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Transcriptome Analysis of Diploid and Autotetraploid *Hemerocallis* Response to Drought Stress

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Abstract: Chromosome doubling in ornamental plants, as shown by our study in daylilies (*Hemerocallis* spp.), has great potential to increase tolerance to abiotic stress. Drought is the most critical growth-limiting factor in a changing climate. Drought tolerance is one of the decisive factors for the survival, productivity, and appearance of perennial ornamental plants. Understanding and elucidating the molecular mechanisms that determine plant response to abiotic stress is essential. De novo transcriptome assembly of diploid and autotetraploid *Hemerocallis* spp. cv. Trahlyta was performed under artificially induced stress to elucidate the molecular mechanisms related to plant response to drought. In daylily mRNA, 237,886 transcripts were detected, and 42.4% of them were identified as annotated unigenes. In the experiment, diploid plants were more stressed, with 2871 upregulated or downregulated DEGs (differentially expressed genes) responding to drought, while tetraploid plants had 1599 DEGs. The proportion of upregulated DEGs differed by 1.3 times between diploid and autotetraploid genotypes, whereas the proportion of downregulated DEGs was 1.8 times greater in diploid plants. Signaling pathways related to the drought response were activated in daylilies, and key candidate genes were identified in both ploidy genotypes. In autotetraploid plants, more drought-related pathways were activated than in diploids—43 and 19, respectively. The most abundant DEGs in both cases were KEGG (Kyoto Encyclopedia of Genes and Genomes), metabolic (ko01100), and biosynthesis of secondary metabolites (ko01110) pathways. Summarizing the data, it was found that autotetraploid plants of the daylily have a wider potential for adaptation to drought stress. Therefore, they adapt faster and better to adverse drought conditions by activating alternative signaling pathways. The comparative transcriptome analysis of diploid and autotetraploid plants allows us to understand the molecular mechanisms of drought resistance and it is also essential for daylily breeding programs to develop drought-resistant genotypes in the future.

Keywords: drought; abiotic stress; daylily; drought resistance signaling pathway; RNA-Seq; de novo transcriptome

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1. Introduction

Biotic and abiotic stress factors are significant problems for ornamental plants because these plants should maintain their attractive features during the vegetative season [1–10]. Plant morphological, physiological, biochemical, and molecular changes occur due to water deficits, influencing plant functioning and growth. Drought reduces photosynthesis and plant output. Water stress also affects chlorophyll concentration, which is necessary for photosynthesis and is influenced by changes in CO_2 conductance, stomatal movement, and lipid peroxidation. Improving the antioxidant system is essential for drought tolerance and avoiding the harmful effects of drought-induced reactive oxygen species (ROS) [5]. Drought stress is one of the most critical growth-limiting factors in a changing climate [11]. Tolerance for drought is one of the decisive factors for the survival, productivity, and appearance

of perennial ornamental plants [12–14]. Drought tolerance refers to plants' ability to live or develop in water-stressed environments due to dehydration avoidance, tolerance, or recovery. Tolerant plants endure drought better due to physiological, biochemical, and molecular mechanisms, signifying appropriate plant selection attributes in low-water-availability scenarios [5].

Polyploidy in flowering plants has important ecological and evolutionary implications. It is associated with greater resistance to severe settings due to higher genetic variety and duplicated genes. On the other hand, the establishment or long-term survival of these species frequently corresponds to substantial global climatic/geologic shifts or major stress [15]. Studies have shown that plant ploidy affects growth, development, and resistance to stress conditions [15]. To cope with drought stress, plants undergo various morphological, physiological, biochemical, and molecular changes [16]. The tolerance of abiotic stresses on the genome duplication of various plants was investigated. The ploidy level of plants has an impact on water deficit [17], photosynthesis regulation [18], anatomical changes [19], water transport capacity [20], antioxidative enzyme activity and the regulation of osmolyte content [21], stomatal function and transpiration rates [22], and ROS and ABA homeostasis [23].

Daylilies (*Hemerocallis* L.) are a monocot, easy-to-grow perennial plant known for their broad usage in ornamental greenery [24], medicine [25], and culinary activities [26,27]. The genus *Hemerocallis* contains 15 to 19 species native to east Asia [28], mainly growing in grasslands and mountain habitats [29]. Nearly 99 thousand daylily cultivars are registered by the American Hemerocallis Society [30]. Daylilies naturally occur only as diploids and triploids [31]. One of the common ways to make plants more resistant to biotic and abiotic stresses is whole genome duplication (WGD) [19,32,33]. Tetraploid daylilies were artificially induced by using chemical antimitotic agents [34–38]. Currently, hybridizers focus on tetraploid daylily breeding programs with 69.8% of newly registered cultivars in the last five years being tetraploids [30].

It is well known that the ploidy level of daylilies impacts their morphological and physiological characteristics [34]. However, the differences between diploid and tetraploid plants' responses to stress signaling mechanisms and how ploidy affects the functioning of protective systems at the molecular level have yet to be discovered. More research is needed on gene functions and metabolic pathways in daylilies (*Asphodelaceae*). A comparative analysis of the de novo assembled transcriptomes of diploid *H. fulva* was undertaken by X. Cai and colleagues [39]. Annotation of transcripts was carried out, and the molecular mechanisms underlying the drought response of seedlings in vitro was studied.

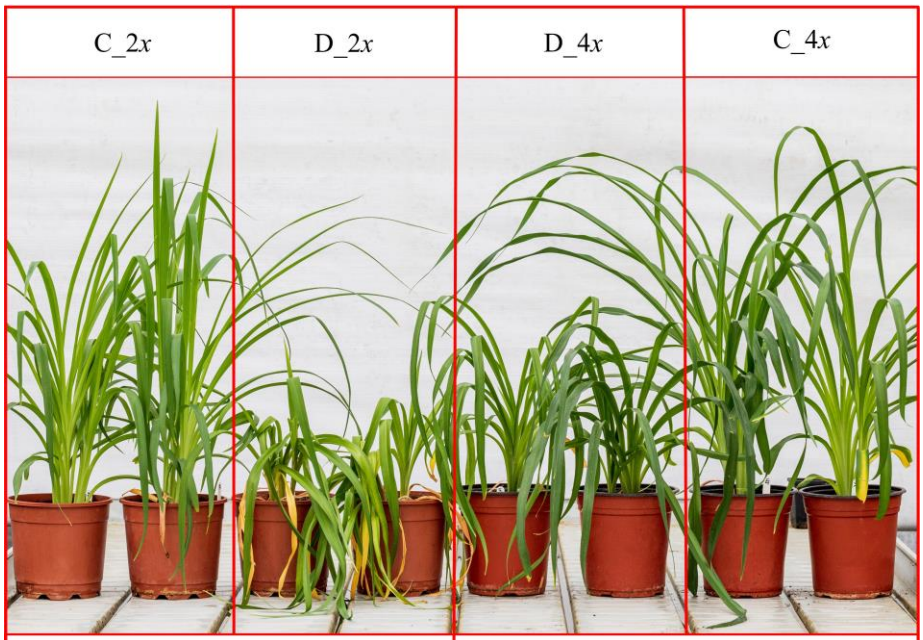
The aim of this study is to understand and elucidate changes in the molecular mechanisms in diploid and autotetraploid plants of daylily cv. Trahlyta by simulating drought stress in a greenhouse environment.

