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# Molecular Responses to Drought and Waterlogging Stresses of Kiwifruit (*Actinidia chinensis* var. *deliciosa*) Potted Vines

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**Abstract:** Environmental extremes, such as drought and flooding, are becoming increasingly common, resulting in significant crop losses. The aim of the present study was to understand the molecular response induced by drought and waterlogging conditions, and to link these responses to the physiological adaptation of plants. For this purpose, leaf RNA expression was analyzed in potted kiwifruit plants by Illumina Next Generation Sequences. Stressed plants showed an impairment of all physiological parameters (leaf-gas exchange and stem-water potential) with a more evident effect in waterlogging condition than in drought condition. However, the impact on the transcriptome in waterlogged plants was less intense than in drought stress. Drought affected several metabolic pathways, among which "plant hormone signal transduction", "protein processing in endoplasmic reticulum", and "mitogen-activated protein kinase signaling pathway" were the most representative in terms of number of genes involved. The genes involved in the biosynthesis of phenylpropanoids were positively influenced by both drought and waterlogging. Finally, waterlogging stimulated secondary metabolisms by upregulating the genes responsible for the biosynthesis of terpenoids and flavonoids, such as stilbenoids. The obtained results show that the two contrasting stress conditions share several common physiological responses and molecular mechanisms.

**Keywords:** water stress; RNA-seq; gene expression; stem-water potential; leaf-gas exchange; metabolic pathway



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## 1. Introduction

Climate change, mainly due to global warming, is increasing the frequency and intensity of extreme weather events leading to conditions of drought or waterlogging in orchards. Both these conditions are detrimental to plants, resulting in a reduction of plant performance and yield [1]. Drought stress occurs in plants when there is low soil-water availability for a prolonged period. On the other hand, an excess of water in the soil leads to the saturation of soil pores, reducing oxygen availability and impairing gas exchange in tissues. In response to water deficit, roots' anatomy and architecture change to enhance water- and nutrient-uptake, and to provide mechanical strength to the root system [2]. In addition, plants reduce stomatal and cuticular conductance and evaporative surfaces [3], leading to a substantial reduction of net photosynthesis [4]. Moreover, drought stress is associated with the reduction of water potential that is lowered by osmotic adjustment and synthesis of low molecular-weight proteins, amino acids [5], and compatible sugars [6] that protect plants from damage caused by water deficiency [7]. Phytohormones play a crucial role in modulating the negative effects of drought stress in fruit crops [8], namely abscisic acid (ABA), a key regulator of plant adaptation to drought stress. The accumulation of ABA in *Arabidopsis thaliana* (L.) Heynh. activates downstream signals leading to stomatal closure, thereby limiting excessive water loss in plants [9]. Excessive water in the soil may lower

oxygen to 1–5% percent, resulting in hypoxia conditions for roots [10], which negatively affects plant growth and fruit production [11].

At the whole-plant level, hypoxia causes a reduction in photosynthesis, stomatal conductance, transpiration and leaf-water potential [12]. Moreover, leaf-chlorophyll concentration, hormonal imbalances, and impairment in the uptake of water and minerals have also been observed [13]. These responses can change energy consumption, cellular metabolism, and gene expression with effects on apricot growth and development [14]. Symptoms of root hypoxia stress often do not occur until several days or weeks after the onset of waterlogging [11], and comprise leaf chlorosis, curling, defoliation, leaf abscission, marginal browning, necrosis, yellowing, root rotting, wilting, and fruit abscission [15].

In temporary stress conditions, plants are able to recover with a timing that depends on species, soil [16], and severity of the stress, otherwise prolonged waterlogging, as well as drought, result in the death of most cultivated plants. However, kiwifruit plants are extremely sensitive to waterlogging stress, and plant growth is reduced even after one day of exposure to anoxic conditions [17]. When roots were exposed to more than four days of waterlogging, vine growth did not recover even after aeration was restored to the roots [17]. In kiwifruit, stomata closure is one of the first symptoms of waterlogging [17]. In vines exposed to waterlogging for more than three days, no recovery of stomatal activity was observed [17]. Other studies indicate that in *Actinidia chinensis* var. *deliciosa* waterlogging damage became irreversible between 7 to 11 days of continuous exposure to an excess of water [18].

Symptoms of waterlogging and drought stress in leaves are similar, and thus they can mislead growers that often keep watering waterlogged plants to recover them from an incorrectly identified drought stress. In addition to the study of the physiological response to water stress, the analysis of gene expression in response to stress shed light on how plants adapt to the environment, providing useful information for developing tools to mitigate the stress in orchard conditions, or to induce plants with tolerance. Since roots are the first organ exposed to waterlogging and drought stress, several experiments focused on the study of gene expression in kiwifruit rootstocks [19,20]. However, elucidating the leaf response may provide a further level of understanding for adaptation to water stress (e.g., stomata closure and photo-dissipation) occurring in the canopy. Furthermore, since leaves are easier and faster to sample and process than roots, the transcriptome study at leaf-level may open new possibilities for an early detection of stress by identifying gene markers activated prior to symptoms' occurrence, and allow us to discriminate drought from waterlogging response directly in field conditions. The results obtained by transcriptome sequencing in *Prunus* spp. [21] and in *Citrus* sp. [13] in response to root hypoxia gave valuable information on species and genotype tolerance to drought and waterlogging, even highlighting common mechanisms of response mainly involving carbon metabolism, nutrient uptake and transport, and hormone synthesis/signaling. Despite several studies on kiwifruit vines, responses to waterlogging and drought stresses have focused on the transcriptional changes in roots [19,20], and knowledge of the responses in leaves is limited.

The aim of the present experiment was to associate drought and waterlogging physiological responses to transcriptional changes, highlighting the molecular mechanism underling kiwifruit plants' responses. We hypothesized that genes responding to waterlogging are different from the genes affected by drought stress in comparison to control leaves.

## 2. Materials and Methods

### *2.1. Plant Material and Treatments*

The study was carried out in summer 2022 at the experimental station of the Department of Agricultural and Food Science of the University of Bologna (Italy), on the north side of the metropolitan area of the city, on 12, one-year-old, potted, self-rooted 'Hayward' (*Actinidia chinensis* var. *deliciosa* (A.Chev.)) plants. The plants were transplanted on March 2022 into 70 L pots filled with clay–loam soil and trained to a single cane.

Irrigation started at the beginning of May in order to maintain soils at field-water capacity. Starting from 17th June, four pots were inserted into plastic containers (40 cm large, 60 cm long, and 30 cm high) filled with tap water (waterlogging = WL), so that plants were immersed in 30 cm of water. Plants were regularly irrigated and maintained in the waterlogging status during the entire vegetative season. On 25th July, four of the remaining plants were stressed by stopping irrigation for 2 days (drought = D); control pots were regularly irrigated by returning the water lost by evapotranspiration.

Leaf-gas exchange measurements and transcriptome analyses of leaf samples were conducted on 27th July (10 days after WL and 2 days after D).

### *2.2. Plant Physiological Measurements*

Leaf-gas exchange was measured on one well-lit, mature, fully expanded leaf per plant, using an open circuit gas-exchange system equipped with an LED light source (LI-6400, LI-COR, Lincoln, NE, USA). In detail, the leaf's net photosynthesis rate (Pn), stomatal conductance (gs), intercellular $CO_2$ concentration (Ci), and transpiration rate (E) were measured. Light intensity inside the chamber was maintained at the natural PAR (photosynthetically active radiation) values experienced by the leaves immediately before the measurements.

Stem water potential (Ψw) was measured with a pressure chamber on one leaf per plant, previously wrapped with aluminum foil and enclosed in a plastic bag at least one hour before excision to allow moisture equilibration with the soil [22,23]. Pressure was gently applied using a pump-up pressure chamber (PMS Instrument Company, Albany, OR, USA).

Physiological data were statistically analyzed in a completely randomized experimental design with 4 biological replicates per treatment; analysis of variance was performed for these data, and when they showed statistically significant ($p \leq 0.05$) effects, means were separated by the Student Newman–Keuls (SNK) test.

### *2.3. RNA Isolation and Sequencing*

The same leaf used for gas-exchange measurements was sampled, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Leaves were ground in liquid nitrogen, and RNA was extracted for each biological replication using the Spectrum Plant Total Rna Kit (Merck KGaA, Darmstadt, Germany) following the manufacturer's instructions. Illumina sequencing (San Diego, CA, USA) and statistical analysis of RNA-seq data were performed by Novogene Co., Ltd. (Cambridge, UK) after checking total RNA quantity and quality on an Agilent 5400 Fragment Analyzer System (Santa Clara, CA, USA).

