

## Article

# Physiological and Proteomic Responses of Pitaya to PEG-Induced Drought Stress

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**Abstract:** Pitaya (*Hylocereus polyrhizus* L.) is highly tolerant to drought stress. Elucidating the response mechanism of pitaya to drought will substantially contribute to improving crop drought tolerance. In the present study, the physiological and proteomic responses of the pitaya cultivar ‘Zihonglong’ were compared between control seedlings and seedlings exposed to drought stress (−4.9 MPa) induced by polyethylene glycol for 7 days. Drought stress obviously enhanced osmolyte accumulation, lipid peroxidation, and antioxidant enzyme activities. Proteomic data revealed drought stress activated several pathways in pitaya, including carbohydrate and energy metabolism at two drought stress treatment time-points (6 h and 3 days). Other metabolic pathways, including those related to aspartate, glutamate, glutathione, and secondary metabolites, were induced more at 3 days than at 6 h, whereas photosynthesis and arginine metabolism were induced exclusively at 6 h. Overall, protein expression changes were consistent with the physiological responses, although there were some differences in the timing. The increases in soluble sugar contents mainly resulted from the degradation and transformation of insoluble carbohydrates. Differentially accumulated proteins in amino acid metabolism may be important for the conversion and accumulation of amino acids. GSH and AsA metabolism and secondary metabolism may play important roles in pitaya as enzymatic and nonenzymatic antioxidant systems. The enhanced carbohydrate and energy metabolism may provide the energy necessary for initiating the above metabolic pathways. The current study provided the first proteome profile of this species exposed to drought stress, and may clarify the mechanisms underlying the considerable tolerance of pitaya to drought stress.

**Keywords:** pitaya; proteomics; glutathione; drought stress; antioxidant enzymes



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## 1. Introduction

Drought, which can be defined as the absence of adequate moisture for normal plant growth, is increasingly becoming a serious global environmental problem [1]. Arid and semiarid regions make up approximately a third of the total land area worldwide. Global warming, deforestation, and urbanization will likely increase the frequency and severity of drought conditions in many regions [2]. Drought severely limits the geographical distribution and productivity of crops, resulting in substantial yield losses [3]. Therefore, there is a growing demand for new crop varieties with enhanced drought tolerance.

Plants have developed complex mechanisms for sensing water availability and reprogramming their metabolism and growth in response to drought stress, which leads to various morphological, physiological, and molecular changes [4]. The morphological changes mainly include decreased shoot growth, stomatal closure, increased rooting depth, and leaf senescence [5,6]. The primary physiological changes involve the photosynthetic system, osmotic regulatory substances, antioxidant enzymes, and endogenous

hormones [6,7]. Molecular studies have resulted in the identification and cloning of some drought-responsive genes. These genes can be classified into the following two groups: genes encoding functional proteins that directly protect plants against environmental stresses, and genes encoding regulatory proteins (e.g., signaling molecules and transcription factors that control gene expression) [8]. Recently, various techniques such as genomics, transcriptomics, and proteomics have been used to understand the complicated mechanisms of plant drought tolerance [5]. By genomic and transcriptome techniques, many candidate genes and pathways associated with drought-stress tolerance have been identified [3,8]. Because cellular processes are regulated by post-translational modifications, protein–protein interactions, and enzymatic activities, gene-expression analyses should be complemented by additional approaches [9]. Accordingly, proteomic studies have been performed to elucidate stress-tolerance mechanisms [10–13]. Additionally, parallel reaction monitoring (PRM), which was recently developed for targeted mass spectrometry, has been used to validate the accuracy and reliability of proteomic data [14]. Protein expression profiles of many crops, such as tobacco (*Nicotiana tabacum* L.) [10], soybean [*Glycine max* (L.) Merr.] [11], rice (*Oryza sativa* L.) [12], wheat (*Triticum aestivum* L.) [13], and cassava (*Manihot esculenta* Crantz) [5] have been reported under drought conditions. To further characterize the mechanisms underlying plant drought resistance, researchers are increasingly relying on a combination of physiology and proteomics [15,16]. Physiological and proteomic responses of contrasting alfalfa (*Medicago sativa* L.) varieties to PEG-induced osmotic stress have been studied by Zhang et al. [6]. Phenological, morpho-physiological, and proteomic responses of *Triticum boeoticum* Boiss. to drought stress were recorded by Moosavi et al. [15]. Chen et al. reported that physiology and proteomics analyses provided comprehensive insights into the overall and variety-specific mechanisms underlying drought response in tobacco, and that protein profiles were consistent with the physiological performances [16]. These studies revealed more proteins and metabolic pathways that respond to drought stress, and provided important information for elucidating the mechanism network of plant response to drought at the system biology level. To date, however, proteomics studies on pitaya are rare. Moreover, physiological and proteomic analyses to understand the response mechanism of pitaya to drought stress have not been reported.

Pitaya (*Hylocereus polyrhizus* L.), also known as dragon fruit, is a member of the family Cactaceae [17]. The pitaya cultivation area is expanding rapidly in many tropical and subtropical areas worldwide because it produces a nutritionally valuable fruit with an exotic appearance, striking colors, and health-promoting properties [18]. Moreover, pitaya is a highly drought-tolerant plant [19], making it an excellent species for mining plant drought-tolerance proteins. Previous studies on pitaya plant responses to drought stress mostly involved physiological and biochemical analyses, with some applying transcriptomic and microarray technologies to detect drought-related expressed sequence tags [20]. However, there are no reports regarding proteome-level analyses of pitaya responses to drought conditions. The objective of this study was to decipher the response mechanism of pitaya to drought. The stems of pitaya seedlings regarding their osmolyte accumulation, degree of lipid peroxidation, antioxidant enzyme activities, and protein abundance changes in response to drought stress simulated using polyethylene glycol (PEG) 6000 were analyzed. The results of this study provide insights into the drought-tolerance mechanisms of pitaya.

## 2. Materials and Methods

### 2.1. Plant Materials and Growth Conditions

Pitaya cultivar ‘Zihonglong’ was provided by the Key Laboratory of Plant Resources Conservation and Germplasm Innovation in the Mountainous Region (Ministry of Education), Institute of Agro-Bioengineering, Guizhou University, China. To ensure the uniformity of the examined plant material, pitaya stem buds from the same clone were rapidly micropropagated and maintained in a growth chamber under controlled conditions (25 °C; 14 h day/10 h night cycle; 60 ± 5% relative humidity; and 300 µmol m<sup>-2</sup> s<sup>-1</sup> light intensity) as previously described [19]. After 4 weeks of growth, rooted seedlings similar

in size (7–8 cm tall) were transferred to a nutrient solution developed by Hoagland and Arnon [21]. The nutrient solution was changed every 3 days during the hydroponic culture period and was aerated using an air pump to provide plants with sufficient O<sub>2</sub>.

## 2.2. Stress Treatments

To simulate drought stress, 6-week-old pitaya seedlings were transferred to a nutrient solution with an osmotic potential of  $-0.49$  MPa. The solution was prepared using 20% PEG 6000. Seedlings not subjected to drought stress (0 MPa) were used as the control plants. At specific post-treatment time-points (0, 6, 12, and 18 h, as well as 1, 3, 5, and 7 days), six stems of each time-point from stressed and the corresponding control pitaya seedlings were collected (three biological replicates), immediately frozen in liquid nitrogen, and stored at  $-80$  °C before analyzing their physiological parameters. Based on physiological responses, 6 h and 3 days were selected as the optimal treatment durations for investigating pitaya proteomic responses to drought stress. Therefore, pitaya seedlings exposed to drought stress for 6 h and 3 days were designated as OS6H and OS3D, respectively, with the corresponding controls designated as NS6H and NS3D, respectively. A total of 12 stems of the control and drought-stressed (NS6H, NS3D, OS6H, and OS3D, each for three biological replicates) pitaya seedlings were collected for a quantitative 4D label-free proteomics analysis. Six stems of the controls and drought-stressed (3 for NS6H and 3 for OS6H) pitaya seedlings were collected for verification of the differentially accumulated proteins (DAPs) via PRM. The drought experiment design for pitaya is shown in Figure S1.

## 2.3. Physiological Measurements

The soluble sugar content was measured using a commercial assay kit (catalog number KT-1-Y) according to the method of Buysse and Merckx [22]. The soluble protein content was measured using a commercial assay kit (catalog number BCAP-1-W) by a Coomassie Blue dye-binding method with bovine serum albumin as the standard [23]. The free proline content was determined by a commercial assay kit (catalog number PRO-1-Y) according to the acid ninhydrin method [24]. The malondialdehyde (MDA) was measured with thiobarbituric acid reaction according to Castrejón and Yatsimirsky [25] as described in a commercial assay kit (catalog number MDA-1-Y). The superoxide anion (O<sub>2</sub><sup>-</sup>) content was determined by a commercial assay kit (catalog number SA-1-G) following the method of Lin et al. [26]. The hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) level was measured using a commercial assay kit (catalog number H2O2-1-Y) according to Sima et al. [27]. The superoxide dismutase (SOD) activity was measured using a commercial assay kit (catalog number SOD-1-W) according to García-Triana et al. [28]. The glutathione reductase (GR) activity was assayed using a commercial assay kit (catalog number GR-1-W) as described by Foster and Hess [29]. The ascorbic peroxidase (APX) activity was assayed using a commercial assay kit (catalog number APX-1-W) using the method of Ullah et al. [30]. All commercial assay kits were purchased from Suzhou Keming Biotechnology Co., Ltd., Suzhou, China. Each index was conducted with three technique replicates and three biological replicates.