2. Materials and Methods

2.1. Plant Material, Cultivation, and Treatment

Daylily plants with identical genomes are the preferred choice for the comparative analysis of molecular response to stress in diploid and tetraploid plants. Daylily cv. Trahlyta, registered by F. Childs in 1982, is highly used in breeding programs, with 166 registered offsprings [30]; moreover, an autotetraploid clone of cv. Trahlyta was implemented that is identical to the diploid form, which was obtained by treating the meristem with antimitotic agents. The ploidy of an autotetraploid cv. Trahlyta was tested by flow cytometry, and its genetic identity was approved by SSR markers [40]. Plants of *Hemerocallis* cv. Trahlyta (2*x*) and autotetraploid cv. Tet. Trahlyta (4*x*) were grown in a greenhouse in the Department of Orchard Plant Genetics and Biotechnology at the Institute of Horticulture, LAMMC, under water deficiency (D) and unstressed conditions (C) (Figure 1). Mature daylily cv. Trahlyta and autotetraploid Trahlyta divisions were planted in 3-litre containers with peat and perlite mixture (3:1) and grown for two years to eliminate transplanting factors. Prior to the experiment all containers with plants were soaked in water to provide similar

conditions. Then the soil moisture was measured using an HH2 soil moisture meter (Delta-T Devices Ltd., Cambridge, UK) every three days. The control group of plants moisture was maintained at 45% by watering them once a week. The drought treatment group was not watered, and samples of control and stressed plant leaf were collected simultaneously when the soil moisture was constantly less than 10%. Simultaneously, the plant height (cm), the number of yellow leaves (%), the stress balance indexes of the chlorophyll (Chl), flavonols (Flav), and nitrogen balance (NBI) were evaluated (measurements using Dualex 4 Scientific® (FORCE-A, Orsay, France)) (Figure 1).



Treatment	Average differences (control − treatment) and standard deviation				
	Plant height, cm	Yellow leaves, %	Chl	Flav	NBI
C_2*x* vs. D_2*x*	11 ± 0.35	16.35 ± 2.28	−8.97 ± 0.45	−0.072 ± 0.004	19.81 ± 3.23
C_4*x* vs. D_4*x*	3 ± 0.50	0.954 ± 1.89	−7.21 ± 0.61	0.021 ± 0.006	−60.19 ± 4.58

Figure 1. Morphological and physiological average differences between diploid (2*x*) and autotetraploid (4*x*) plants of daylily cv. Trahlyta under drought stress conditions (10% soil moisture). Chl—chlorophyll, Flav—flavonol, NBI—nitrogen balance index.

2.2. RNA Isolation, cDNA Library Preparation and Sequencing

Leaf disks of the centre of the fourth leaf were collected in three biological replications from control and drought-stressed plants of both ploidy groups. The samples were instantly frozen into liquid nitrogen and held until RNA extraction with a GeneJET Plant RNA Purification Mini Kit (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's recommendations. The RNA concentration and quality were measured using an Implen GmbH spectrophotometer (Implen, Munich, Germany). A quantity of 4 µg of qualified RNA per sample was sent to Novogene (Cambridge, UK) for mRNA library preparation (poly-A enrichment) and RNA sequencing. Next-generation sequencing (NGS) was performed on an Illumina 6000 NovaSeq PE150 (6 Gb raw data per sample) platform.

2.3. De Novo Transcriptome Analysis

In the de novo transcriptome analysis, data from 24 replicates (three biological replicates per treatment and two techniques) were used from diploid and autotetraploid *Hemerocallis* spp. cv. Trahlyta. The raw transcriptome read data are available in the SRA database with BioProject accession number PRJNA993133: Daylily drought transcriptome analysis.

2.4. Functional Annotation of Unigenes

Low-quality raw reads were filtered: reads containing adaptors, N > 10%, and with a Q score of more than 50% bases less than five were removed (Table 1). Trinity was used to reconstruct the de novo transcriptome [41]. Corset, a command-line software application, was used to cluster the assembled transcripts [42], and redundancy was eliminated. The longest transcripts from each cluster were selected as unigenes.

Table 1. Quality control (QC) data summary of the diploid and autotetraploid genome reads of *Hemerocallis* spp. cv. Trahlyta.

Ploidy	Sample	Raw Reads	Raw Bases, Gb	Clean Reads	Clean Bases, Gb	Q20	GC pct, %
2*x*	C	20,677,729 (±1,491,722)	6.2 (±0.4)	19,834,903 (±2,072,326)	6.0 (±0.6)	96.80 (±0.74)	45.78 (±0.64)
	D	28,653,149 (±2,942,249)	8.6 (±0.9)	27,341,411 (±3,482,860)	8.2 (±1.0)	96.31 (±0.06)	44.73 (±0.92)
4*x*	C	22,504,410 (±644,583)	6.8 (±0.2)	21,555,141 (±438,035)	6.5 (±0.2)	96.71 (±0.76)	46.21 (±1.12)
	D	25,293,629 (±465,420)	7.6 (±0.2)	23,803,623 (±195,953)	7.1 (±0.1)	94.47 (±1.25)	45.93 (±0.38)

Note. 2*x*—diploid; 4*x*—autotetraploid; C—control group; D—drought; Q20—the percentage of bases whose Q Phred values are greater than 20; GC pct (%)—the percentage of all bases with G and C base numbers.

2.5. Gene Functional Annotation

Seven public databases were used to provide functional annotation of all the assembled unigenes: the Non-Redundant Protein Sequence Database (NR), the Nucleotide Sequence Database (NT), Gene Ontology (GO), Swiss-Prot, the Kyoto Encyclopedia of Genes and Genomes (KEGG), and Pfam and Clusters of Orthologous Groups for Eukaryotic Complete Genomes (KOG). The genes successfully annotated in GO were classified into three main categories: biological process (BP), cellular component (CC), and molecular function (MF). KOG-annotated genes were classified into functional groups. The genes that were successfully annotated in KEGG were classified according to the KEGG pathway they belonged to.

2.6. Differentially Expressed Genes Venn Diagram, Volcano Plot, GO and KEGG Enrichment Analysis

A Venn diagram was created with the BioVinci software 3.0.0 (Bio Turning, San Diego, CA, USA), showing the number of represented genes that are differently expressed in each group, with the overlapping regions indicating the number of genes expressed in the two groups. The total number of genes expressed within a group is represented by the sum of numbers in each circle, and the overlap shows genes expressed in common between groups with Fpkm > 0.3 as the criterion. Volcano plots were generated using DESeq2 software [43] to infer the overall distribution of the differentially expressed genes. The x-axis represents the fold change in gene expression across different samples, while the y-axis represents the statistical significance of the differences. Differences that are statistically significant are represented by red or green dots. Gene ontology (GO) is a significant bioinformatics categorization system that aims to standardize the presentation of gene features across all species. It is divided into three main sections: cellular components, molecular function, and biological process. Functional enrichment analysis was performed using the R package [44]. The enrichment process used GOseq for terms with padj < 0.05. Additional GO enrichment analysis was implemented using the NovoSmart program with a p-value < 0.05. The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to systematically analyse gene function, and analysis of the genomic information databases was performed using KOBAS [45], showing the genes and gene expressions as a whole network.