Sequencing libraries were constructed with the Novogene NGS RNA Library Prep Set (PT042)* for Illumina and sequenced on an Illumina NovaSeq 6000 platform with PE150. RNA-seq raw reads were processed to obtain high-quality clean reads by removing the adapter, reads with "N" > 10%, and reads when low quality nucleotides (base quality less than 5) constituted more than 50 percent of the read.

Clean reads were then mapped onto the kiwifruit reference genome and the manually curated *A. chinensis* var. *chinensis* Red5 genome [24] with the HISAT2 algorithm (v 2.0.5 [25]). Transcripts were assembled by StringTie software (v1.3.3) and differentially expressed gene (DEG) analysis was performed using the DESeq2 R package [26]. The resulting *p*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with a corrected *p*-value (padj) below 0.05 were considered differentially expressed.

The enrichment analysis of DEGs was performed to find out which biological functions or pathways were significantly associated with them. ClusterProfiler 3.8.1 [27] was used for enrichment analysis, including GO (Gene Ontology, http://www.geneontology.org/, accessed on 31 March 2023) and KEGG (Kyoto Encyclopedia of Genes and Genomes, (http://www.genome.jp/kegg/, accessed on 31 March 2023)) enrichment. The GO terms and the KEGG pathways with padj < 0.05 were considered significantly enriched.

Transcriptome data were deposited in NCBI databases under the accession number PRJNA1076811.

## 3. Results

### *3.1. Physiological Response to Drought and Waterlogging*

After 10 days of waterlogging or 2 days of drought, leaves showed a strong reduction of net photosynthesis with a more evident effect of waterlogging than drought (Table 1). Both types of water stress induced a reduction in stomatal conductance and transpiration in comparison to the control, while intercellular $CO_2$ concentration was only significantly increased by waterlogging plants in comparison to drought, and the control showed similar values (Table 1). Leaves for both treatments had higher stem-water potential than control leaves (Table 1).

**Table 1.** Effect of soil water availability on the leaf's net photosynthesis rate ($P_n$-µmol $CO_2$ $m^2$ $s^{-1}$), stomatal conductance ($g_s$-µmol $H_2O$ $m^2$ $s^{-1}$), intercellular $CO_2$ concentration ($C_i$-µmol $CO_2$ $m^2$ $s^{-1}$), transpiration rate (E-µmol $H_2O$ $m^2$ $s^{-1}$), and stem-water potential (Ψw-MPa).

| TREATMENT | Pn | gs | $C_i$ | E | Stem $\Psi_w$ |
|---|---|---|---|---|---|
| Control | 13.2 a | 0.174 a | 244 b | 4.09 a | −0.438 a |
| Waterlogging | 0.155 c | 0.063 b | 480 a | 0.787 b | −0.563 a |
| Drought | 3.64 b | 0.036 b | 266 b | 1.27 b | −1.18 b |
| Significance | *** | ** | * | *** | *** |

*, **, ***: effect significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Values in the same column followed by the same letter are not statistically different according to the Student Newman–Keuls test ($p \leq 0.05$).

### *3.2. Overview of Sequencing Data and Statistical Summary of DEGs*

Illumina RNA-Seq generated 73.6 million raw reads and 72.8 million clean reads on average for each leaf sample. The Q30 percentages ranged from 93.5 to 94%, while the GC contents were around 45.4% and 46.2%. The mapping of the clean reads on the reference genome using HISAT2 v2.0.5 software allowed us to map 82% of the clean reads, and 78.5% of them mapped onto a unique position in the genome. In total, 33,044 genes were analyzed, and the gene expression was estimated by Fragments Per Kilobase of transcript sequence per Millions of base pairs sequenced (FPKM). In a first analysis of gene expression (FPKM > 1 as the threshold to determine whether one gene is expressed; padj < 0.05), it was observed that more than 66% of annotated genes were commonly expressed among the three treatments (Figure 1); the number of shared expressed genes between drought and the control was low, while it was four-fold higher between the control and waterlogging. Drought induced the highest number of specifically expressed genes, while in waterlogging a lower number of unique, differentially expressed genes was detected (Figure 1), suggesting a less differential molecular response for this treatment.

This result was further confirmed when differentially expressed genes (DEG) were analyzed. Only 260 genes (0.8% of all annotated transcripts present in the reference genome) were differentially expressed between waterlogging and the control (Figure 2; log2Fold Change $\geq$ 2), while a stronger effect was detected following drought, with 5195 (15.7% of all annotated transcripts) DEGs compared to the control (Figure 2). The number of downregulated DEGs between the control and waterlogging treatments was lower than those upregulated (Figure 2). Conversely, in the comparison between the control and drought, a higher number of downregulated than upregulated DEGs (Figure 2) were in evidence.

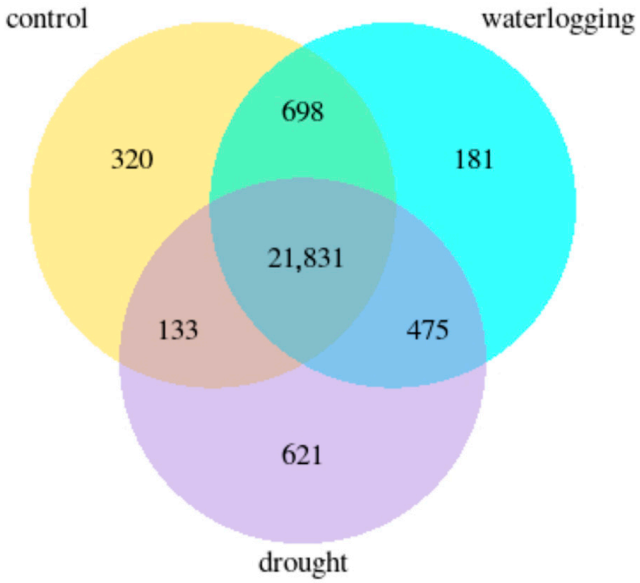



**Figure 1.** Venn diagram of leaves' expressed genes for each treatment (FPKM > 1).

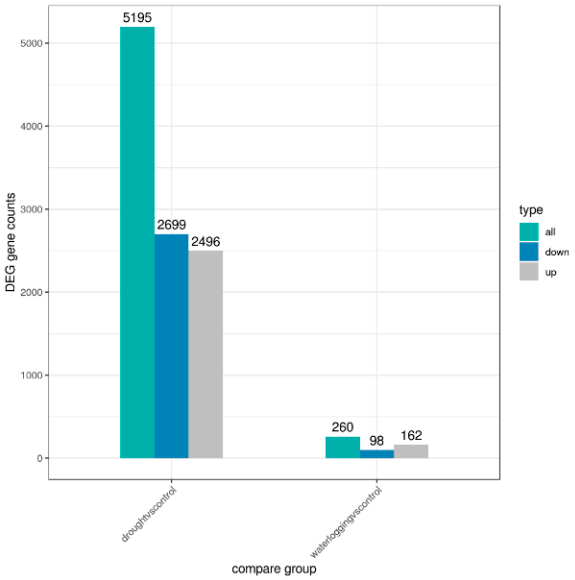
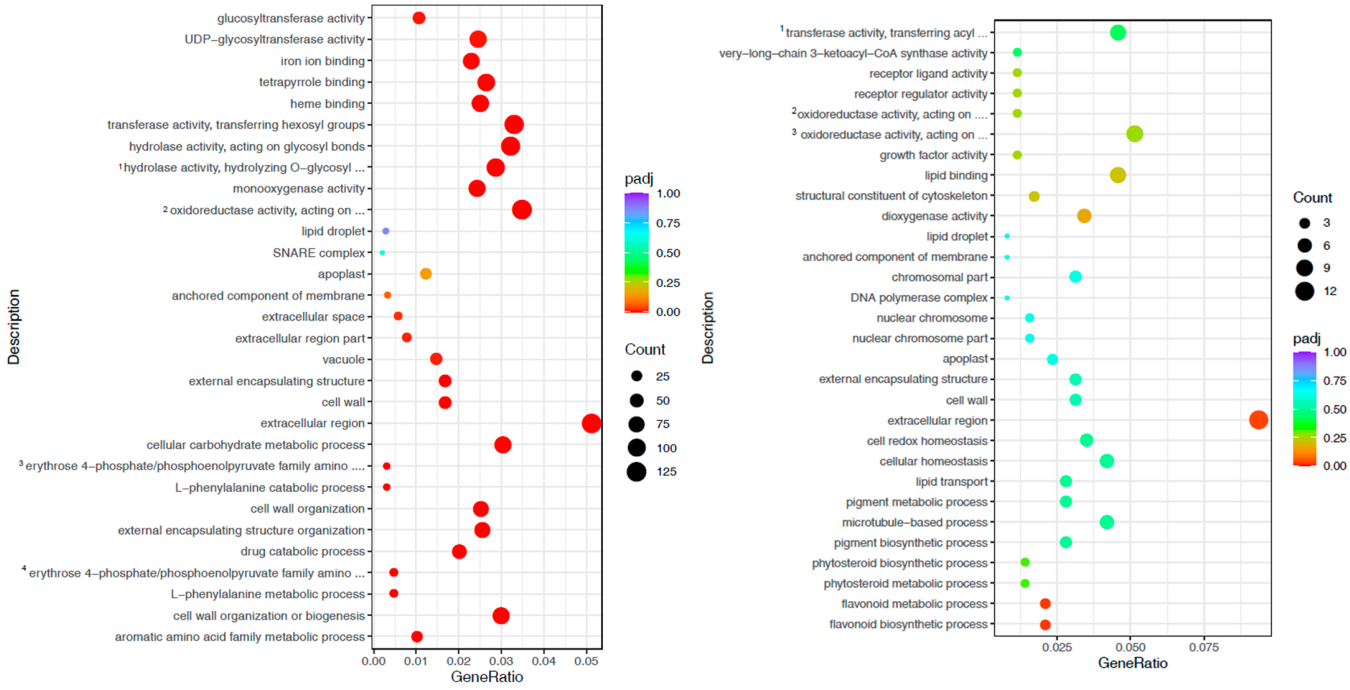