## 2.4. Protein Extraction

Total proteins were extracted from the stems of controls and drought-stressed pitaya seedlings according to a slightly modified version of a method described by Zhao et al. [31]. Samples were ground to a powder in liquid nitrogen. The powder was transferred to lysis buffer supplemented with 1% Triton X-100, 10 mM dithiothreitol, and 1% protease inhibitor cocktail in a 5 mL centrifuge tube and then sonicated three times on ice using a high-intensity ultrasonic processor (Scientz Biotechnology Co., Ningbo, China). After adding an equal volume of Tris-saturated phenol (pH 8.0), the mixture was vortexed for 5 min before centrifugation ( $5500 \times g$ , 10 min, 4 °C). The phenol phase was transferred to a new centrifuge tube and precipitated with five volumes of methanol saturated with ammonium sulfate, which was followed by overnight incubation at  $-20$  °C. After centrifuging at  $5500 \times g$  for 10 min at 4 °C, the supernatant was discarded. The remaining precipitate was

washed once with ice-cold methanol and then three times with ice-cold acetone. Finally, proteins were redissolved in 8 M urea and quantified using a BCA Protein Assay kit (Pierce, Rockford, IL, USA).

### 2.5. Trypsin Digestion

Before the trypsin digestion, the volume of the protein solutions was adjusted so all samples were at the same concentration. To precipitate the proteins, samples were slowly mixed with 20% trichloroacetic acid (final concentration) using a vortexer and then incubated at 4 °C for 2 h. The precipitate was pelleted by centrifugation (4500× g, 5 min, 4 °C) and washed two or three times with precooled acetone. The pelleted proteins were then resuspended using 200 mM triethylammonium bicarbonate. Trypsin was added at a 1:50 trypsin-to-protein mass ratio for overnight digestion at 37 °C. The resulting peptides were reduced with 5 mM dithiothreitol for 30 min at 56 °C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness.

### 2.6. LC-MS/MS Analysis

The peptides were dissolved in solvent A (0.1% formic acid and 2% acetonitrile in water) and then loaded onto a homemade reversed-phase analytical column (25 cm long, 100 µm internal diameter). Peptides were separated on a nanoElute UHPLC system (Bruker Daltonics, Inc., Billerica, MA, USA) using solvent B (0.1% formic acid in acetonitrile) and the following gradients: 4–22% over 70 min, 22–30% in 14 min, increased to 80% in 3 min, and then held at 80% for 3 min, all at a constant flow rate of 450 nL/min. The peptides were subjected to capillary electrophoresis followed by an analysis using the timsTOF Pro (Bruker Daltonics, Inc., Billerica, MA, USA) mass spectrometry system. The electrospray voltage was 1.6 kV. Precursors and fragments were analyzed at the TOF detector, with an MS/MS scan range of 100 to 1700 m/z. The timsTOF Pro system was operated in the parallel accumulation serial fragmentation (PASEF) mode. Precursors with charge states 0–5 were selected for fragmentation, and 10 PASEF-MS/MS scans were acquired per cycle. Dynamic exclusion was set to 30 s.

### 2.7. Database Search

The resulting MS/MS data were processed using the Maxquant search engine (version 1.6.6.0). Tandem mass spectra were used to screen the *H. polyrhizus* transcriptome database (comprising 24,810 sequences), which has not yet been published. Trypsin/P was specified as the cleavage enzyme, with up to two missed cleavages allowed. The mass tolerance for precursor ions was set as 20 ppm in the first search and 20 ppm in the main search. The mass tolerance for fragment ions was set as 20 ppm. Carbamidomethylation of Cys was specified as a fixed modification, whereas acetylation of protein N-terminals and oxidation of Met were specified as variable modifications. The false-discovery rate was adjusted to <1% and the minimum score for peptides was set as >40.

### 2.8. Data Analysis

Physiological status of samples were investigated and presented with quantified data. Statistical analysis was carried out with DPS 7.05 software (China) and Microsoft Excel 2007. Significant differences were assessed by one-way ANOVA followed by post hoc Duncan's multiple range tests ( $p < 0.05$  or  $p < 0.01$ ).

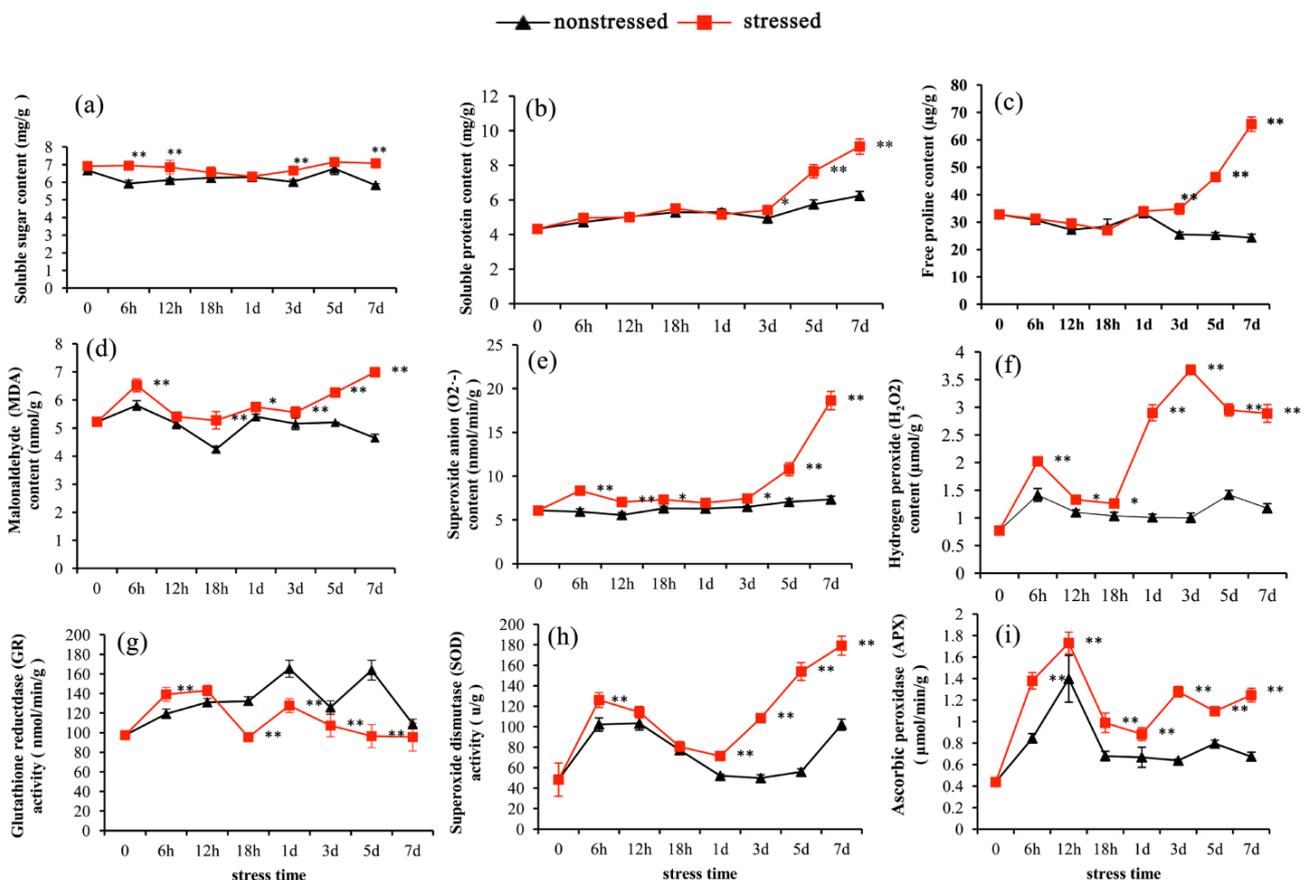
Gene ontology (GO) functional annotations were completed using the UniProt-GOA database (<http://www.ebi.ac.uk/GOA/>, accessed on 2 July 2020), and the unannotated proteins were predicted using the InterProScan software. Proteins were classified into the three main GO categories (biological process, cellular component, and molecular function). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.kegg.jp/>, accessed on 2 July 2020) was used to identify enriched pathways. The protein contents of the stressed plants and the controls were compared. The significance of any difference was assessed using the two-sample two-tailed T-test. The protein ratio was calculated by the

mean of three biological replicates. Each index was measured in triplicate. A  $p$ -value  $< 0.05$  and a protein ratio  $> 1.2$  were set as the criteria for upregulation, whereas a  $p$ -value  $< 0.05$  and a protein ratio  $< 0.83$  were set as the criteria for downregulation. The DAPs annotated with enriched GO terms or assigned to KEGG pathways were analyzed using the two-tailed Fisher's exact test.