2.7. Protein–Protein Interaction Analysis

PPI (protein–protein interaction) analysis was performed using de novo transcriptome analysis data of the differential expression of genes by searching the STRING protein interaction database for each protein's interaction with other proteins based on score. Analysis was carried out using the CYTOSCAPE program [46]. Clusters were made based on up- and downregulated proteins indicated by different colors. Hierarchical interactions were assigned and GO and KEGG enrichments were performed using *Arabidopsis thaliana* as a reference for protein function analysis.

3. Results

3.1. De Novo Transcriptome Sequencing and Quality Control of Bio-Project Data

In order to elucidate the molecular mechanisms underlying the drought stress in diploid and tetraploid daylilies, we synthesized twelve cDNA libraries from leaf samples of C_2*x*, D_2*x*, C_4*x*, and D_4*x* and generated de novo RNA-sequencing data. More than 277 million high-quality clean reads were generated from RNA extracted from daylily leaves and were used in the de novo transcriptome assembly of *Hemerocallis* spp. cv. Trahlyta diploid and autotetraploid plants. A total of 87.4 Gbases of raw reads data were generated for four groups (Table 1). Clean reads accounted for 95.3% of the samples, which ranged from 5.6 to 8.9 Gbases, and had a Q20 percentage of 93.66–97.65 and a GC percentage of 44.07–47.49. More reads were generated in water-deficit plant leaves in both ploidy groups.

A total of 237,886 transcripts were assembled, and 100,861 non-redundant unigenes were generated. A total of 42,091 unigenes (41.73%) had a sequence length of up to 500 bp, and 31,073 unigenes (30.81%) had a length of 501–1000 bp. In addition, 17,049 (16.90%) had a 1001–2000 bp length, and 10,648 unigenes (10.56%) had a sequence longer than 2000 bp. As a result, as the sequence length increased, the number of transcripts assembled in this project decreased. The same tendency was observed with unigenes too (Figure 2). The unigene length distribution varied from 301 to 14,681 amino acids, and the mean length was 936.

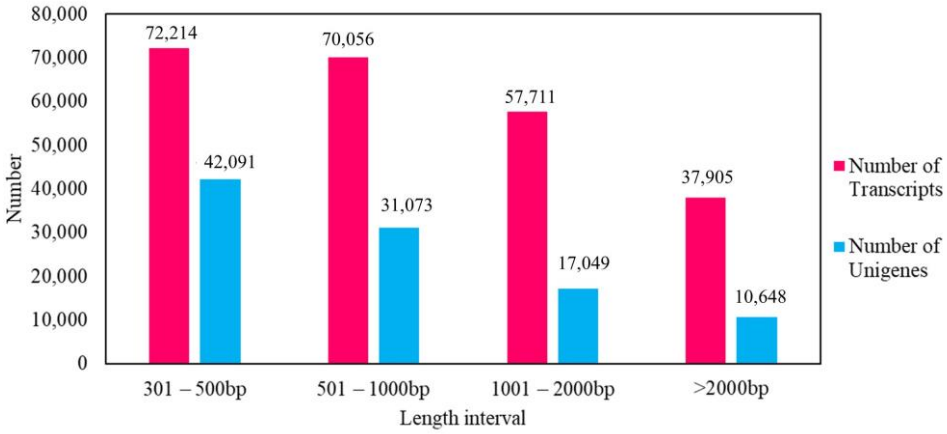


Figure 2. The assembled transcripts and unigene length distribution from the diploid and autotetraploid transcriptome of *Hemerocallis* spp. cv. Trahlyta.

3.2. Annotation of Unigenes in Hemerocallis spp. *Transcriptome*

The assembled 100,861 unigenes in the *Hemerocallis* spp. cv. Trahlyta transcriptome were annotated into seven databases: GO, KO, KOG, NR, NT, PFAM, and SwissProt (Figure 3A). The most unigenes, 38,185 (38%), were in the de novo assembled transcriptome annotated according to the NR database. According to the databases, GO, PFAM, SwissProt, and NT were identified as containing 26%, 26%, 25%, and 21% unigenes, respectively. Finally, the functionally annotated genes were poorly represented according to KO and KOG (12% and 7%, respectively).

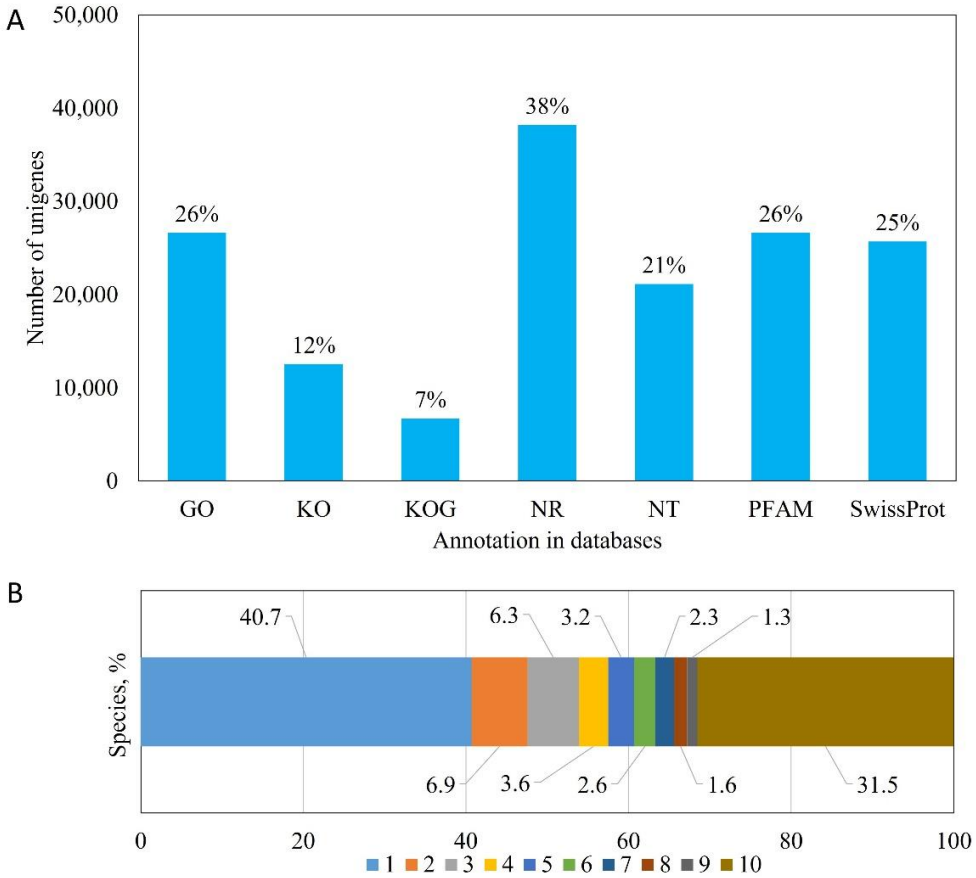


Figure 3. Number of unigenes of diploid and autotetraploid plants of daylily cv. Trahlyta annotated into seven databases (**A**), and the identity of plant species by *Hemerocallis* spp. gene annotation according to the NR database (**B**). Species: 1—*Asparagus officinalis*; 2—*Elaeis guineensis*; 3—*Phoenix dactylifera*; 4—*Vitis vinifera*; 5—*Ananas comosus*; 6—*Musa acuminata*; 7—*Dendrobium catenatum*; 8—*Ensete ventricosum*; 9—*Apostasia shenzhenica*; 10—other species.