**Figure 2.** Differentially expressed genes (DEGs) between control and drought (left), and waterlogging and control (right). All = number of all DEGs; down = number of DEGs that were downregulated; up = number of DEGs that were upregulated (padj < 0.05).

### *3.3. Gene Ontology and Kyoto Encyclopaedia of Genes and Genome-Enrichment Analyses*

In the Gene Ontology (GO), the number of main functional categories of genes affected by waterlogging (Figure 3a) were lower in comparison to those observed in drought (Figure 3b). Among the shared GO classes, the "extracellular region", which included genes that were not attached to the cell surface, was significantly affected by both stresses and resulted in the greatest representation in the waterlogging vs. the control (12 genes differentially expressed: 6 up- and 6 downregulated), and was one of the most represented in the drought vs. control comparison (125 genes differentially expressed: 55 up- and 70 downregulated).



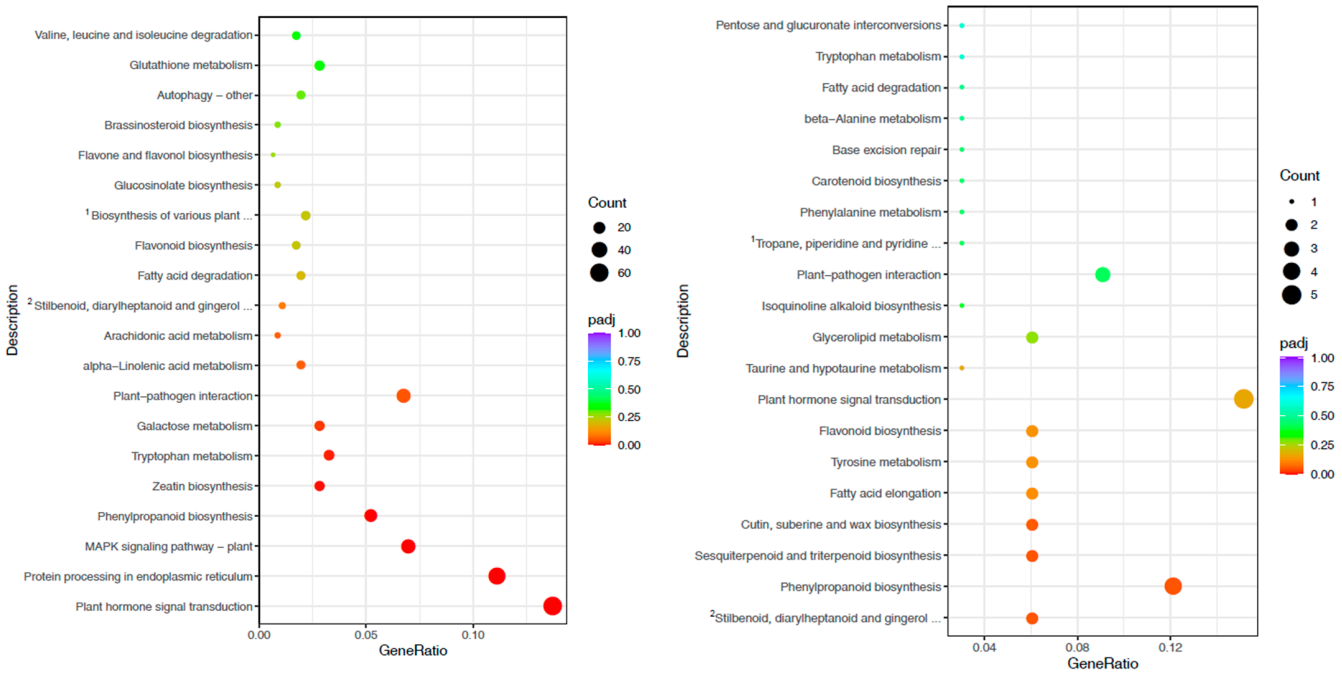

(**a**) (**b**)

**Figure 3.** Gene Ontology (GO) enrichment scatter plot of top 30 significantly enriched terms in the GO enrichment analysis. (**a**). drought vs. control ([1] hydrolase activity, hydrolyzing O-glycosyl compounds; [2] oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen; [3] erythrose 4-phosphate/phosphoenolpyruvate family amino acid catabolic process; [4] erythrose 4-phosphate/phosphoenolpyruvate family amino acid metabolic process); (**b**). waterlogging vs. control ([1] transferase activity, transferring acyl groups other than amino-acyl groups; [2] oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water; [3] oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen). Gene ratio = ratio between the number of differentially expressed genes in each GO term and all differentially expressed genes that can be found in the GO database; padj = adjusted *p*-values $\leq 0.05$ are significant; count = the number of differentially expressed genes concerning this term.

Related to the "cellular component", other functional categories linked to the cellular integrity, such as "cell wall" and "external encapsulating structure", were represented in both stress conditions, but were differentially expressed in comparison to the control only in drought, where 41 genes were differentially expressed (7 up- and 34 downregulated, for both cellular components). Unlike drought stress, waterlogging significantly affected the biological process involving flavonoid metabolism and biosynthesis, with three genes differentially expressed (Figure 3b). Conversely, drought was able to modify several genes involved in carbohydrate and amino acid metabolisms, but also genes involved both in the biological process and molecular function. The highest proportion related to the photosynthetic process were iron, tetrapyrrole, and heme, binding with 84 (42 up and 42 down), 97 (51 up and 46 down), and 92 (52 up and 41 down) DEGs, respectively (Figure 3a). Moreover, drought stress also showed a different gene expression for the cellular carbohydrate metabolic process that was mainly downregulated (62 genes versus 27 upregulated) and for several biological processes that induce changes in cellular organization (cell wall organization and biogenesis, vacuole, etc.).

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis allows a detailed identification of significantly enriched metabolic pathways or signal transduction pathways associated with differentially expressed genes between waterlogging and drought, both in comparison to the control. Several metabolic pathways were upregu-

lated by drought (Figure 4a), among which "plant hormone signal transduction", "protein processing in endoplasmic reticulum", and "mitogen-activated protein kinase (MAPK) signaling pathway–plant" were the most representative in terms of number of genes involved (63, 51 and 32, respectively). The biosynthesis of phenylpropanoid was positively affected from both drought and waterlogging, with a more evident effect as consequence of water deficit (43 genes) than excess (94 genes). In addition, waterlogging stimulated secondary metabolisms by upregulating the genes responsible for the biosynthesis of terpenoids, stilbenoids, and triterpenoids (Figure 4b).




(**a**) (**b**)

**Figure 4.** Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment scatter plot of top 20 significantly upregulated enriched terms in the KEGG enrichment analysis. (**a**). drought vs. control ([1] biosynthesis of various plant secondary metabolites; [2] stilbenoid, diarylheptanoid and gingerol biosynthesis) (**b**). waterlogging vs. control ([1] tropane, piperidine and pyridine alkaloid biosynthesis; [2] stilbenoid, diarylheptanoid and gingerol biosynthesis). Gene ratio = ratio between the number of differentially expressed genes in each pathway and all differentially expressed genes that can be found in the KEGG database; padj = adjusted *p*-values $\leq 0.05$ are significant; count = the number of differentially expressed genes concerning this term.