### 3. Results

#### 3.1. Physiological Responses of Pitaya to Drought Stress

The soluble sugar content of pitaya at 6 and 12 h, as well as 3 and 7 days, after starting the drought stress treatment were 1.17, 1.12, 1.11, and 1.21 times higher than the corresponding control levels, respectively (Figure 1a). Compared with the controls, there were no significant differences in the soluble protein and free proline contents before the 3-day time-point, but there was a significant increase afterward (Figure 1b,c). The soluble protein content of the stressed samples was 4.33 mg/g at the beginning of the treatment period and increased to 9.09 mg/g after 7 days (Figure 1b). The free proline content of the stressed samples was 32.77  $\mu$ g/g at the beginning of the treatment period and increased to 65.71  $\mu$ g/g after 7 days (Figure 1c).



**Figure 1.** Changes in the soluble sugar (a), soluble protein (b), free proline (c), MDA (d),  $O_2^-$  (e),  $H_2O_2$  (f), GR (g), SOD (h), and APX (i) contents in pitaya plants under the control and stressed conditions at different time-points. Asterisks indicate significant differences (\*  $p < 0.05$ , \*\*  $p < 0.01$ ) from the control. Data are presented as the mean  $\pm$  SE of three biological replicates ( $n = 3$ ).

Regarding the lipid peroxidation levels of pitaya, compared with the controls, the MDA and  $O_2^-$  contents of the stressed pitaya samples initially increased and then decreased before increasing again during the stress treatment period. Specifically, the MDA and  $O_2^-$  contents of the stressed pitaya samples at 6 h were approximately 1.12 and 1.40 times higher, respectively, than the corresponding contents of the controls ( $p < 0.01$ ). After a

slight decrease, the MDA and O<sub>2</sub><sup>-</sup> contents began to increase again after 3 days of the drought-stress treatment (Figure 1d,e). The H<sub>2</sub>O<sub>2</sub> content had a wavelike fluctuating trend as the duration of the drought-stress treatment increased. The H<sub>2</sub>O<sub>2</sub> content of stressed pitaya samples first peaked at 6 h (approximately 1.42 times higher than the control level;  $p < 0.01$ ), after which it tended to decrease before increasing to a second peak that was higher than the first one at the 3-day time-point (approximately 3.67 times higher than the control level;  $p < 0.01$ ) and then decreasing again (Figure 1f).

Drought stress significantly affected antioxidant enzyme activities. As the treatment duration increased, the GR activities were significantly higher and lower in the stressed pitaya samples than in the controls at 6 and 18 h, respectively, implying that GR was sensitive to drought stress (Figure 1g). The SOD activities of the stressed pitaya samples first peaked at 6 h (approximately 1.23 times higher than the control level;  $p < 0.01$ ), after which it began to increase again after 1 day (Figure 1h). The APX activities had a wavelike fluctuating trend over the period of the drought-stress treatment. Specifically, the APX activities of the stressed pitaya samples first peaked at 6 h (approximately 1.63 times higher than the control level;  $p < 0.01$ ) and then tended to decrease before increasing to a second peak at 3 days (approximately 2.00 times higher than the control level;  $p < 0.01$ ) (Figure 1i).

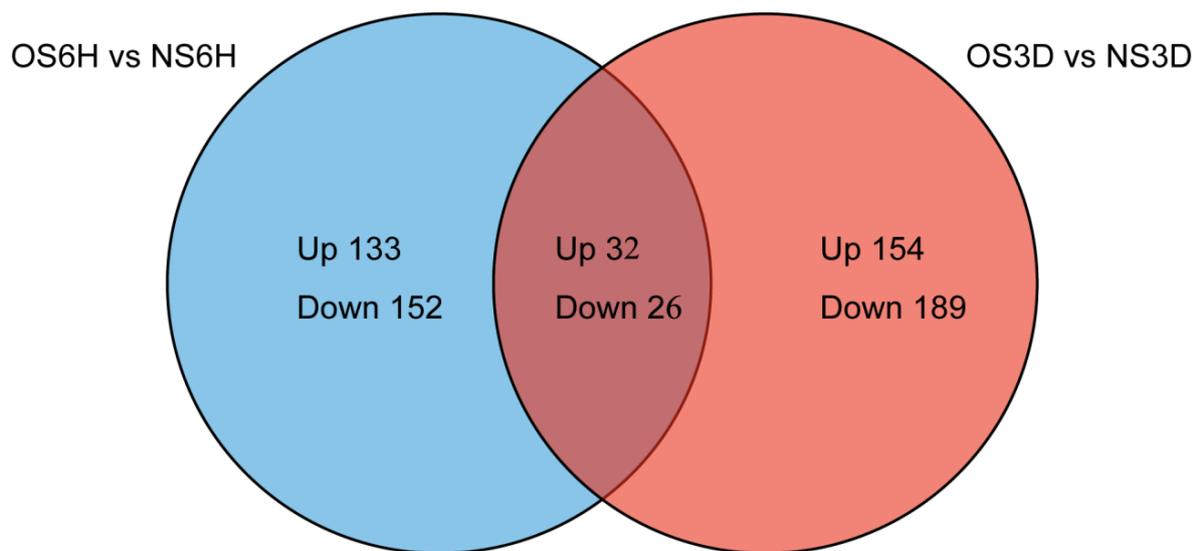
### 3.2. Primary Data Analysis and Protein Detection

To obtain a global overview of the molecular responses of pitaya to drought stress, the proteomes of the stressed and the control stems were compared. Based on the physiological analysis described above, stem samples were collected from stressed and control plants at the 6 h and 3-day time-points for 4D label-free proteomics analysis. A total of 3,107,303 spectra were generated, including 371,016 spectra corresponding to known spectra, 43,489 peptides, 41,084 unique peptides, 5929 identified proteins, and 5269 quantified proteins. The distribution of the number of peptides defining each protein is presented in Figure S2. Most of the proteins included at least two peptides. To clarify the functions of the 5269 quantified proteins, they were annotated according to GO terms, predicted functional domains, KEGG pathways, and KOG functional classifications. Specific details regarding all identified proteins are listed in Table S1.

### 3.3. Identification and Functional Analysis of DAPs

To identify DAPs, the results of the 4D label-free proteomics analysis were compared as follows: OS6H vs. NS6H and OS3D vs. NS3D. A total of 686 DAPs were identified in these two comparisons. Of these DAPs, 58 (32 upregulated and 26 downregulated) were common to both comparisons, 285 (133 upregulated and 152 downregulated) were specific to the OS6H vs. NS6H comparison, and 343 proteins (154 upregulated and 189 downregulated) were exclusive to the OS3D vs. NS3D comparison (Tables S2 and S3). A Venn diagram was used to illustrate the numbers of significantly upregulated and downregulated proteins in both comparisons (Figure 2). These results reflected the significant differences in the pitaya activities between the stressed and control samples at the two time-points.

The GO annotation and enrichment analysis indicated the identified DAPs were related to 13 biological processes, 9 cellular components, and 10 molecular functions. The number of upregulated proteins annotated with GO terms associated with the prevalent biological processes (e.g., cellular process, metabolic process, response to stimulus, and biological regulation) and the main cellular components (e.g., cell, organelle, membrane, and cell junction) was higher for the OS3D vs. NS3D comparison than for the OS6H vs. NS6H comparison (Figure 3a,b).