The identities of plant species determined by *Hemerocallis* spp. annotation of genes according to the NR database are shown in Figure 3B. Following the NR annotations, the highest matching percentage of *Hemerocallis* spp. unigenes was with *Asparagus officinalis* in 40.7% of instances (Figure 3B). The assembled unigenes of cv. Trahlyta showed some identity with several other monocots—6.9% with *Elaeis guineensis*, 6.3% with *Phoenix dactylifera*, 3.2% with *Ananas comosus*, and 3.6% with the eudicot *Vitis vinifera*. Weak identity was shown between the daylily unigenes and other monocot plants, *Musa acuminata*, *Dendrobium catenatum*, *Ensete ventricosum*, and *Apostasia shenzhenica*, of 2.6%, 2.3%, 1.6%, and 1.3%, respectively.

The GO, KEGG, and KOG databases were used to annotate the functions of the *Hemerocallis* spp. unigenes. According to GO, 104,968 unigenes of cv. Trahlyta were allocated to 41 functional groupings after being classified into three functional categories (biological process, cellular component, and molecular function) (Figure S1A). A total of 52,366 daylily unigenes were linked to genes involved in 24 biological processes. In total, 23,343 and 29,259 unigenes were found in the cellular component and the molecular function categories, respectively.

The KOG database predicted and categorised the functions of the daylily unigenes (Figure S1B). In total, 7556 unigenes were divided into 25 categories based on ancestral proteins. KOG allocated *Hemerocallis* spp. unigenes to each category. According to the KOG classification data, three dominating categories of unigenes appeared. The post-translational modification, protein turnover, and chaperones group (O) consisted of 992 unigenes (13.13%); the general function prediction-only group (R) included 835 unigenes

(11.05%); and the translation ribosomal structure and biogenesis group (J) included 700 unigenes (9.26%). However, the most minor groups were extracellular structures (W) and cell motility (N), with three unigenes each.

Based on the KEGG database, all the annotated unigenes (13,826) were classified into 18 functional KEGG pathways and assigned to five ontologies (Figure S1C). A total of 1610 were assigned to cellular processes (red), 1493 to environmental information processing (dark blue), 2793 to genetic information processing (light blue), 5501 to metabolism (green), and 2429 to organismal systems (yellow) via the KEGG database. The five metabolic pathways were further subdivided into 33 subcategories. A total of 39.79% of the unigenes were assigned to the metabolism pathways. A total of 1391 unigenes were associated with signal transduction metabolism, 1097 with translation, and 1019 with carbohydrate metabolism.

3.3. Differentially Expressed Genes (DEGs) to Drought Stress Response in Diploid and Autotetraploid Daylily Leaves

A Venn diagram was used to show the number of consensus and differently expressed unigenes between plants under drought stress and in normal-irrigated conditions (Figure 4). The expression level of 74,639 unigenes in the diploid and 67,498 in the autotetraploid genotypes of cv. Trahlyta was obtained. A total of 62,646 unigenes expressed independently of drought stress were found among the diploids (D_2*x* vs C_2*x*) and 51,687 among the autotetraploids (C_4*x* vs D_4*x*) (Figure 4A). Response to drought stress identified 30,422 unigenes in the diploid and 28,055 in the autotetraploid genotypes. Depending on the ploidy, different numbers of expressed unigenes responded to stress (Figure 4B). A total of 14,589 unigenes associated with drought tolerance were characteristic for the diploid and 8405 for the autotetraploid samples. It was determined that only 3840 transcripts were expressed in both genotypes under drought stress conditions.

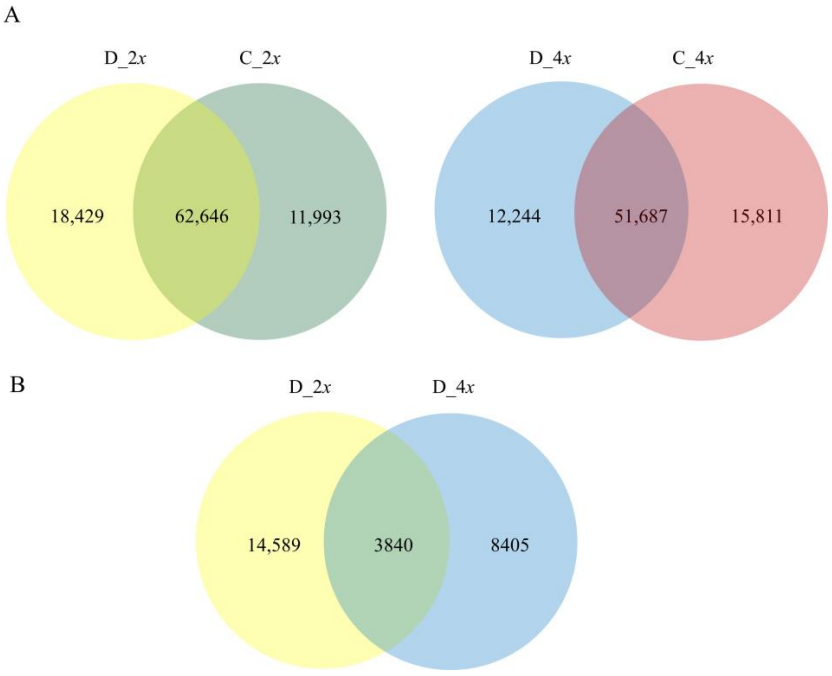


Figure 4. Venn diagram of differential expression gene (DEG) analysis of diploid (2*x*) and autotetraploid (4*x*) daylilies under drought stress (D) and for not-stressed plants (C): (**A**) diploid (D_2x vs. C_2x) and autotetraploid (C_4*x* vs. D_4*x*) DEGs; (**B**) DEGs-only response to drought stress in diploid and autotetraploid (D_2*x* vs. D_4*x*) plants.

The up or downregulated DEGs with significant value in the drought stress of diploid and autotetraploid daylilies were identified. There were 2341 and 1599 DEGs found in response to drought stress in the diploid and autotetraploid daylilies, respectively (Figure 5, Table S1). In the diploids, 1170 genes were downregulated, while 1171 were upregulated,

and in the autotetraploids, 661 were downregulated and 938 were upregulated. The expression level remained unchanged (false) for 94.24% (95,051) of the genes in the diploid and 90.68% (91,460) of the genes in the autotetraploid plants.