Both drought and waterlogging induced the downregulation of genes involved in the biosynthesis of flavonoids (Figure 5). Drought stress also led to the down regulation of genes responsible for starch and sucrose metabolism (37), carbon fixation (20), co-factor biosynthesis (49), and amino sugar and nucleotide sugar metabolism (27). Similarly, the biosynthesis of amino acids (57), in particular of phenylalanine, tyrosine, and tryptophan (18), was negatively affected by drought (Figure 5a). In waterlogging only, the downregulation of the biosynthesis of unsaturated fatty acids was observed, and it was limited to a small number of genes involved (29); no more genes were differentially downregulated for this treatment (Figure 5).

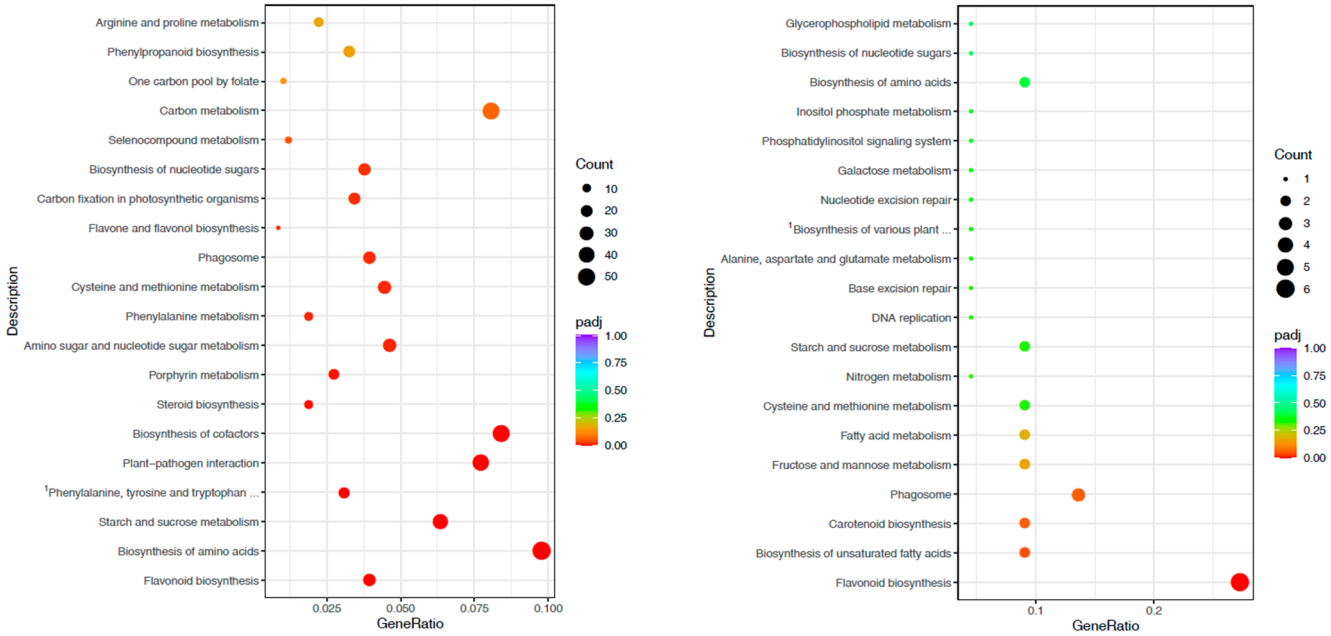



**Figure 5.** Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment scatter plot of top 20 significantly downregulated enriched terms in the KEGG enrichment analysis. (**a**). drought vs. control ([1] phenylalanine, tyrosine and tryptophan biosynthesis); (**b**). waterlogging vs. control ([1] biosynthesis of various plant secondary metabolites). Gene ratio = ratio between the number of differentially expressed genes in each pathway and all differentially expressed genes that can be found in the KEGG database; padj = adjusted *p*-values $\leq 0.05$ are significant; count = the number of differentially expressed genes concerning this term.

### *3.4. Pathways Involved in Drought and Waterlogging Stress Response*

As reported above, several biological process, cellular components, and molecular functions were influenced by the water stress conditions considered. However, in order to shed light on the gene changes that led to the physiological response (Table 1), we focused on the following metabolic pathways: carbon and sugar metabolism, carbon fixation, photosynthesis, hormone signaling, and the secondary metabolites pathway.

#### 3.4.1. Effect of Drought and Waterlogging on Genes Involved in Secondary Metabolites Pathways

Several pathways involved in secondary metabolite production were differentially regulated under drought and waterlogging. The biosynthesis of flavonoids showed several differentially expressed genes both in drought (31) and waterlogged plants (8; Figure 6). In both water conditions, the number of detected upregulated genes was lower in comparison to the downregulated ones. In particular, both stresses caused the upregulation of two genes involved in stilbene biosynthesis (two isoforms of spermidine hydroxy cinnamoyl transferase, CEY00_Acc29568, CEY00_Acc10082, and CEY00_Acc00879) and the downregulation of several genes involved in the synthesis of different flavonoid compounds, namely chalcone isomerase (CEY00_Acc03848, CEY00_Acc27670, and CEY00_Acc03638), dihydroflavonol 4-reductase (CEY00_Acc19353 and CEY00_Acc01005), anthocyanidin synthase (CEY00_Acc28876 and CEY00_Acc16762), and anthocyanidin reductase (CEY00_Acc09639 and CEY00_Acc17426; Figure 6); while only in drought-stressed plants, there was found a downregulation in chalcone synthase (CEY00_Acc00260, CEY00_Acc24966, CEY00_Acc02004, and CEY00_Acc08970), and flavonol synthase (CEY00_Acc24372 and CEY00_Acc11493) genes. Similar to what was observed for flavonoids, phenylpropanoid biosynthesis (Figure S1A) was also affected by drought, with 43 different transcripts in comparison to the control, while only four differentially expressed genes were detected following waterlog-

ging (Figure S1B). In particular, all the genes detected in waterlogging were shared with drought and were upregulated—these were spermidine hydroxycinnamoyl transferase, 5-O-(4-coumaroyl)-D-quinate 3′-monooxygenase, and two members of the peroxidase family, which together led to the stimulation of the production of lignin. Moreover, several differentially expressed genes in comparison to the control and involved in other secondary metabolite biosynthesis were found following drought, namely "stilbenoid, diarylheptanoid and gingerol biosynthesis" (7 genes), "biosynthesis of various plant secondary metabolites" (18 genes), and "flavone and flavonol biosynthesis" (8 genes). On the other hand, waterlogging induced the upregulation of two genes involved in the biosynthesis of sesquiterpenes and triterpenoids (Figure S2).