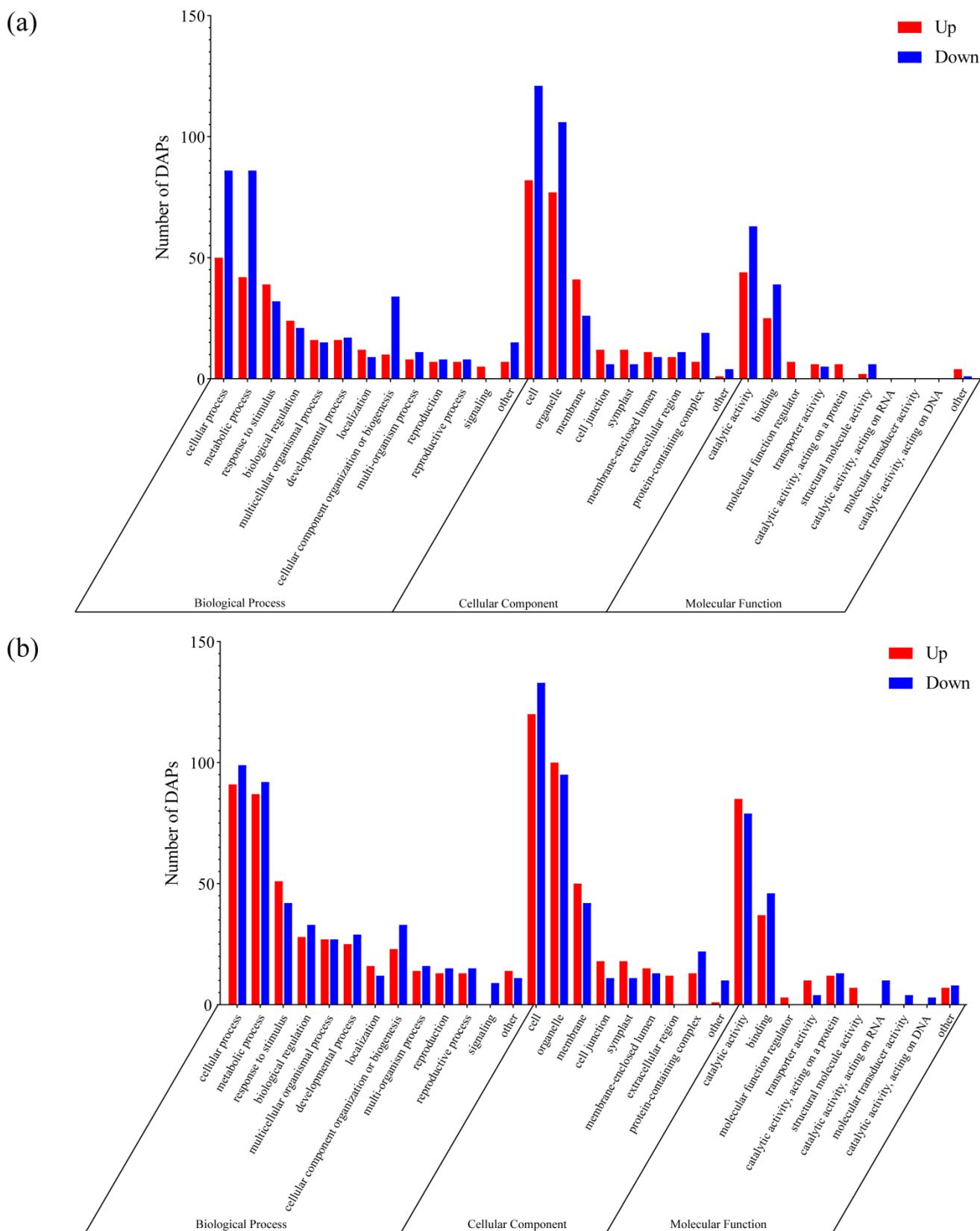


**Figure 2.** Graphical representation and functional cataloging of DAPs. The Venn diagram presents the number of significantly upregulated and downregulated proteins in pitaya under the drought-stress conditions induced by PEG.

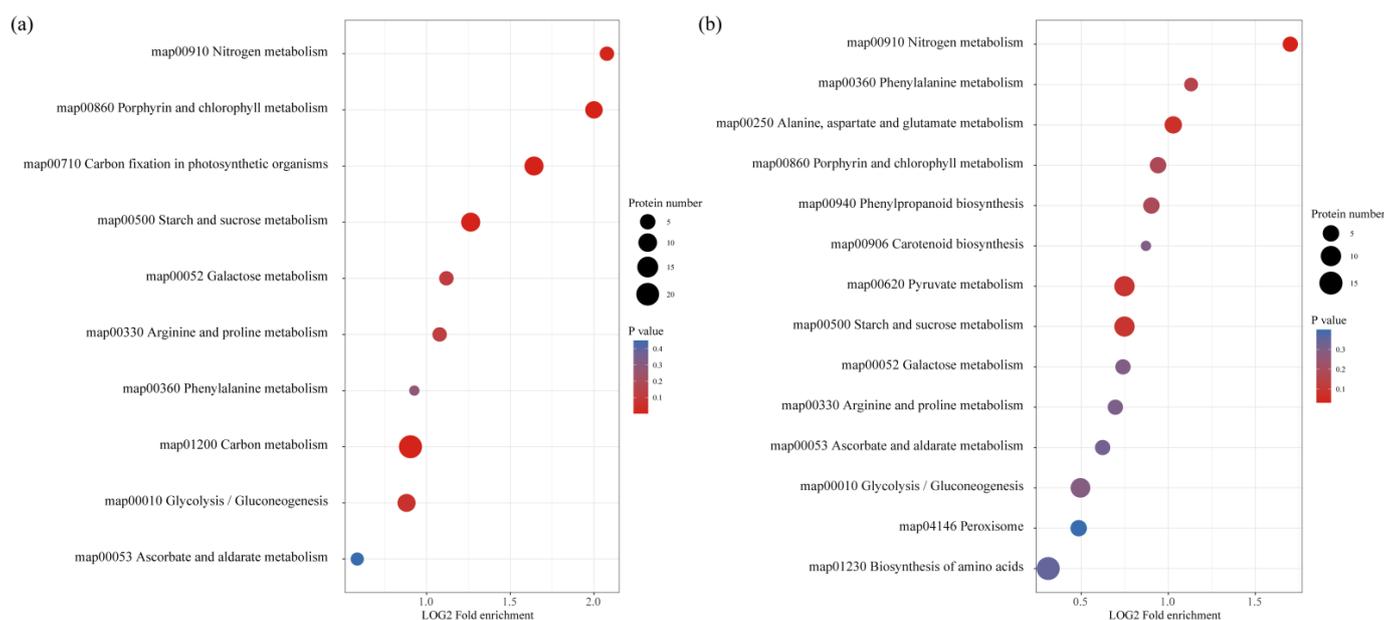
The enriched KEGG pathways among the DAPs revealed by the OS6H vs. NS6H and OS3D vs. NS3D comparisons are presented in Figure 4a,b. Among the DAPs in the OS6H vs. NS6H comparison, the four most enriched pathways were carbon metabolism, starch and sucrose metabolism, carbon fixation in photosynthetic organisms, and porphyrin and chlorophyll metabolism (Figure 4a). Of the DAPs in the OS3D vs. NS3D comparison, the three most enriched pathways were starch and sucrose metabolism; pyruvate metabolism; and alanine, aspartate, and glutamate metabolism (Figure 4b). The following eight pathways were enriched among the DAPs in both comparisons: nitrogen metabolism, porphyrin and chlorophyll metabolism, starch and sucrose metabolism, galactose metabolism, arginine and proline metabolism, phenylalanine metabolism, glycolysis/gluconeogenesis, and ascorbate (AsA) and aldarate metabolism. The DAPs related to energy, carbohydrates, amino acids, glutathione (GSH), AsA, and secondary metabolism are examined in greater detail in the following sections.

#### 3.4. DAPs Involved in Carbohydrate Metabolism

Thirty-eight proteins were predicted to be associated with carbohydrate metabolism, with 16 involved in starch and sucrose metabolism, 17 involved in glycolysis, 3 involved in galactose metabolism, and 2 involved in the citrate cycle (TCA cycle) (Table 1). Most of the glycolysis-related enzymes, such as enolase (PGH1), triosephosphate isomerase, phosphoglucomutase (PGMP), glyceraldehyde-3-phosphate dehydrogenase (GAPC), ATP-dependent 6-phosphofructokinase 6 (PFK6), and pyruvate decarboxylase (PDC), were significantly upregulated in pitaya in response to drought stress. Additionally, the accumulation of some key enzymes involved in starch and sucrose metabolism, including isoamylase 3 (ISA3), 4-alpha-glucanotransferase (DPEP), inactive beta-amylase (BAM9), and alpha-amylase 3 (AMY3), increased significantly and promoted starch degradation in pitaya under drought-stress conditions. Interestingly, aldehyde dehydrogenase family 3 members ALDH3F1 and ALDH3H1 exhibited the opposite changes in abundance. A similar difference was detected in the changes to the accumulation of a probable starch synthase 4 (SS4) and starch synthase 1 (SS1).



**Figure 3.** Gene ontology (GO) analysis of DAPs in pitaya stems under drought-stress conditions at 6 h (a) and 3 days (b). Red and blue bars represent upregulated and downregulated proteins, respectively.



