, and the normal growth and development of plants are restored in the rewatering after drought treatment.

5. Conclusions

In this study, various approaches were used to clarify the mechanism by which “Zhangji” strawberry plants respond to different levels of drought stress. At the physiological level, strawberry plants can adapt to drought stress by regulating the water content in their plant body; for example, by reducing their respiration rate. Transcriptome analysis revealed that genes involved in oxidoreductase activity and in sulfur and nitrogen metabolism were up-regulated, as well as starch and sucrose. The results of the metabolomic analysis showed that hormones such as SA, ABA, and ($\pm$)-JA in strawberry plants increased. Strawberry plants secrete various endogenous growth hormones to maintain their normal growth under drought stress. Rehydration after drought restores the normal growth and development of strawberry plants. Our findings provide new insights into the pathways that mediate the response to different levels of drought stress in “Zhangji” strawberry plants and will aid the development of new drought-resistant strawberry varieties.

Author Contributions: Investigation, C.W.; Data curation, X.W.; Writing—original draft, R.S.; Writing—review & editing, L.J. and J.W.; Visualization, C.W.; Supervision, L.J.; Project administration, J.W.; Funding acquisition, J.W. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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