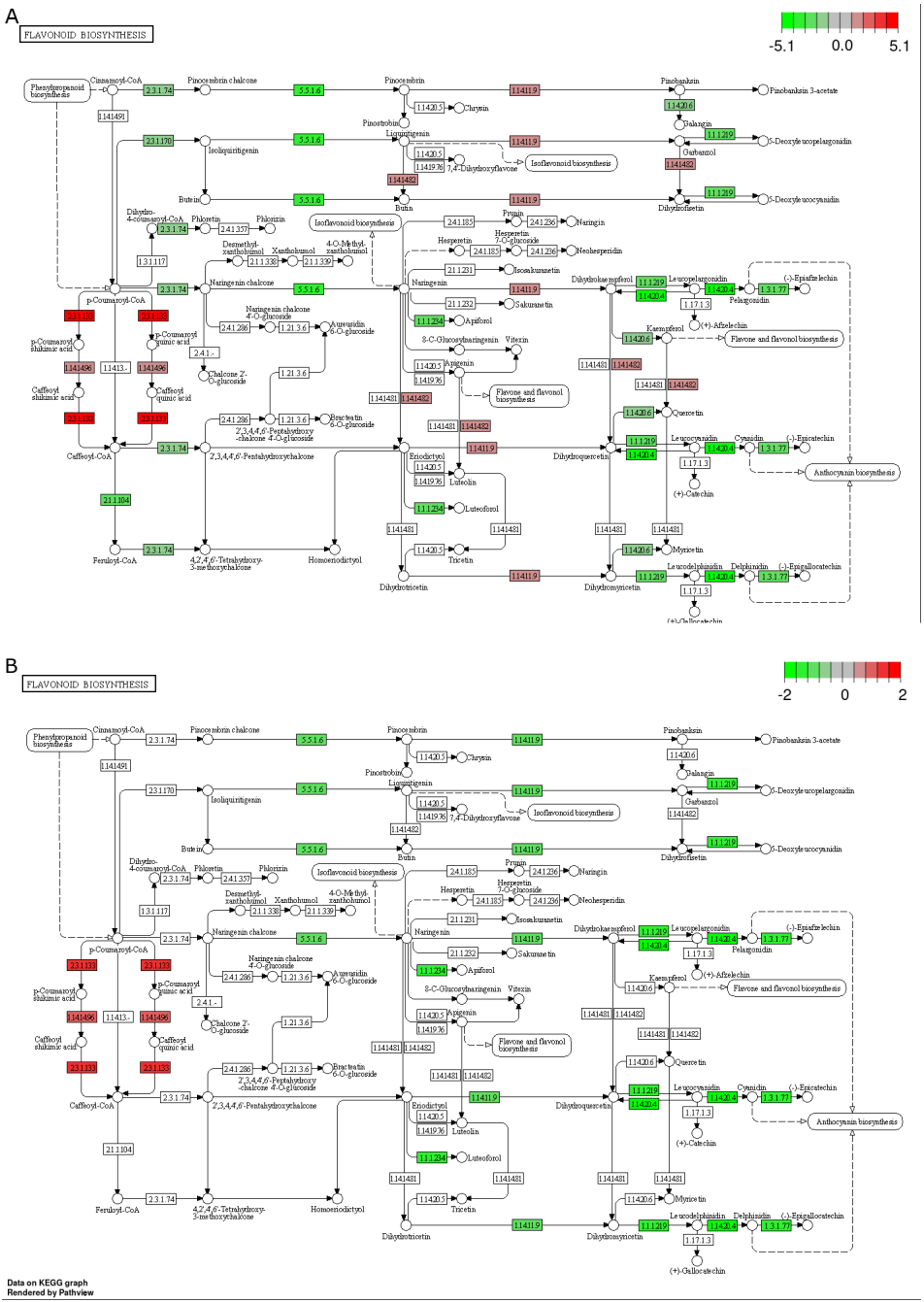



**Figure 6.** Differentiated genes in control vs. drought stress (**A**) and control vs. waterlogging (**B**) in flavonoid metabolism. The green box represents downregulated genes, the red box upregulated genes, and the white box not differentially expressed; padj < 0.05.

### 3.4.2. Effect of Drought and Waterlogging on Genes Involved in Carbon and Sugar Metabolism, Carbon Fixation, and Photosynthesis

In waterlogging conditions, no differentially expressed genes were found in comparison to the control. In relation to drought stress, we focused on the starch and sucrose metabolism (Figure 7) that showed 51 differentially expressed genes, among which 37 were down- and 14 upregulated. Most of the downregulated genes were related to enzymes that are involved in the metabolism of glucose, like glucose-6-phosphate isomerase (CEY00_Acc04867) and hexokinase (CEY00_Acc32432 and CEY00_Acc10665), or that contribute to starch production, like the 1,4-alpha-glucan branching enzyme (CEY00_Acc16922) and granule-bound starch synthase (CEY00_Acc03251). In addition, amylase (β-CEY00_Acc15000, CEY00_Acc28118, CEY00_Acc28117, CEY00_Acc09124, α - CEY00_Acc31412, CEY00_Acc06307, and CEY00_Acc23514), some transferase as 4-alpha-glucanotransferase (CEY00_Acc13256), sucrose phosphate synthase (CEY00_Acc11414 and CEY00_Acc15447), glucose-1-phosphate adenylyl transferases §(CEY00_Acc03134), as well as trehalose-phosphatase/synthase (CEY00_Acc02730, CEY00_Acc01464, CEY00_Acc, CEY00_Acc21372, CEY00_Acc06522, CEY00_Acc20715, CEY00_Acc25163, CEY00_Acc29021, CEY00_Acc10556, CEY00_Acc19227, and CEY00_Acc06511) were downregulated (Figure 7). On the other hand, those upregulated were mainly linked to the increased transcription of β-glucosidase (CEY00_Acc23018, CEY00_Acc13317, and CEY00_Acc09335) and β-fructofuranosidase (CEY00_Acc27954 and CEY00_Acc22611; Figure 7).

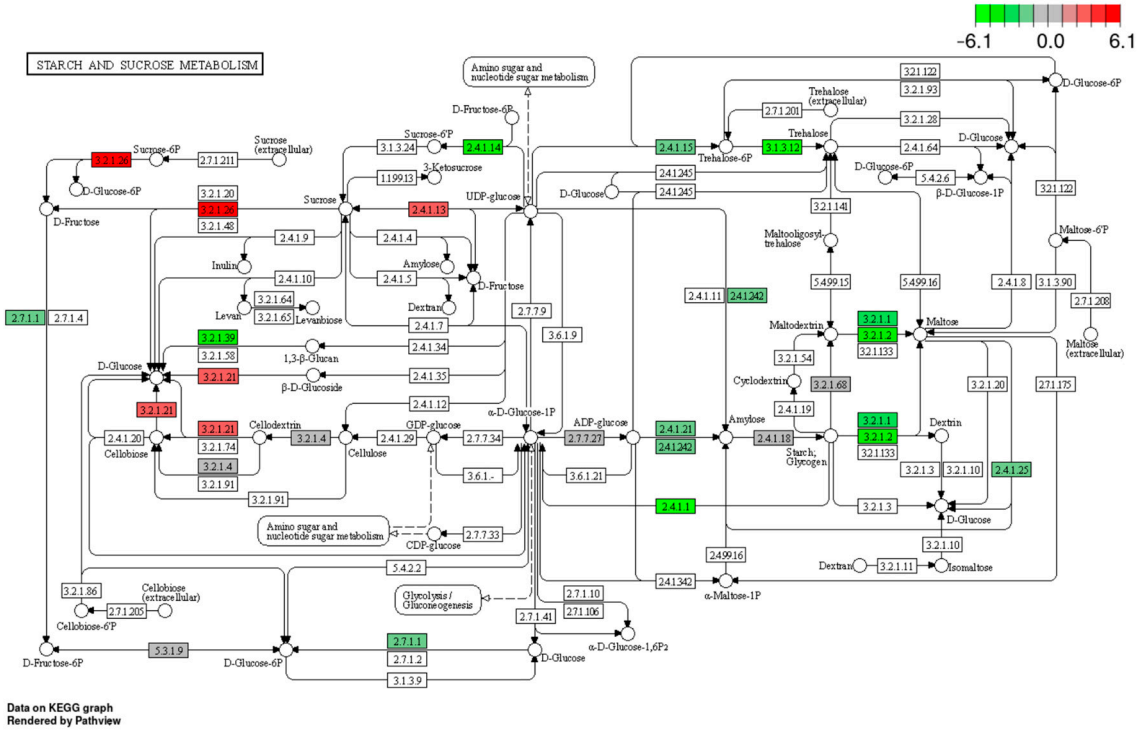



**Figure 7.** Differentially expressed genes in the control vs. drought stress conditions relating to starch and sucrose metabolism. Green boxes represent downregulated genes, red boxes upregulated genes. Genes in grey and white boxes were not differentially expressed; padj < 0.05.

Moreover, in the pathway related to "carbon fixation in photosynthetic organisms", a downregulation of several genes like phosphoenolpyruvate carboxylase (CEY00_Acc26501, CEY00_Acc19136, and CEY00_Acc28561), malate dehydrogenase (CEY00_Acc27472 and CEY00_Acc19122), fructose-bisphosphate aldolase (CEY00_Acc27551), ribose 5-phosphate isomerase (CEY00_Acc28440, CEY00_Acc09423, and CEY00_Acc17197), and ribulose bisphosphate carboxylase (CEY00_Acc28506) was observed (Figure 8).