**Figure 4.** KEGG analysis of DAPs in pitaya stems under drought-stress conditions at 6 h (a) and 3 days (b).

### 3.5. DAPs Involved in Energy Metabolism

Included among the DAPs were 21 proteins predicted to be associated with energy metabolism, with 10 involved in oxidative phosphorylation, 7 involved in carbon fixation in photosynthetic organisms, and 4 involved in photosynthesis (Table 2). Mitochondrial uncoupling protein 1 (PUMP1) and external alternative NAD(P)H-ubiquinone oxidoreductase B2 (NDB2) associated with oxidative phosphorylation, as well as NADP-dependent malic enzyme-related proteins and phosphoenolpyruvate carboxylase (PPC) associated with carbon fixation in photosynthetic organisms, were upregulated in both comparisons. In contrast, NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 9 (CIB22), V-type proton ATPase subunit G1 (VATG1), and acyl carrier protein 2 (MTACP2), associated with oxidative phosphorylation, were upregulated DAPs only in the OS6H vs. NS6H comparison. Additionally, V-type proton ATPase subunit a3 (VHA-a3) and V-type proton ATPase subunit E (VATE), associated with oxidative phosphorylation, were upregulated DAPs only in the OS3D vs. NS3D comparison. Two DAPs, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 6 (At3g12260) and plasma membrane ATPase 4 (PMA4), associated with oxidative phosphorylation, were downregulated in the OS3D vs. NS3D comparison. The photosynthesis-related DAPs, which were specific to the OS6H vs. NS6H comparison, included an antenna protein (chlorophyll a-b binding protein 7 (LHCB7)) and two photosystem I reaction center subunits (subunit IV (PSAE-1) and subunit VI (PSAH)), which were upregulated, and protease Do-like 2 (DEGP2), which was downregulated. Overall, energy metabolism was enhanced in pitaya under drought-stress conditions.

### 3.6. DAPs Involved in Amino Acid Metabolism

Of the DAPs induced by drought stress, 14 (5 upregulated and 9 downregulated) were involved in amino acid metabolism (Table 3). Glutamine synthetase (GLN2) was downregulated in both comparisons. Some key enzymes were detected as DAPs only in the OS6H vs. NS6H comparison, including ornithine aminotransferase ( $\delta$ -OAT), NO-associated protein 1 (NOA1), and spermine synthase (SPMS), which are associated with arginine and proline metabolism. Furthermore, the following proteins were differentially abundant only in the OS3D vs. NS3D comparison: proline-rich receptor-like protein kinase (PERK1), involved in proline metabolism; hydroxyphenylpyruvate reductase (HPPR) and arogenate dehydratase/prephenate dehydratase 6 (ADT6), involved in phenylalanine metabolism; and glutamate decarboxylase (GAD), gamma-aminobutyrate transaminase 3 (GABA-TP3),

aspartate aminotransferase 3 (ASP3), and glutamine synthetase nodule isozyme, involved in aspartate and glutamate metabolism.

### 3.7. DAPs Involved in GSH and AsA Metabolism

Eleven DAPs were revealed to be involved in GSH and AsA metabolism, including seven involved in AsA metabolism and five involved in GSH metabolism (Table 4). Regarding the AsA metabolism-related proteins, probable 2-oxoglutarate-dependent dioxygenase (At5g05600) and L-ascorbate peroxidase T (APXT) were upregulated DAPs in both comparisons. However, the downregulated proteins UDP-glucose 6-dehydrogenase 4 (UGD4) and UDP-glucose 6-dehydrogenase 5 (UGD5) were detected only in the OS6H vs. NS6H comparison. Additionally, L-galactose dehydrogenase (LGALDH) and L-ascorbate oxidase homolog (Bp10) were detected only in the OS3D vs. NS3D comparison. Moreover, the following four proteins associated with GSH metabolism were upregulated DAPs only in the OS3D vs. NS3D comparison: glutathione S-transferase T1 (GSTT1), IN2-1 homolog B (GSTZ5), and two glucose-6-phosphate 1-dehydrogenase 6 (G6PD6) proteins.

### 3.8. DAPs Involved in Secondary Metabolism

The DAPs induced by drought stress also contributed to secondary metabolism (Table 5). Specifically, a probable tocopherol O-methyltransferase (VET4), involved in tocopherol synthesis, as well as caffeic acid 3-O-methyltransferase (COMT1), involved in phenylpropanoid synthesis, were upregulated DAPs in both comparisons. Superoxide dismutase (Cu–Zn) (SODCP) in the antioxidation system was an upregulated DAP only in the OS6H vs. NS6H comparison. Seven DAPs were exclusive to the OS3D vs. NS3D comparison, of which peroxidase 4 (GSVIVT00023967001), involved in phenylpropanoid synthesis, as well as a probable carotenoid cleavage dioxygenase 4 (CCD4) and lycopene beta-cyclase (LCY1), involved in carotenoid biosynthesis, were upregulated, whereas omega-hydroxypalmitate O-feruloyl transferase (HHT1) and a probable cinnamyl alcohol dehydrogenase (CAD6), involved in phenylpropanoid synthesis; anthocyanidin-3-O-glucoside rhamnosyltransferase (RT), involved in flavonoid synthesis; and anthocyanidin 3-O-glucosyltransferase 2 (FGT), involved in anthocyanin biosynthesis, were downregulated.

Table 1. DAPs are involved in carbohydrate metabolism.

| Protein ID                    | Description  | Protein Abbreviation | OS6H/NS6H Ratio | OS6H/NS6H <i>p</i> -Value | OS3D/NS3D Ratio | OS3D/NS3D <i>p</i> -Value |
|-------------------------------|--|----------------------|-----------------|---------------------------|-----------------|---------------------------|
| Glycolysis                    |  |                      |                 |                           |                 |                           |
| TRINITY_DN1285_c0_g1_m.2918   | Pyrophosphate—fructose 6-phosphate 1-phosphotransferase subunit beta | PPF-BETA             | 1.2232          | 0.028736                  | ns              | 0.491619                  |
| TRINITY_DN1345_c0_g1_m.3326   | Enolase  | PGH1                 | 1.2113          | 0.023062                  | ns              | 0.176205                  |
| TRINITY_DN1541_c1_g1_m.4614   | Triosephosphate isomerase  | –                    | 1.2205          | 0.048792                  | ns              | 0.190237                  |
| TRINITY_DN22699_c0_g1_m.8480  | Plastidial pyruvate kinase 2   | PKP2                 | 0.7949          | 0.012965                  | ns              | 0.071468                  |
| TRINITY_DN680_c2_g1_m.20738   | Fructose-1,6-bisphosphatase  | –                    | 0.6861          | 0.001059                  | ns              | 0.068059                  |
| TRINITY_DN700_c0_g1_m.21125   | Phosphoglucomutase   | PGMP                 | 1.2058          | 0.003547                  | ns              | 0.09038                   |
| TRINITY_DN8402_c0_g1_m.23765  | Aldehyde dehydrogenase family 3 member F1                            | ALDH3F1              | 1.4108          | 0.034248                  | 1.4821          | 0.004249                  |
| TRINITY_DN956_c0_g1_m.25499   | Glyceraldehyde-3-phosphate dehydrogenase                             | GAPC                 | 1.2105          | 0.00537                   | ns              | 0.23787                   |
| TRINITY_DN956_c0_g1_m.25500   | Glyceraldehyde-3-phosphate dehydrogenase                             | GAPC                 | 1.2849          | 0.006127                  | ns              | 0.272781                  |
| TRINITY_DN1378_c0_g1_m.3547   | ATP-dependent 6-phosphofructokinase 6                                | PFK6                 | ns              | 0.052857                  | 2.8748          | 0.008707                  |
| TRINITY_DN188_c0_g2_m.6607    | Aldehyde dehydrogenase family 3 member F1                            | ALDH3F1              | ns              | 0.102474                  | 0.7175          | 0.042614                  |
| TRINITY_DN2017_c0_g1_m.7240   | Pyruvate decarboxylase 4   | PDC4                 | ns              | 0.091026                  | 1.7692          | 0.002958                  |
| TRINITY_DN2017_c0_g2_m.7243   | Pyruvate decarboxylase 1   | PDC1                 | ns              | 0.087881                  | 1.2044          | 0.012331                  |
| TRINITY_DN2514_c0_g1_m.9503   | Aldehyde dehydrogenase family 3 member H1                            | ALDH3H1              | ns              | 0.724813                  | 0.6052          | 0.012778                  |
| TRINITY_DN5539_c0_g1_m.18129  | Alcohol dehydrogenase class-3  | –                    | ns              | 0.526004                  | 1.2465          | 0.00285                   |
| TRINITY_DN785_c0_g1_m.22777   | Hexokinase-3   | At1g50460            | ns              | 0.97966423                | 0.6069          | 0.002177                  |
| Starch and sucrose metabolism |  |                      |                 |                           |                 |                           |
| TRINITY_DN120_c1_g1_m.2258    | Beta-fructofuranosidase, soluble isoenzyme I                         | INV*DC4              | 1.2139          | 0.025473                  | ns              | 0.36858184                |
| TRINITY_DN1281_c0_g1_m.2874   | 4-alpha-glucanotransferase   | DPEP                 | 1.3783          | 0.001518                  | ns              | 0.065598773               |
| TRINITY_DN2311_c0_g1_m.8701   | Alpha-1,4 glucan phosphorylase L isozyme                             | –                    | 1.2988          | 0.015376                  | 1.6666          | 0.033337                  |
| TRINITY_DN243_c0_g1_m.9202    | Glucose-1-phosphate adenyltransferase large subunit 1                | AGPS1                | 1.3654          | 0.002383                  | ns              | 0.065961158               |
| TRINITY_DN243_c0_g2_m.9203    | Glucose-1-phosphate adenyltransferase large subunit 1                | AGPS1                | 1.4417          | 0.005241                  | ns              | 0.082784957               |
| TRINITY_DN3421_c0_g1_m.12709  | Probable starch synthase 4   | SS4                  | 0.8081          | 0.000869                  | ns              | 0.436338494               |
| TRINITY_DN396_c0_g2_m.14182   | Isoamylase 3   | ISA3                 | 1.2597          | 0.038832                  | 2.2276          | 0.005774                  |
| TRINITY_DN558_c0_g2_m.18286   | Glucan endo-1,3-beta-glucosidase 1                                   | At1g11820            | 0.7347          | 0.009098                  | ns              | 0.258131706               |
| TRINITY_DN6728_c0_g1_m.20594  | Inactive beta-amylase 9  | BAM9                 | 2.8986          | 5.89E-05                  | ns              | 0.422387234               |
| TRINITY_DN8071_c0_g1_m.23172  | Glucose-1-phosphate adenyltransferase small subunit                  | AGPB1                | 1.3814          | 0.013501                  | ns              | 0.152380603               |
| TRINITY_DN2107_c0_g1_m.7698   | Probable alpha,alpha-trehalose-phosphate synthase                    | TPS11                | ns              | 0.107875285               | 0.6784          | 0.047779                  |
| TRINITY_DN409_c0_g3_m.14564   | Alpha-glucosidase  | –                    | ns              | 0.145618967               | 1.3875          | 0.047325                  |
| TRINITY_DN483_c0_g2_m.16576   | Alpha-1,4 glucan phosphorylase L isozyme,                            | –                    | ns              | 0.241229313               | 1.2924          | 0.04298                   |
| TRINITY_DN4852_c0_g1_m.16596  | Alpha-amylase 3  | AMY3                 | ns              | 0.629154053               | 1.4422          | 0.043718                  |
| TRINITY_DN6640_c0_g1_m.20450  | Glucose-1-phosphate adenyltransferase large subunit                  | AGPS1                | ns              | 0.11514614                | 1.3167          | 0.030977                  |
| TRINITY_DN7735_c0_g1_m.22545  | 4-alpha-glucanotransferase   | DPEP                 | ns              | 0.15889801                | 1.3818          | 0.01198                   |
| TRINITY_DN8896_c0_g1_m.24535  | Starch synthase 1  | SS1                  | ns              | 0.999352844               | 1.4676          | 0.046376                  |