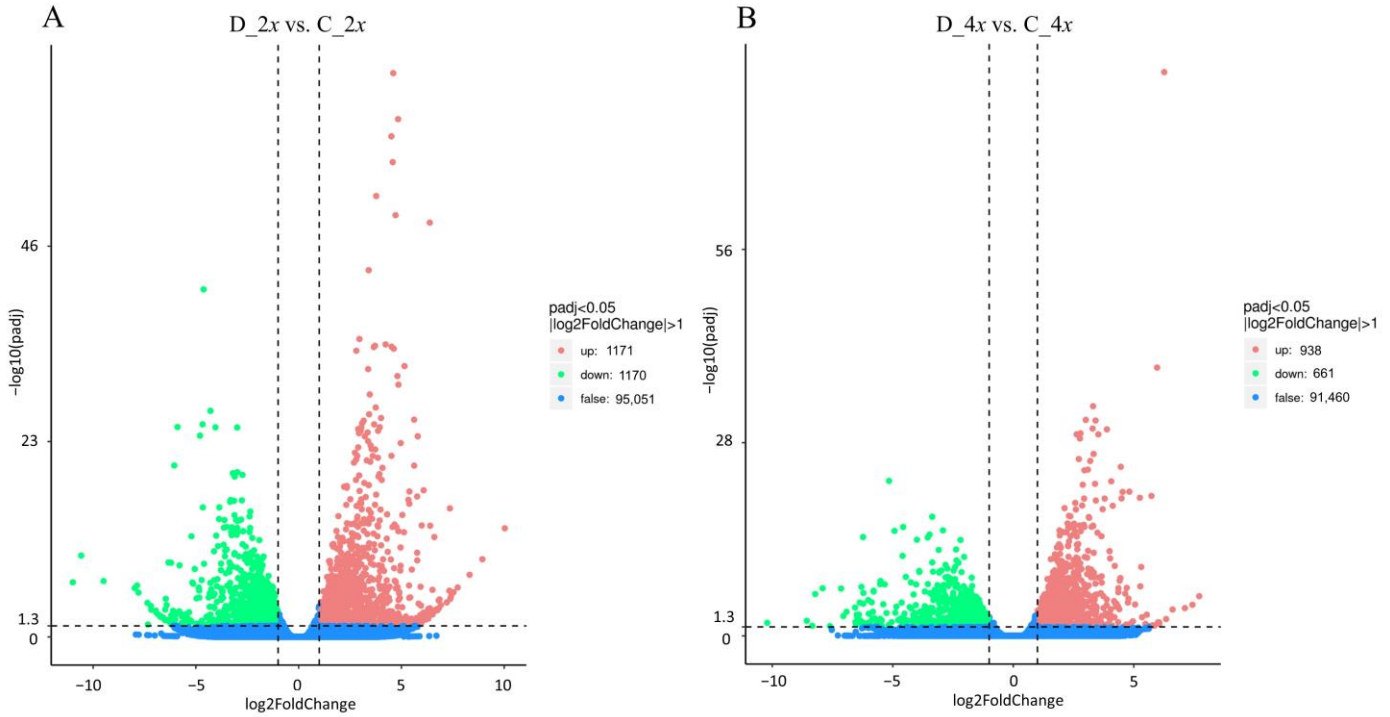


Figure 5. The number of differentially expressed genes (DEGs) in response to drought for diploid (**A**) and autotetraploid (**B**) *Hemerocallis* spp. cv. Trahlyta.

Figure 6 shows the enriched gene pathways identified by GO (functional categories grouping). Twenty functional pathways with reliable expression of specific genes were identified in the diploid and autotetraploid daylily leaves following drought stress. The diploid plants in stressed conditions were significantly expressed in four GO enrichments: carbohydrate metabolic process; transferase activity, transferring glycosyl groups; cellular protein modification process; and hydrolase activity, acting on glycosyl bonds. All were mainly downregulated. Autotetraploids were assigned to 10 GO enrichments. Three were the same as the diploids, and cellular protein modification processes were found only for the diploids. Oxidoreductase activity and DNA-binding transcription factor activity genes were mainly upregulated, while cytosol, lipid metabolic process, transferase activity, transferring acyl groups, external encapsulating structure, and cell wall genes were mainly downregulated.

GO enrichment analysis was used to conduct a thorough examination of the potential functions of DEGs using NovoSmart with a p-value < 0.05. These DEGs were assigned into 75 GO terms, including cellular component (20 subcategories), molecular function (22 subcategories), and biological process (33 subcategories) (Table S2). In the cellular component, most genes were significantly enriched in the photosynthetic membrane (GO:0034357), the photosystem (GO:0009521), and the cytosol (GO:0005829). Within the molecular function category, the predominantly enriched GO terms were hydrolase activity acting on glycosyl bonds (GO:0016798), tetrapyrrole binding (GO:0046906), transferase activity transferring alkyl or aryl (other than methyl) groups (GO:0016765), transferase activity transferring glycosyl (GO:0016757) and acyl (GO:0016746) groups, and carbon–oxygen lyase activity (GO:0016835). Only two subcategories, cellular carbohydrate metabolic process (GO:0044262) and cell wall organization (GO:0071555), were enriched in the biological process category. Among these enriched GO terms, two upregulated and 13 downregulated terms were screened out. Most of the upregulated subcategories

were associated with cellular components and molecular function. Four (GO:0034357; GO:0009521; GO:0016765 and GO:0016667) downregulated subcategories of diploid plants and seven (GO:0005829; GO:0030312; GO:0016757; GO:0016746; GO:0016835; GO:0044262 and GO:0071555) of autotetraploid plants were only assigned according to the ploidy of plants, while two (GO:0016798 and GO:0046906) appeared in both ploidy groups.

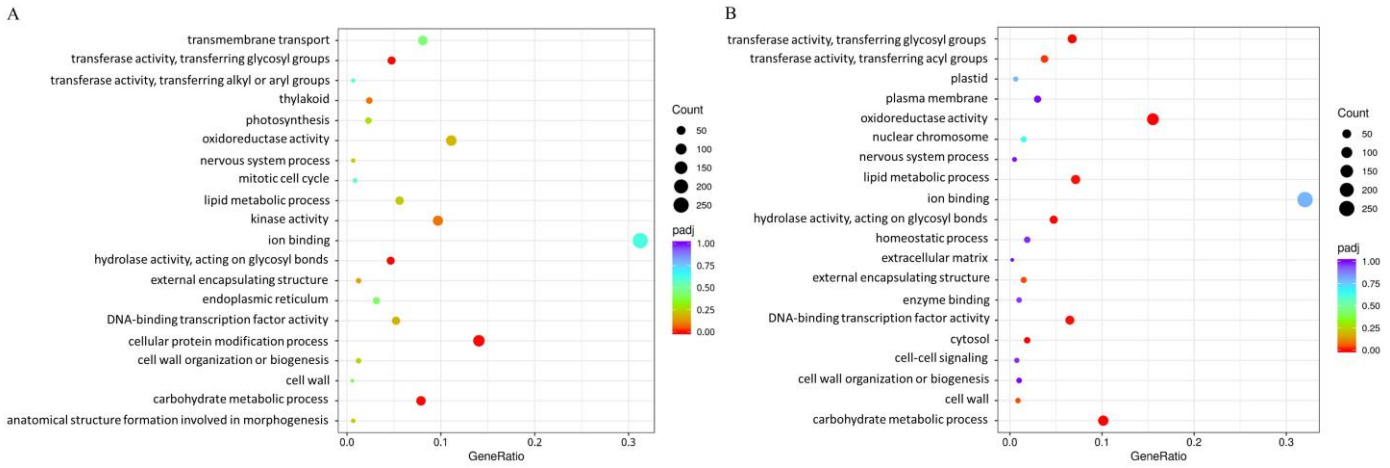


Figure 6. GO-enriched gene pathways involving differentially expressed genes (DEGs) following abiotic stress (drought) for diploid (**A**) and autotetraploid (**B**) *Hemerocallis* spp. cv. Trahlyta.