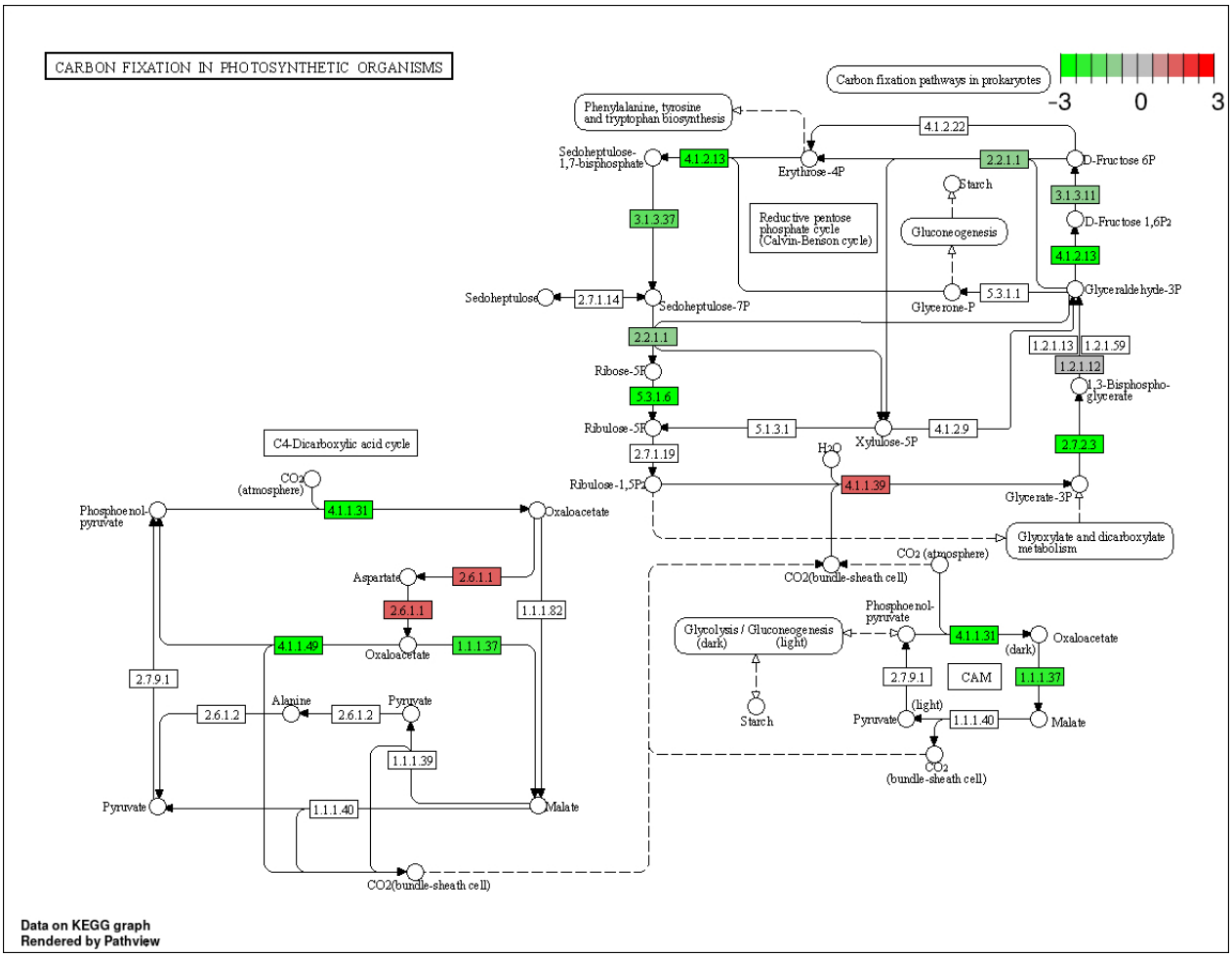



**Figure 8.** Differentially expressed genes in control vs. drought stress conditions relating to carbon fixation in photosynthetic organisms. Green boxes represent downregulated genes, red boxes upregulated. Genes in white boxes were not differentially expressed; padj < 0.05.

The observed downregulation of genes involved in carbon fixation following drought can be considered a direct effect of the general downregulation of the photosynthetic process. Indeed, several components of the light-dependent reactions of photosynthesis (photosystem I and II, cytochrome b6/f, electron transport chain, and ATP-ase complex) were downregulated in comparison to the control (Figure 9).

### 3.4.3. Effect of Drought and Waterlogging on Genes Involved in Hormone Metabolism

The responsiveness of the plant to the main plant hormones (auxins, cytokines, gibberellins, abscisic acid, ethylene, brassinosteroids, jasmonic acid, and salicylic acid) was affected by drought and waterlogging starting from several pathways (diterpenoid, cysteine and methionine, α−linolenic acid, phenylalanine metabolism, and brassinosteroid biosynthesis). In particular, water deficiency caused the differential expression of 97 genes (63 up- and 34 downregulated) in comparison to the control, while in waterlogging only 5 upregulated genes were detected. As previously seen for phenylpropanoid biosynthesis, even in this case, the five genes detected for waterlogging were common to drought, and represented the genes involved.

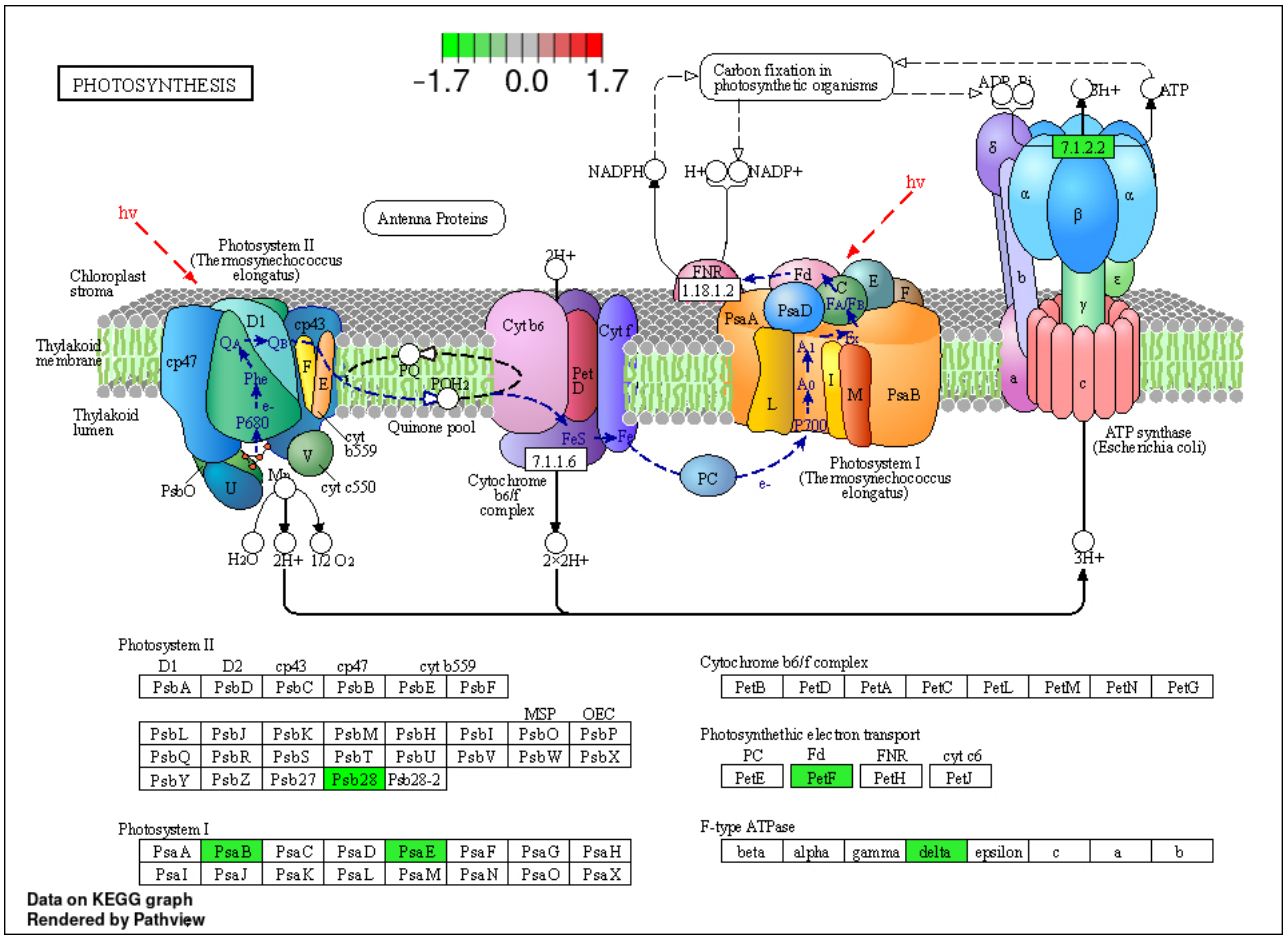



**Figure 9.** Differentially expressed genes in control vs. drought stress conditions relating to photosynthesis. Green boxes represent downregulated genes. Genes in white boxes were not differentially expressed; padj < 0.05.