Table 1. Cont.

| Protein ID                   | Description   | Protein Abbreviation | OS6H/NS6H Ratio | OS6H/NS6H <i>p</i> -Value | OS3D/NS3D Ratio | OS3D/NS3D <i>p</i> -Value |
|------------------------------|---|----------------------|-----------------|---------------------------|-----------------|---------------------------|
|                              | Citrate cycle (TCA cycle)                           |                      |                 |                           |                 |                           |
| TRINITY_DN12082_c0_g1_m.2243 | Citrate synthase                                    | –                    | ns              | 0.664702                  | 1.2021          | 0.020504                  |
| TRINITY_DN3909_c0_g2_m.14018 | ATP-citrate synthase alpha chain protein 1          | ACLA-1               | ns              | 0.137801                  | 1.2304          | 0.002227                  |
|                              | Galactose metabolism                                |                      |                 |                           |                 |                           |
| TRINITY_DN455_c0_g1_m.15769  | Probable galactinol–sucrose galactosyltransferase 6 | RFS6                 | 1.255           | 0.02696                   | ns              |                           |
| TRINITY_DN4902_c0_g1_m.16720 | Probable galactinol–sucrose galactosyltransferase 6 | RFS6                 | 1.3             | 0.044023                  | ns              |                           |
| TRINITY_DN327_c0_g1_m.12252  | Alpha-galactosidase 3                               | AGAL3                | ns              |                           | 1.4405          | 0.029031                  |

Note: “–” = not found or did not exist; “ns” = no significant difference. Pitaya seedlings exposed to drought stress for 6 h and 3 days were designated as OS6H and OS3D, respectively, with the corresponding controls designated as NS6H and NS3D, respectively.

Table 2. DAPs are involved in energy metabolism.

| Protein ID                    | Description  | Protein Abbreviation | OS6H/NS6H Ratio | OS6H/NS6H <i>p</i> -Value | OS3D/NS3D Ratio | Os3d/NS3d <i>p</i> -Value |
|-------------------------------|--|----------------------|-----------------|---------------------------|-----------------|---------------------------|
|                               | Oxidative phosphorylation                                    |                      |                 |                           |                 |                           |
| TRINITY_DN130_c0_g2_m.3091    | NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 9  | CIB22                | 1.215           | 0.006                     | ns              | 0.481                     |
| TRINITY_DN284_c10_g1_m.10791  | V-type proton ATPase subunit G 1                             | VATG1                | 2.006           | 0.022                     | ns              | 0.355                     |
| TRINITY_DN5440_c0_g1_m.17954  | External alternative NAD(P)H-ubiquinone oxidoreductase B2    | NDB2                 | 1.295           | 0.024                     | ns              | 0.406                     |
| TRINITY_DN7109_c0_g2_m.21284  | Mitochondrial uncoupling protein 1                           | PUMP1                | 1.285           | 0.006                     | 1.2331          | 0.016                     |
| TRINITY_DN1470_c0_g1_m.4137   | V-type proton ATPase subunit a3                              | VHA-a3               | ns              | 0.059                     | 1.592           | 0.034                     |
| TRINITY_DN30311_c0_g1_m.11465 | V-type proton ATPase subunit E                               | VATE                 | ns              | 0.039                     | 1.224           | 0.025                     |
| TRINITY_DN5440_c0_g1_m.17953  | External alternative NAD(P)H-ubiquinone oxidoreductase B2    | NDB2                 | ns              | 0.084                     | 1.202           | 0.014                     |
| TRINITY_DN6767_c0_g2_m.20656  | Acyl carrier protein 2                                       | MTACP2               | 1.264           | 0.003                     | ns              | 0.131                     |
| TRINITY_DN10196_c0_g1_m.265   | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6 | At3g12260            | ns              | 0.293                     | 0.826           | 0.014                     |
| TRINITY_DN517_c0_g3_m.17409   | Plasma membrane ATPase 4                                     | PMA4                 | ns              | 0.15                      | 0.726           | 0.022                     |
|                               | Carbon fixation in photosynthetic organisms                  |                      |                 |                           |                 |                           |
| TRINITY_DN1003_c0_g1_m.71     | NAD-dependent malic enzyme 62 kDa isoform                    | –                    | 1.2388          | 0.005                     | ns              | 0.196015                  |
| TRINITY_DN1003_c0_g1_m.72     | NAD-dependent malic enzyme 62 kDa isoform                    | –                    | 1.2197          | 0.004638                  | 1.2602          | 0.018536                  |
| TRINITY_DN1199_c0_g1_m.2179   | NAD-dependent malic enzyme 59 kDa isoform                    | –                    | 1.2055          | 0.012349                  | ns              | 0.187842                  |
| TRINITY_DN21851_c0_g1_m.8123  | NADP-dependent malic enzyme                                  | –                    | 1.4738          | 0.028002                  | ns              | 0.184188                  |

Table 2. Cont.

| Protein ID                   | Description   | Protein Abbreviation | OS6H/NS6H Ratio | OS6H/NS6H <i>p</i> -Value | OS3D/NS3D Ratio | Os3d/NS3d <i>p</i> -Value |
|------------------------------|---|----------------------|-----------------|---------------------------|-----------------|---------------------------|
| TRINITY_DN54_c0_g1_m.18065   | Phosphoenolpyruvate carboxylase                     | PPC16                | 1.3809          | 0.006254                  | 2.0725          | 0.015201                  |
| TRINITY_DN5722_c0_g1_m.18543 | Phosphoenolpyruvate carboxylase 1                   | PPC1                 | 1.5942          | 0.029038                  | 2.5273          | 0.005758                  |
| TRINITY_DN5722_c0_g1_m.18545 | Phosphoenolpyruvate carboxylase 1<br>Photosynthesis | PPC1                 | 1.7533          | 0.024815                  | 1.471           | 0.019766                  |
| TRINITY_DN3928_c2_g1_m.14056 | Chlorophyll a-b binding protein 7                   | LHCB7                | 1.311           | 0.048                     | ns              | 0.216                     |
| TRINITY_DN2207_c0_g1_m.8225  | Photosystem I reaction center subunit IV            | PSAE-1               | 1.24            | 0.003                     | ns              | 0.244                     |
| TRINITY_DN4734_c0_g1_m.16295 | Photosystem I reaction center subunit VI            | PSAH                 | 1.422           | 0.048                     | ns              | 0.091                     |
| TRINITY_DN2097_c1_g1_m.7621  | Protease Do-like 2                                  | DEGP2                | 0.746           | 0.007                     | ns              | 0.39                      |

Note: “–” = not found or did not exist; “ns” = no significant difference. Pitaya seedlings exposed to drought stress for 6 h and 3 days were designated as OS6H and OS3D, respectively, with the corresponding controls designated as NS6H and NS3D, respectively.