During drought stress in autotetraploid plants, more KEGG pathways were activated than in diploids—43 and 19, respectively (Figure 7). The most abundant appearance of DEGs in both cases was in the metabolic (ko01100) and biosynthesis of secondary metabolites (ko01110) pathways. The same number of upregulated genes activated mitogen-activated protein kinase (MAPK) signaling pathways in the diploid and autotetraploid plants, while the diploids showed more downregulated genes. Phenylpropanoids, having a wide variety of functions as structural and signaling molecules, showed a similar count of downregulated genes in the diploid and autotetraploid plants—12 and 11, respectively. The plant hormone signal transduction pathway was activated only in diploid plants with 26 downregulated genes and only four upregulated. In photosynthesis, the antenna proteins pathway was downregulated in diploid plants. Protein processing in the endoplasmic reticulum pathway was activated with 26 upregulated genes in autotetraploid plants. In the longevity regulating pathway, 14 genes in autotetraploid plants upregulated multiple species. Other biosynthesis pathways activated in autotetraploid plants are detailed in Figure 7B.

3.4. Protein–Protein Interactions (PPI) Network during Drought Stress

In plants, proteins do not function in isolation from cells but as part of a network. This study constructed a potential protein–protein interactions (PPI) network between overlapping differently expressed proteins (DEPs) using CYTOSCAPE to interact and link between different proteins. The network included 212 and 118 nodes and 326 and 166 edges in the diploid and autotetraploid plants, respectively. The red nodes represent 38 and 47 upregulated proteins, whereas the green nodes represent 86 and 29 downregulated proteins in the diploid and autotetraploid plants, respectively (Figure 8).

Figure 7. Pathway enrichment analysis of up- and downregulated genes (n = x) in abiotic stress (drought) for diploid (**A**) and autotetraploid (**B**) *Hemerocallis* spp. Cv. Trahlyta in the Kyoto Encyclopedia of Genes and Genomes (KEGG).

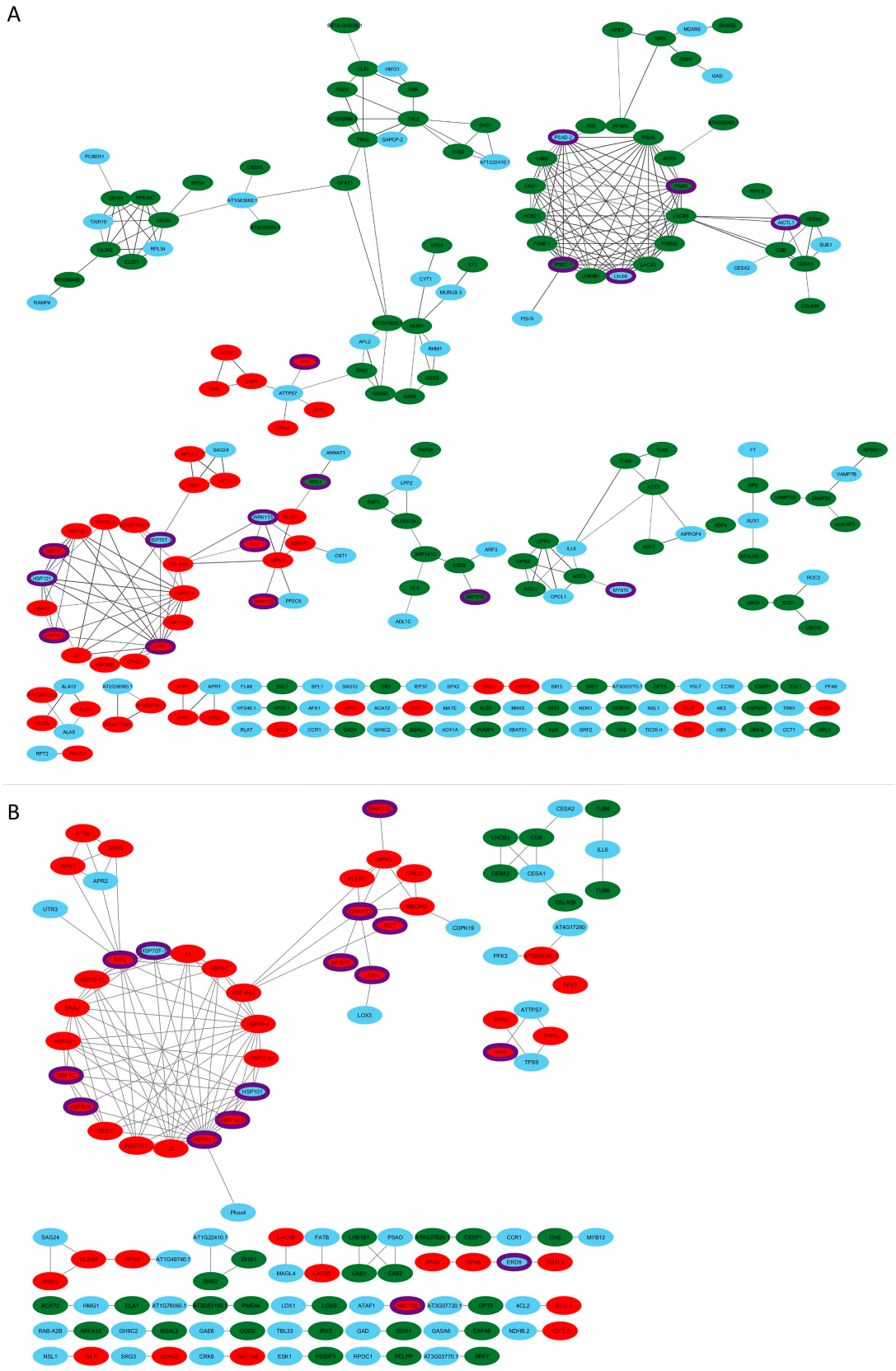


Figure 8. PPI network constructed with the up- and downregulated DEGs in diploid (**A**) and autotetraploid (**B**) plants during drought stress conditions. The red dots represent DEGs of upregulated miRNAs, the green dots represents DEGs of downregulated miRNAs, and the blue dot is the base. The purple border represents proteins and transcription factors linked with the drought stress response.

The autotetraploid plants have only one light-harvesting chlorophyll b-binding protein 3 and cellulose synthase proteins. In diploid plants, the heat-shock cluster has one downregulated protein, NDL1, which is an AGB1/AGG dimmer interacting protein responsible for water deficit. Figure 8 demonstrates that a large proportion of the proteins identified in both the diploid and autotetraploid plant leaves were associated with heat-shock proteins, while light-harvesting and chlorophyll a/b-binding associated proteins were essential in diploids but were not as active in autotetraploids.