Both stresses induced the upregulation of two genes involved in the auxin pathway—the auxin responsive GH3 gene (GH3) and the SAUR-like auxin-responsive gene (SAUR)—even with a higher significance in waterlogging than in drought (Figure 10). Moreover, drought induced the upregulation of one auxin influx carrier (belonging to the AUX1-LAX family) and of two members of the auxin-responsive proteins (AUX/IAA; Figure 10). In the pathways of signal transduction induced by abscisic acid (ABA), both stressing conditions led to the upregulation of the protein phosphatase 2C (PP2C) and of two abscisic acid (ABA) responsive element binding factors (ABF). Moreover, following drought, all the genes involved in the signal transduction induced by ABA were upregulated, except one member of the abscisic acid receptor PYR/PYL (Figure 10).

Concerning the other hormones, as previously described, no effect of waterlogging was detected (Figure S3B). Following drought, a general downregulation of the genes involved in cytokinin and jasmonic acid responses were, instead, detected (Figure S3A), while a general induction was found for ethylene response (with the upregulation of the ethylene response sensor 2 (ETR), EIN3-binding F box protein 1, and ethylene response factor 1), and for the salicylic acid-mediated response (with the upregulation of the pathogenesis-related protein 1, PR1; Figure S3A). A less clear behavior was detected for the brassinosteroid-induced response, with the upregulation of some genes at the beginning and the down regulation of others at the end of the hormone-signal transduction (Figure S3A).

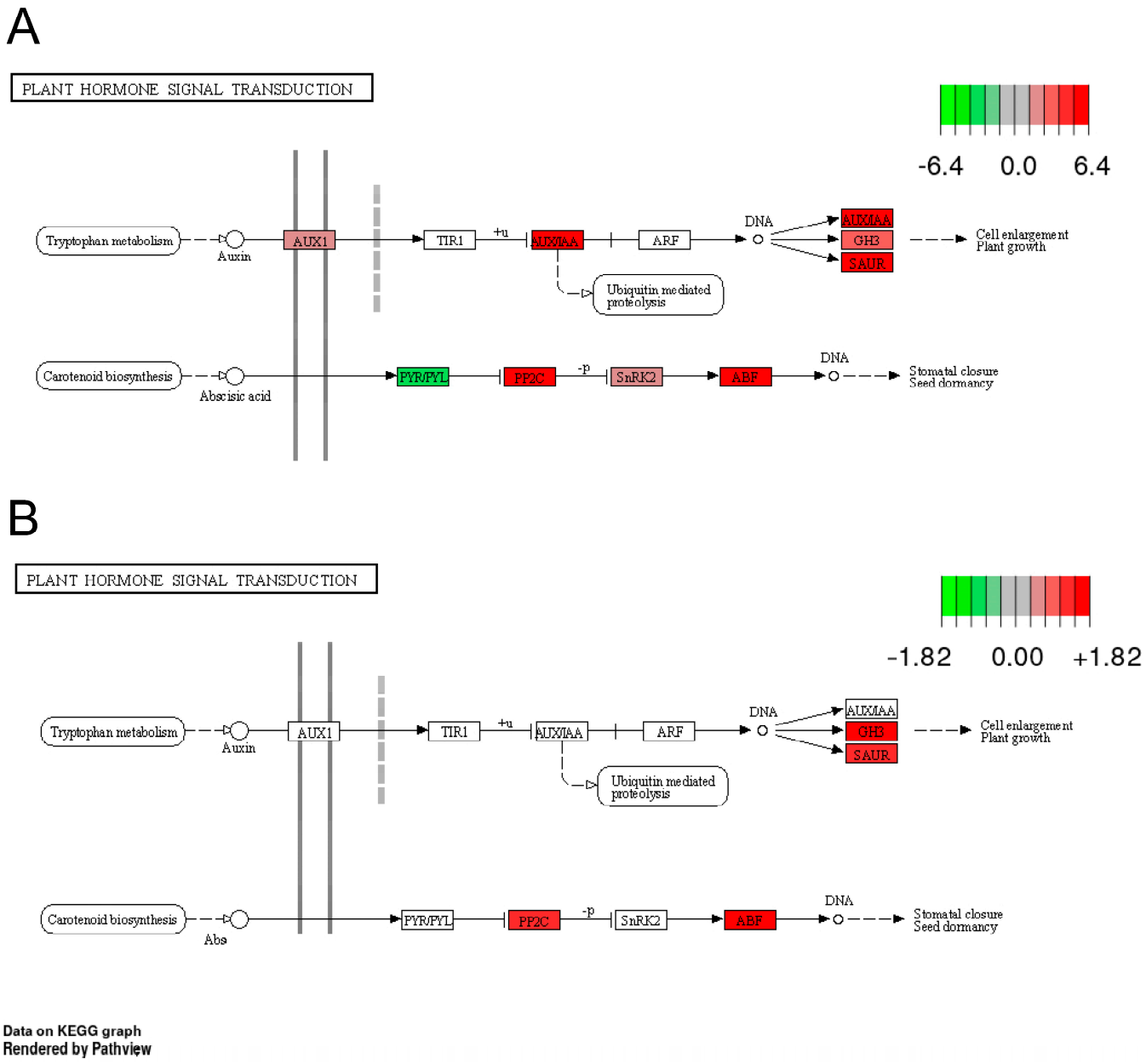



**Figure 10.** Differentiated genes in control vs. drought stress (**A**) and control vs. waterlogging (**B**) in plant hormone signal transduction. The green box represents downregulated genes, the red box upregulated genes, and the white box not differentially expressed genes; padj < 0.05.

## 4. Discussion

The results demonstrate the unique and differential response of kiwifruit leaf under two extreme and contrasting water regimes, drought and waterlogging. Several mechanisms, such as hormonal signaling, photosynthesis, respiration, and secondary metabolite production were implicated in plant responses to water excess or deficit showing different impacts on the transcriptome, with nonetheless similar physiological behavior. In the present experiment, we observed a higher impact on differential gene expression under drought as compared to under waterlogging as also evidenced on soybeans [28,29].

In general, drought stress induced the upregulation of several genes linked to the GO categories "plant hormone" and "MAPK signaling transduction", as well as "protein processing in endoplasmatic reticulum" which, in our experimental conditions, could together allow the plant to respond to water deficiency. The MAP kinases are involved in a wide variety of physiological processes mainly linked to signal transduction caused by

wounding, pathogens, and abiotic stresses [30], and their upregulation could consequently play a critical role in enhancing stress resistance [31]. Similarly, genes belonging to the category "protein processing in endoplasmatic reticulum" are involved in the processing of misfolded proteins, which are retained within the endoplasmic reticulum and are directed toward degradation through the proteasome. The accumulation of misfolded proteins in the endoplasmatic reticulum is, in general, linked to severe stress conditions and, in these situations, the protective mechanisms activated by the unfolded protein response (UPR) is not sufficient to restore normal endoplasmatic reticulum function and cells could die by apoptosis. It was reported that drought stress causes accumulation of misfolded or unfolded proteins that induce endoplasmic reticulum homeostasis imbalance, resulting in a higher load on secretory proteins [32]. In similar situations, the overexpression of genes involved in the UPR signaling can lead to the stimulation of the expression of specific genes, which regulate the response to stress [33].

On the other hand, a reduced number of GO categories were significantly influenced by waterlogging. In detail, phenylpropanoids, terpenoids, stilbenoids, and triterpenoids biosynthesis genes were upregulated while, as also for drought, the flavonoid pathway genes were downregulated.

Secondary metabolites are required for protection, stress tolerance, competition, and species interaction [34,35]. Among them, flavonoids were those that showed differentially expressed genes both in drought and waterlogging conditions. Flavonoids are one of the most important plant secondary metabolites with multiple functions in response to stress, and they are composed of six subgroups: chalcones, flavones, flavan-3-ols, flavandiols, anthocyanins, and proanthocyanidins or condensed tannins [36,37].

Chalcone isomerase is one of the genes involved in the first-step biosynthetic pathway of flavonoids; its down regulation, as a consequence of both the stressing conditions, together with the reduced expression of flavanone 4-reductase, dihydroflavonol 4-reductase, anthocyanidin synthase, and anthocyanidin reductase could bring about reduced activation of the flavonoid branch of the phenylpropanoid pathway that led to flavan-3-ols production [38,39]. Even if in other species, as in sorghum and corn, the role of flavan-3-ols as antioxidants under drought stress has been well described [40], other secondary metabolite compounds probably play a key role in kiwifruit leaves' response to drought.