Table 3. DAPs are involved in amino acid metabolism.

| Protein ID                   | Description                                    | Protein Abbreviation | OS6H/NS6H Ratio | OS6H/NS6H <i>p</i> -Value | OS3D/NS3D Ratio | OS3D/NS3D <i>p</i> -Value |
|------------------------------|--|----------------------|-----------------|---------------------------|-----------------|---------------------------|
|                              | Aspartate and glutamate metabolism             |                      |                 |                           |                 |                           |
| TRINITY_DN1488_c1_g1_m.4241  | Glutamine synthetase                           | GLN2                 | 0.7957          | 0.026414                  | ns              | 0.97597626                |
| TRINITY_DN1404_c3_g1_m.3689  | Glutamate decarboxylase                        | GAD                  | ns              | 0.0013574                 | 1.3966          | 0.027208                  |
| TRINITY_DN294_c0_g1_m.11144  | Gamma aminobutyrate transaminase 3             | GABA-TP3             | ns              | 0.683342268               | 1.2024          | 0.010061                  |
| TRINITY_DN7757_c0_g1_m.22593 | Aspartate aminotransferase 3                   | ASP3                 | ns              | 0.141381977               | 1.272           | 0.00091                   |
| TRINITY_DN1488_c1_g1_m.4239  | Glutamine synthetase                           | GLN2                 | ns              | 0.064775788               | 0.8262          | 0.037899                  |
| TRINITY_DN15906_c0_g1_m.4931 | Glutamine synthetase nodule isozyme            | –                    | ns              | –                         | 0.7607          | 0.027319                  |
|                              | Arginine and proline metabolism                |                      |                 |                           |                 |                           |
| TRINITY_DN1617_c1_g1_m.5135  | Ornithine aminotransferase                     | DELTA-OAT            | 1.2603          | 0.008374713               | ns              | 0.258228322               |
| TRINITY_DN809_c0_g2_m.23215  | Proline-rich receptor-like protein kinase      | PERK1                | ns              | 0.160140522               | 0.7234          | 0.001426311               |
| TRINITY_DN6277_c0_g2_m.19711 | NO-associated protein 1                        | NOA1                 | 0.8235          | 0.019199161               | ns              | 0.055348656               |
| TRINITY_DN7062_c0_g1_m.21204 | Spermine synthase                              | SPMS                 | 0.7974          | 0.014501                  | ns              | 0.35623713                |
|                              | Phenylalanine metabolism                       |                      |                 |                           |                 |                           |
| TRINITY_DN10229_c1_g1_m.315  | Hydroxyphenylpyruvate reductase                | HPPR                 | ns              | 0.735219447               | 1.2086          | 0.003324                  |
| TRINITY_DN3220_c0_g1_m.12107 | Probable enoyl-CoA hydratase 1                 | ECHIA                | 0.8204          | 0.031414137               | ns              | 0.293016083               |
| TRINITY_DN185_c0_g1_m.6424   | Histidinol-phosphate aminotransferase          | HPA                  | ns              | 0.024259888               | 0.8195          | 0.015681                  |
| TRINITY_DN2397_c0_g1_m.9007  | Arogenate dehydratase/prephenate dehydratase 6 | ADT6                 | ns              | 0.081337319               | 0.7012          | 0.012254                  |

Note: “–” = not found or did not exist; “ns” = no significant difference. Pitaya seedlings exposed to drought stress for 6 h and 3 days were designated as OS6H and OS3D, respectively, with the corresponding controls designated as NS6H and NS3D, respectively.

**Table 4.** DAPs are involved in glutathione and ascorbate metabolism.

| Protein ID                   | Description                                   | Protein Abbreviation | OS6H/NS6H Ratio | OS6H/NS6H <i>p</i> -Value | OS3D/NS3D Ratio | OS3D/NS3D <i>p</i> -Value |
|------------------------------|---|----------------------|-----------------|---------------------------|-----------------|---------------------------|
|                              | Ascorbate metabolism                          |                      |                 |                           |                 |                           |
| TRINITY_DN1613_c0_g1_m.5115  | UDP-glucose 6-dehydrogenase 4                 | UGD4                 | 0.6064          | 0.006265                  | ns              | 0.151395367               |
| TRINITY_DN1613_c0_g2_m.5117  | UDP-glucose 6-dehydrogenase 5                 | UGD5                 | 0.8276          | 0.020249                  | ns              | 0.671819139               |
| TRINITY_DN1595_c0_g1_m.4962  | L-galactose dehydrogenase                     | LGALDH               | ns              | 0.701474521               | 1.291           | 0.036156                  |
| TRINITY_DN3248_c0_g1_m.12163 | Probable 2-oxoglutarate-dependent dioxygenase | At5g05600            | 1.8511          | 0.006704                  | 4.1204          | 0.0000578                 |
| TRINITY_DN893_c0_g1_m.24609  | L-ascorbate peroxidase T                      | APXT                 | ns              | 0.841331205               | 1.2801          | 0.0015628                 |
| TRINITY_DN893_c0_g1_m.24610  | L-ascorbate peroxidase T                      | APXT                 | 1.2609          | 0.039256535               | ns              | 0.056852039               |
| TRINITY_DN427_c0_g2_m.15091  | L-ascorbate oxidase homolog                   | Bp10                 | ns              | 0.562150148               | 1.2159          | 0.013967466               |
|                              | Glutathione metabolism                        |                      |                 |                           |                 |                           |
| TRINITY_DN4975_c0_g1_m.16910 | Glutathione S-transferase T1                  | GSTT1                | ns              | 0.117978                  | 1.6817          | 0.0466                    |
| TRINITY_DN178_c0_g1_m.6079   | Protein IN2-1 homolog B                       | GSTZ5                | ns              | 0.262995961               | 1.3097          | 0.011018                  |
| TRINITY_DN2046_c0_g1_m.7392  | Glucose-6-phosphate 1-dehydrogenase 6         | G6PD6                | ns              | 0.019788019               | 1.2133          | 0.009388                  |
| TRINITY_DN2046_c0_g1_m.7393  | Glucose-6-phosphate 1-dehydrogenase 6         | G6PD6                | ns              | 0.321337379               | 1.255           | 0.04952                   |

Note: “ns” = no significant difference. Pitaya seedlings exposed to drought stress for 6 h and 3 days were designated as OS6H and OS3D, respectively, with the corresponding controls designated as NS6H and NS3D, respectively.

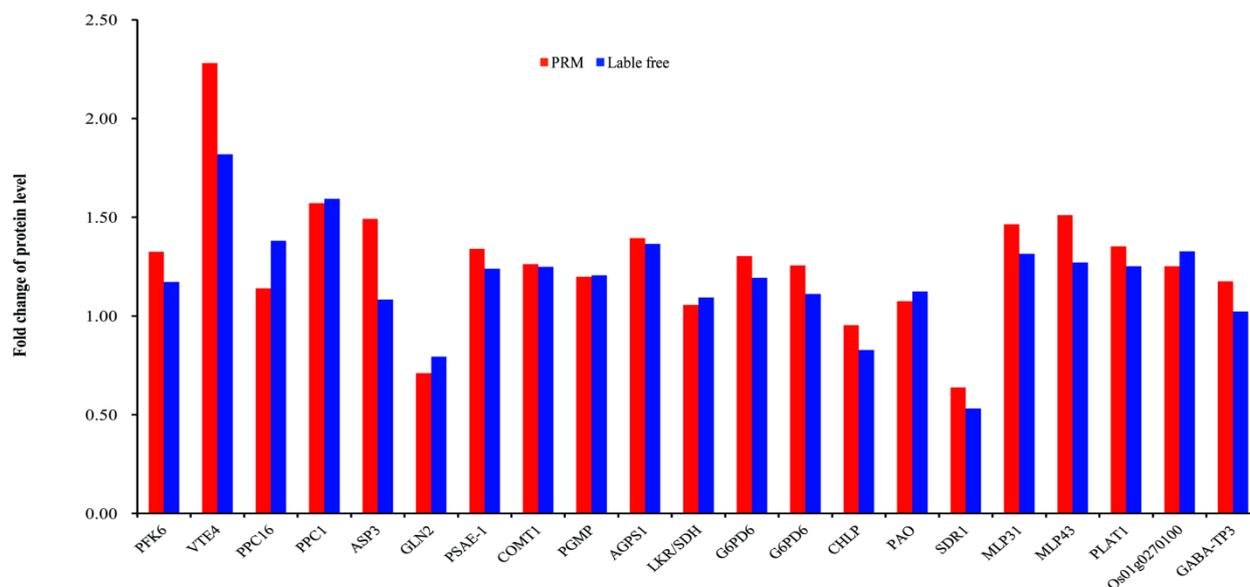
**Table 5.** DAPs are involved in secondary metabolism.