Based on hierarchical protein interactions in diploid plants (Figure S2), PSAD-2 and Lhcb6 proteins were based on the complex with mostly downregulated proteins that resulted in PSBO-1 and PSAN synthesis. These proteins regulate photosystem subunits. In comparison, autotetraploid plants' hierarchical protein interactions (Figure S3) start with HSP70T-1 and HSP101 proteins, resulting in BIP2, HSP90.1, HSFB2A, and HSP18.2 downregulated protein synthesis. These proteins regulate luminar binding and heat-shock proteins. Similar interactions appear in diploid plants involving NUT, NDL1, and ARFB1B.

Protein enrichment was performed using CYTOSCAPE, which revealed 137 and 92 pathways in diploid and autotetraploid plants, respectively (Table S3). Based on GO analysis, functional distribution analysis was performed, and the DEPs identified in the drought conditions of diploid and autotetraploid plants were selected as the drought-induced proteins. In terms of the cellular component, branch cytoplasm, photosystem I, plastid, photosystem, and chloroplast terms were the top five most significantly enriched in both ploidy plants ($p < 0.05$).

4. Discussion

4.1. De Novo Transcriptome Sequencing of Daylily cv. Trahlyta

The adaptability of daylilies to different environmental conditions has made them a popular choice among gardeners. In recent years, the focus has shifted towards studying autotetraploid varieties. For this purpose, the ploidy level is altered by diploid genotypes [19,32,33]. Autotetraploid plants generally exhibit certain advantages over diploid plants. This multiplication of genetic material (resulting in a total of 44 chromosomes) often leads to larger flowers, stronger stems, and increased vigour compared to their diploid counterparts [36]. Due to the redundancy of genetic information, autotetraploids also demonstrate higher adaptability and resilience to environmental stresses [19]. The cell size of the autotetraploid genotypes of *Hemerocallis* is extensive in all cases studied [38]. The autotetraploid cv. Trahlyta (Tet. Trahlyta), converted by breeder J. Gossard, is extensively utilized in hybridization processes, improving the price and agronomic value of conversions. In this study, we investigated the advantages for drought adaptation of cv. Trahlyta with autotetraploid characteristics over diploids.

Transcriptome analysis is a powerful tool for investigating living organisms' gene expression patterns and regulatory networks [47]. The autotetraploid and diploid daylily analysis revealed significant gene expression differences between the variants. The *Hemerocallis* spp. genus does not have a reference genome yet. Therefore, de novo assembly of daylilies was conducted; a total of 237,886 transcripts were assembled, and 100,861 non-redundant unigenes were identified (Figure 2). Only 38% of them were annotated in the non-redundant (NR) database (Figure 3A). The matching degree with the monocot *Asparagus officinalis* was the highest among the homologous sequences of other plant species (40.7% of unigenes annotated according to NR) (Figure 3B). The characteristics of the daylily unigenes based on the GO, KOG, and KEGG pathways classifications in this study (Figure S1) show many similarities with other plant transcriptome studies [28,39,48].

We conducted a de novo assembly of daylily cv. Trahlyta transcriptomes after mRNA NGS from diploid and autotetraploid leaves under drought stress and normal irrigation conditions. The autotetraploids showed differences in terms of responding faster and more effectively in resistance to drought as a general interpretation of the experimental results relating to simulated stress in the greenhouse (Figure 1). The ploidy level of cv. Trahlyta was associated with increased cell size (cell size difference was 0.8), transcriptome diversity,

and altered gene expression patterns (Tables 1 and S1, Figures 4 and 5). These factors allow them to store more resources and use them more efficiently, which can be crucial during periods of limited availability. The ploidy level can contribute to improved characteristics; however, it is important to note that the specific advantages may vary depending on the environmental conditions in which the plants are growing.

4.2. Ploidy Effect on DEGs and Pathways

The quantity of expressed unigenes in response to drought stress (Figure 4) clearly demonstrated the high mobilization of diploid resources to abiotic stress in a diploid plant. Regardless of ploidy, 3840 unigenes for drought stress were identified in cv. Trahlyta samples. Diploids showed a higher number of differentially expressed genes (DEGs) in response to stress that were increased than autotetraploids. Diploids showed almost 1.5 times the number of DEGs as autotetraploids. Diploid genes were 1.3 times more upregulated and 1.8 times more downregulated than autotetraploids (Figure 5). This advantage enables autotetraploids to maintain optimal physiological functioning for extended periods, even in harsh or unpredictable environments. The presence of additional gene copies in autotetraploids can lead to altered gene expression patterns [19]. This phenomenon, known as dosage compensation, can result in the upregulation of beneficial genes or the downregulation of harmful ones [49].

Functional enrichment analysis highlighted pathways related to growth, development, and stress response as being significantly enriched in the autotetraploid variants. Autotetraploids showed differences in DEGs in GO (Figure 6, Table S2). The molecular function of the ion binding was activated in both cases during drought stress (at the unigenes level, it was greater in the diploid) (Figure 6). It is known that the ion-binding pathway in plants during drought stress is a complex and highly regulated process [50]. These regulations help maintain cellular homeostasis in plants during stress. Ion channels are dynamically regulated during drought stress to prevent excessive ion leakage. Increased levels of ROS under drought stress affect the ion-binding pathways. By expressing more activated and upregulated genes, diploid daylilies contain higher quantities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). In our study, most of the upregulated subcategories were associated with cellular components and molecular function (Table S2). It was indicated that cytosol (GO:0005829) and transferase activity transferring glycosyl groups (GO:0016757) may be more significant in autotetraploid daylilies than in diploid.

Moreover, four (GO:0034357; GO:0009521; GO:0016765 and GO:0016667) downregulated subcategories in diploid plants and seven (GO:0005829; GO:0030312; GO:0016757; GO:0016746; GO:0016835; GO:0044262 and GO:0071555) in autotetraploid plants were assigned only according to the ploidy of the plants. During drought stress, plant photosynthesis is adversely affected due to reduced stomatal conductance and limited CO_2 availability [51]. Diploid daylilies activated the photosynthetic modification pathways to adapt to water-deficient conditions, and all the unigenes were downregulated. Protein processing in the endoplasmic reticulum pathway was activated with 26 upregulated genes in autotetraploid plants that prevented cells from dying by apoptosis. In autotetraploid plants, 14 genes were upregulated in the longevity regulating pathway, including those involved in multiple species, transactivating genes involved in resistance to oxidative stress and energy metabolism, DNA damage repair, glucose metabolism, autophagy, and protein chaperone protection.

The autotetraploid plants showed an overall upregulation of gene expression compared to the diploid plants in the activated KEGG pathways (Figure 7), giving them an advantage over the diploid genotype. The diploids showed more downregulated genes than upregulated ones, and the count of downregulated genes in the diploids was higher than in the tetraploids. By fine-tuning the gene expression in various pathways, autotetraploids can enhance their adaptation to environmental changes. Tetraploid plants' ability to withstand abiotic stresses can have an impact on their capacity to transport water, the

way they deal with water shortages and regulate photosynthesis, their morphological alterations and osmolyte content, their ability to control stomata function and regulate transpiration rates, and their ability to control ROS and ABA homeostasis [17–23]. Most abundant with DEGs in both cases were the KEGG metabolic (ko01100) and biosynthesis of secondary metabolites (ko01110) pathways (Figure 7). Drought stress disrupted the physiological and biochemical processes within plant cells, leading to reduced growth [52]. However, daylilies have developed intricate metabolic and biosynthesis of secondary metabolites pathways to counteract the adverse effects of drought stress and sustain their survival. Mitogen-activated protein kinase (MAPK) signaling pathways were activated in diploid and autotetraploid plants with the same number of upregulated genes, while diploids showed more downregulated genes. The MAPK signaling pathway is essential for appropriate cellular responses and adaptation to drought stress in autotetraploid plants and was less destructive than in the diploid plants [52].