Shikimate O-hydroxycinnamoyl transferase and spermidine hydroxycinnamoyl transferase, upregulated by both stresses, take part in the monolignol pathway [41] that leads to the production of the stress-induced lignin polymer [42]. It was previously demonstrated ([43] and literature here cited) that drought stress affects both the xylem anatomy and lignification of trees due to the modification by stressed cells of their pattern of lignin deposition by strengthening the cell wall. Both drought and waterlogging can lead to wilting and increased lignification that would contribute to improved mechanical support of the plant aerial structure, as well as water transport [43]. Lignification could also help to reduce cell expansion and plant growth during this phase, thereby favoring reallocation of carbon resources to other defense mechanisms [43].

Waterlogging also induced the upregulation of several genes involved in terpenes and terpenoids biosynthesis, consistent with the role in mitigating the effects of oxidative stress by modulating the oxidative status of plants [44].

According to several researchers, [45–47] under drought stress the reduction of photosynthetic activity leads to changes in carbohydrate metabolism in leaves [48]. Drought stress resulted in the downregulation of several genes involved in photosynthesis, carbohydrate metabolism and, in particular, of the one related to starch accumulation. According to their form, carbohydrates serve diverse functions. For example, starch is the major source of energy reserves, whereas sucrose, fructose, and glucose are solutes for cell osmotic potential. In addition, sucrose also has a role in stabilizing proteins and membranes after stress damage [49–51]. Plants can regulate cells' osmotic potential by fine-tuning the rate of starch accumulation and its ratio with soluble sugars that are osmotically active [52]. In our experiment, drought induced the downregulation of genes involved in starch production

(1,4-alpha-glucan branching enzyme, granule-bound starch synthase) and the metabolism of glucose (glucose-6-phosphate isomerase; hexokinase). This was also followed by a reduction in the expression of genes involved in starch degradation such as β and α amylase, and sucrose phosphate synthase.

Moreover, in the pathway related to carbon fixation in photosynthetic organisms, there was a downregulation of several genes like phosphoenolpyruvate carboxylase, malate dehydrogenase, aldolase superfamily proteins, ribose 5-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and ribulose bisphosphate carboxylase. All these enzymes take part in the Calvin cycle that is the most common $CO_2$ fixation process in C3 plants. Drought stress, by inducing stomatal closure, leads to a decrease in $CO_2$ availability, thus impairing the efficiency of the Calvin cycle, which is crucial for photosynthetic responses and metabolic pathways.

On the other hand, upregulated genes were mainly linked to the increased expression of β-glucosidase. Previous studies on beans [53], wheat [54], and tobacco [55] evidenced an increase in β-glucosidase abundance under water deficit. This protein is present in all living cells and plays fundamental biological roles in several processes such as degradation of cellulose and other carbohydrates [56]. It is suggested that glycosyl hydrolases enzymes utilize cell-wall polysaccharides as an alternate carbon source under sugar depletion in the cytoplasm during dehydration [57,58]. These results, consequently, fit with the general concept that drought stress might affect biochemical metabolism in the cell wall. Moreover, in previous experiments [59], it was found that β-glucosidase is responsible for the production of the free form of abscisic acid. The data presented by Dietz and co-authors [59] provide evidence that β-glucosidase of barley leaves has a specific function in ABA-dependent root:shoot signaling in plants. The phytohormone ABA has a critical role in regulating the plant response to water stress [60,61]. Its concentration increases in roots in response to drought stress [61,62], resulting in stomatal closure and a consequent reduction of photosynthetic rates and the accumulation of protective proteins and metabolites to maintain cellular water status [60,61]. On the other hand, in response to flooding, ABA levels decrease in roots and increase in the aerial organs [63]. A transcriptome study performed on Arabidopsis revealed an upregulation of ABA biosynthesis genes in leaves, and a downregulation in roots under flooding conditions [64]. It was thus suggested that ABA may accumulate in leaves of flooded plants because of reduced translocation of photoassimilates out of leaves, and roots do not act as the source of ABA because most of them die within the first few days of flooding [65]. Thanks to xylem sap analysis, other authors [63] have revealed that an increase in leaves' ABA concentrations was not due to its translocation from roots to shoots, but to an increased production in old leaves and transportation to younger leaves [66]. As for citrus and also grapes, waterlogging can induce substantial ABA accumulation in leaves, which is mainly responsible for the severe stomatal closure following waterlogging in plants [67].

In the present experiment, the highest impact on photosynthesis was found in plants exposed to waterlogging. The reduction in photosynthesis was primarily connected to a significantly lower stomatal conductance, which resulted in lower intercellular $CO_2$ concentration. Both stress conditions induced an upregulation of abscisic acid (ABA) responsive element binding factor (ABF). ABA is the key regulator of stomata opening, and thus the rise in ABF induction suggests that the plants respond to stress by a prompt stomata closure. Stomatal conductance in waterlogged plants was 24-fold lower than in drought-exposed ones. This result corroborates the finding that kiwifruit responds to water excess by a prompt stomata closure within 3 h after the exposure to stress [17]. Moreover, stomata closure impairs evapotranspiration, and thus the plant is unable to dissipate water excess. This phenomenon makes kiwifruit vines extremely sensitive to waterlogging, and permanent damage to the root system occurs within three to five days of exposition [68].

In our experiment, we did not observe a differentiated expression of genes directly related to ABA biosynthesis, however, we found some effects on the carotenoid signaling pathways induced by ABA [69]. Specifically, we measured a downregulation of PYR/PYR

receptors and an upregulation of PPC2, ABF, and SnRK2 (only in response to drought). According to previous research [70–72], the interactions between PYR/PYL/RCAR proteins and their target PP2Cs induce the activation of downstream targets of the group A PP2Cs, including SnRK2 protein kinases that play key roles in the regulation of transcriptional response and stomatal closure, thereby confirming our findings.

Beside ABA, auxin signal transduction was also altered by stress, with a trend of upregulation of genes encoding auxin influx carrier (AUX1), auxin-responsive protein IAA family (AUX/IAA), and SAUR, highlighting the crucial roles of auxin-signaling genes in drought stress responses. Comparable results were previously found in rice [73], *Arabidopsis* [74], and *Davidia involucrata* [75]. In recent years, several researchers demonstrated the potential link between auxin response and abiotic stress [76–78] due to the interaction with other plant hormones like salicylic acid and ABA [79]. In a study on transgenic *Arabidopsis* [80], the protective role of endogenous and exogenous auxin in drought stress resistance through the regulation of root architecture, ABA-responsive genes expression, reactive oxygen species metabolism, and metabolic homeostasis was demonstrated. In addition, in a study on white clover [81], it was demonstrated that exogenous auxin application mitigated plant dehydration symptoms by improving stem dry weight, relative water content, and total chlorophyll content in leaves. This was mainly because exogenous auxins induced the production of other hormones, suggesting that auxins increasing production at leaf-level could have a positive effect on improving the drought tolerance of white clover. In a grape experiment [67], some auxin-induced genes such as GH3 (auxin-conjugating enzyme) were upregulated under waterlogging stress, confirming our findings. Consequently, as also for drought, auxins, by interacting with other hormones, could play a positive role as signaling molecules in response to waterlogging stress.

## 5. Conclusions

Economic returns from kiwifruit cultivation strongly depend on individual fruit sizes and quality, which are directly affected by water availability. For this reason, kiwifruits are usually irrigated and, in some areas, this practice has led to demands for water that exceed local resources, even if more frequently water scarcity is experienced due to climate change. Plant responses toward abiotic stress are complex and involve several different regulatory networks between genes related to hormonal signaling, photosynthesis, respiration, and secondary metabolite production. Comparative transcriptome profiling performed in this study under drought and waterlogging has provided an opportunity to evaluate and differentiate the categorized molecular responses. Surprisingly, despite similar physiological behavior, the two contrasting stress conditions have a quite different impact on the transcriptome, suggesting the strongest effect came from the drought stress in comparison to the flooding. Even if some shared molecular mechanisms were observed, our results suggest that, while the plant actively reacts to water deficit, the response to water excess seems to be passive, with the plant reducing at minimum its metabolic activity. Further studies are required to understand whether, in the short-term, even an excess of water can induce a specific response, in order to identify genes as early markers of stress. Concerning drought, the massive transcriptome reprogramming that emerged for the leaves in the present study suggests that several effects may also be present in fruit, and it would be interesting to ascertain whether, considering climate change and agricultural sustainability, a controlled reduction of the water use for kiwifruit irrigation could yield fruit equally appreciated by consumers.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10080834/s1, Figure S1: differentially expressed genes involved in phenylpropanoid biosynthesis in control vs. drought stress and control vs. waterlogging; Figure S2: differentially expressed genes in control vs. waterlogging in sesquiterpenoid and triterpenoid biosynthesis; Figure S3: differentially expressed genes in control vs. drought stress and control vs. waterlogging in plant hormone signal transduction.

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