The phenylpropanoids biosynthesis pathway, having a wide variety of functions as structural and signaling molecules, showed a similar count of downregulated genes in diploid and autotetraploid plants—12 and 11, respectively. Drought stress in plants can be managed by regulating genes in the phenylpropanoids biosynthesis pathway [53].

Plant hormone signal transduction pathways enable plants to cope with drought stress. The intricate network of hormones, including abscisic acid (ABA), cytokinins, and jasmonic acid (JA), allows plants to modulate their physiology and allocate resources towards stress response mechanisms [54]. Understanding these signaling pathways can aid in developing strategies for enhancing plant resilience to drought stress, ensuring sustainable crop production in arid regions. In our study, the plant hormone signal transduction pathway was deactivated only in the diploid genotype (Figure 7). During drought stress, plant hormones, such as abscisic acid (ABA), are important in modifying the ion-binding pathways. ABA signaling triggers the closure of ion channels, reducing ion flux, and preserving cellular water content. Plants detect water deficiency at their roots and deliver this information to their shoots, which generate abscisic acid (ABA) in their leaves [55]. To prevent water loss, protein kinases, such as MAPKs, detect ABA influx in guard cells that regulate stomatal closure [55]. The JA signaling pathway intersects with ABA signaling. Coordinating plant responses to drought stress and in response to drought, the JA levels in diploids increased, promoting the expression of genes involved in stress tolerance and defence mechanisms. Cytokinins, known for their role in cell division and growth, also played a role in drought stress response. In our case, the cytokinin levels decreased during drought stress in diploid plants, leading to decreased cell division and growth. Consequently, cytokinin signaling was downregulated in the diploids under drought conditions, diverting resources to stress response mechanisms.

4.3. Drought Resistance in Tetraploid Daylilies in PPIs Networks

The constructed protein–protein network revealed key hubs and modules associated with the differentially expressed genes, providing further insights into potential protein interactions and regulatory networks [56]. These interactions can act as key regulatory points in cellular processes, providing a basis for future studies investigating protein function and its roles in polyploidization. Calcium-dependent protein kinases (CDPKs), mitogen-activated protein kinases (MAPKs), HD-zip/bZIP, AP2/ERF, NAC, MYB, and WRKY are examples of regulatory gene products that can induce changes in plants through modulating signal transduction pathways (Figures 8, S2 and S3, Table S3). Summarizing the data, it was found that the autotetraploid plants of daylily have a broader potential for adaptation to abiotic stress. We discovered four protein–protein interaction networks in autotetraploids and seven in diploids, alongside significantly enriched GO terms and KEGG pathways. Heat-shock proteins were crucial in coordinating drought stress responses in the PPI network of daylilies. Chlorophyll a/b-binding proteins and light-harvesting chlorophyll b-binding proteins were the most downregulated, exhibiting high connectivity

in diploid plants. Along the diploid upregulated central cluster were proteins related to the photosynthesis process and ROS production.

In the molecular function category, proteins with catalytic activity, ion binding, protein domain-specific binding, chlorophyll-binding, and small molecule binding terms were the most positively regulated in the diploid plants (Table S3). Autotetraploid plants represented the top five in catalytic activity, adenylyl-sulfate reductase (glutathione) activity, 3-deoxy-7-phosphoheptulonate synthase activity, adenylyl-sulfate reductase activity, and phosphoadenylyl-sulfate reductase (thioredoxin) activity terms. The most highly enriched proteins in the biological process were those involved in the reaction to abiotic stimulus and the metabolism of small molecules, carboxylic and oxalic acids, and organic acids. [56]. In response to drought stress, plants dynamically rearrange their protein interaction networks to activate stress-responsive genes and initiate adaptive responses. These interactions involve a multitude of proteins, including transcription factors, protein kinases, and enzymes, forming complex networks that regulate stress tolerance (Figures S2 and S3). Key proteins involved in the drought stress response, RD26 [50] and DREB2A [55], were not found in our study. In daylilies, TPPI (both ploidy groups), AtCTL1 (only in diploids), ERD9, NAC102, and LOX4 (only in autotetraploids) were observed. Transcription factors modulated the PPIs networks. WRKY transcription factors have been found to interact with various proteins involved in the stress response pathways in daylilies. The transcriptional co-activator MBF1c was also found in the diploid plants. Drought stress-responsive protein kinases play a vital role in activating downstream signaling events. SnRK2 kinases phosphorylate downstream targets, triggering stress responses in plants [57]. Abiotic stresses induce NAC102, LOX4 lipoxygenase 4, and trigger a defence response, and an ERD9 early response to dehydration 9 was found in autotetraploid daylilies.

Understanding the essential proteins, transcription factors, and signaling pathways involved in the PPI network can aid in developing strategies for enhancing drought tolerance in *Hemerocallis* spp. Further research in this field will deepen our understanding of plant stress responses and enable the development of stress-tolerant plant varieties.

5. Conclusions

Chromosome doubling in ornamental plants, as shown by our study in daylilies (*Hemerocallis* spp.), has great potential to increase tolerance to abiotic stress. By activating alternative signaling pathways, autotetraploid plants can respond faster and more efficiently to adverse drought conditions. Analysis of the transcriptome and the protein interaction network of autotetraploid and diploid daylily cv. Trahlyta revealed significant differences in the gene expression patterns in plants of different ploidy. This study paves the way for further investigations of autopolyploidy's genetic and molecular basis in daylily plants. It reveals the value of autotetraploid forms for the development of new cultivars under changing climate conditions to create more drought-tolerant daylily cultivars.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9111194/s1, Figure S1: Characteristics of daylily unigenes based on Gene Ontology (GO) categorization (A), Clusters of Orthologous Group (KOG) Function classification (B), Kyoto Encyclopedia of Genomes (KEGG) pathways classification (C); Figure S2: PPI hierarchical network constructed with the up- and downregulated DEGs in diploid plants during drought stress conditions. The red dots represent DEGs of upregulated miRNAs, the green dot represents DEGs of downregulated, and the blue dot is the base; Figure S3: PPI hierarchical network constructed with the up-and downregulated DEGs in autotetraploid plants during drought stress conditions. The red dots represent DEGs of upregulated miRNAs, the green dot represents DEGs of downregulated, and the blue dot is the base; Table S1: Differentially expressed genes (DEGs) with up and downregulation during drought stress in diploid (2*x*) and autotetraploid (4*x*) daylily cv. Trahlyta; Table S2: GO classification of DEGs identified in diploid (2*x*) and autotetraploid (4*x*) up- and downregulated genes during water deficit in daylily leaves using NovoSmart with p-value <0.05 indicated in green or red colors; Table S3: Enrichment of PPI in diploid (2*x* sheet) and autotetraploid (4*x* sheet) daylily plants during drought stress.

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