| Protein ID                    | Description                                      | Protein Abbreviation | OS6H/NS6H Ratio | OS6H/NS6H <i>p</i> -Value | OS3D/NS3D Ratio | OS3D/NS3D <i>p</i> -Value |
|-------------------------------|--|----------------------|-----------------|---------------------------|-----------------|---------------------------|
|                               | Tocopherol                                       |                      |                 |                           |                 |                           |
| TRINITY_DN1553_c0_g1_m.4682   | Probable tocopherol O-methyltransferase          | VET4                 | 1.82            | 0.014                     | 2.725           | 0.014                     |
|                               | Flavone and flavonol biosynthesis                |                      |                 |                           |                 |                           |
| TRINITY_DN12687_c0_g1_m.2773  | Anthocyanidin-3-O-glucoside rhamnosyltransferase | RT                   | ns              | 0.299                     | 0.729           | 0.017                     |
|                               | Phenylpropanoid biosynthesis                     |                      |                 |                           |                 |                           |
| TRINITY_DN6823_c0_g1_m.20755  | Caffeic acid 3-O-methyltransferase               | COMT1                | 1.249           | 0.049                     | 2.209           | 0.014                     |
| TRINITY_DN10780_c0_g1_m.946   | Omega-hydroxypalmitate O-feruloyl transferase    | HHT1                 | ns              | 0.748                     | 0.374           | 0.000248                  |
| TRINITY_DN8170_c0_g2_m.23343  | Probable cinnamyl alcohol dehydrogenase          | CAD6                 | ns              | 0.178                     | 0.72            | 0.048                     |
|                               | Anthocyanin biosynthesis                         |                      |                 |                           |                 |                           |
| TRINITY_DN142_c0_g2_m.3870    | Anthocyanidin 3-O-glucosyltransferase 2          | FGT                  | ns              | 0.522                     | 0.792           | 0.049                     |
|                               | Carotenoid biosynthesis                          |                      |                 |                           |                 |                           |
| TRINITY_DN2792_c0_g2_m.10610  | Probable carotenoid cleavage dioxygenase 4       | CCD4                 | ns              | 0.264956118               | 1.4016          | 0.038862                  |
| TRINITY_DN92199_c0_g1_m.25001 | Lycopene beta cyclase                            | LCY1                 | ns              | 0.699025813               | 1.3876          | 0.014785649               |
|                               | Antioxidation system                             |                      |                 |                           |                 |                           |
| TRINITY_DN4793_c1_g1_m.16452  | Superoxide dismutase [Cu-Zn]                     | SODCP                | 1.3609          | 0.013743234               | ns              | 0.204307412               |
| TRINITY_DN13770_c0_g1_m.3537  | Peroxidase 4                                     | GSVIT00023967001     | ns              | 0.866                     | 3.076           | 0.043                     |

Note: “ns” = no significant difference. Pitaya seedlings exposed to drought stress for 6 h and 3 days were designated as OS6H and OS3D, respectively, with the corresponding controls designated as NS6H and NS3D, respectively.

### 3.9. Validation of Proteomics Data by PRM

The label-free quantitative proteomics results were validated by PRM, which is a widely used and efficient method for precisely quantifying and verifying an array of target proteins of interest [31]. In this study, 21 DAPs annotated with GO terms and assigned to KEGG pathways were selected for the PRM analysis. Two unique peptides predicted to be stable were chosen for each protein, except for six DAPs, which had only one unique peptide. The relative protein abundance was expressed as the average of the two normalized peptide peak areas (Tables S4 and S5). The PRM results (Figure 5) were consistent with the label-free quantitative proteomics data.



**Figure 5.** Validation of 21 selected DAPs via parallel reaction monitoring. The ordinate represents the OS6H/NS6H ratio. Abbreviated enzyme names are listed in Table S6.

## 4. Discussion

Drought is a major environmental factor affecting crop growth and yield. Treating plants with PEG is an effective method for simulating drought-stress conditions [32]. Pitaya is a widely cultivated crop in dry regions because of its relatively high tolerance to long-term drought stress and its ability to grow in low-quality soils [18]. Therefore, pitaya is a potentially useful research material for elucidating plant responses to drought stress [33]. In the current study, pitaya responses to drought stress induced by PEG were examined. The results provided evidence of the physiological and molecular changes at the protein level in pitaya exposed to drought stress.

### 4.1. Physiological Responses

To cope with drought stress, plants have evolved a range of physiological and biochemical mechanisms [34]. One of the most important mechanisms involves the accumulation of compatible osmolytes, including glycine betaine, soluble sugars, soluble proteins, and free proline [35]. In this study, pitaya plants were exposed to varying durations of drought stress. Compared with the control levels, the soluble protein and proline contents of stressed pitaya did not change significantly in the initial drought-treatment stage, but they increased significantly after three days of drought stress (Figure 1b,c). These results implied that osmoregulation is an important physiological mechanism enabling pitaya to adapt to drought conditions.

As the final product of lipid peroxidation, MDA is widely considered to be an important physiological marker of membrane system injuries induced by stresses [33]. Reactive oxygen species (ROS), such as  $H_2O_2$  and  $O_2^-$ , are some of the most damaging molecules

in plants, capable of inducing changes at the cellular level, leading to membrane damage, protein oxidation, and alterations to DNA [36]. Earlier research concluded that MDA, H<sub>2</sub>O<sub>2</sub>, and O<sub>2</sub><sup>-</sup> levels increased significantly following an exposure to drought stress [6]. The accumulation of ROS can be minimized by an array of nonenzymatic antioxidants (e.g., metabolites such as AsA, alpha-tocopherol, carotenoids, and GSH) and antioxidant enzymes (e.g., SOD and AsA-GSH cycle enzymes, including GR and APX) [37]. In accordance with the findings of Cao et al. [38], the physiological responses of pitaya to drought stress observed in this study included increased lipid peroxidation (as reflected by MDA, O<sub>2</sub><sup>-</sup>, and H<sub>2</sub>O<sub>2</sub> levels) and enhanced antioxidant enzyme activities (e.g., SOD and APX) (Figure 1d–i). Moreover, tocopherol and carotenoid biosynthesis-related proteins were also identified as upregulated DAPs under drought-stress conditions (Table 5).

The ASA–GSH pathway, which maintains the ratios of reduced and oxidized AsA and GSH, affects the scavenging of ROS in plant cells [38]. These ratios are maintained by APX and GR [39]. An earlier investigation demonstrated that GR contents increased during exposure to drought stress [40]. In the present study, compared with the control levels, GR activities were significantly higher and lower in stressed pitaya samples at 6 and 18 h after initiating the drought-stress treatment, respectively (Figure 1g). These results indicated that GR is sensitive to drought stress and may be important for early pitaya responses to drought conditions.

#### 4.2. Carbohydrate Metabolism

Carbon, which is indispensable for energy circulation and survival, is continuously metabolized in plants, even under adverse conditions [41]. Glycolysis is an important metabolic pathway for carbohydrate metabolism in all living organisms, and releases a small amount of energy in the absence of oxygen [42]. In the present study, most of the enzymes involved in glycolysis were upregulated DAPs in response to drought stress (Table 1). Among these enzymes, PFK6 is the rate-limiting enzyme for glycolysis and GAPC is widely considered to be responsive to drought stress [43]. Moreover, GAPC may restrict the accumulation of ROS and promote root growth in plants under drought conditions [42]. Interestingly, drought stress increased the ALDH3F1 content, but had the opposite effect on the accumulation of ALDH3H1. Accordingly, the aldehyde dehydrogenase family 3 members were differentially responsive to carbohydrate depletion, which was consistent with the findings of an earlier study [44] (Table 1). Stress-associated aldehyde dehydrogenases are important for scavenging ROS and catalyzing the oxidation of toxic aldehydes to produce nontoxic carboxylic acids. Additionally, they participate in several pathways and regulate diverse signal transductions [45]. Regarding starch and sucrose metabolism, starch degradation is promoted by the upregulated expression of some key genes, including those encoding ISA3 [46], DPEP [47], BAM9 [48], and AMY3 [49]. A previous study proved that the increases in soluble sugar contents in response to drought stress mainly resulted from the degradation and transformation of insoluble carbohydrates [50]. The activities of these enzymes may increase the soluble sugar content, which was consistent with the results of physiological determination (Table 1, Figure 1a). The drought-stress treatment in the current study had the opposite effects on the contents of two starch synthases (SS4 and SS1). A recent study indicated that SS4 contributed to the initiation of starch granules [48]. However, the exact roles of these enzymes in pitaya plants exposed to drought stress remain uncharacterized. Starch synthase activity affects chain elongation, as well as the branch placement resulting from the balanced activities of starch branching and debranching enzymes [51]. These results reflected the complexity of the changes in the expression of genes associated with the starch and sucrose metabolic pathway in pitaya plants under drought conditions.

#### 4.3. Energy Metabolism

Several proteins associated with oxidative phosphorylation were activated in response to drought stress in pitaya plants (Table 2). Among these proteins, NADH dehydroge-

nase is involved in the classical electron-transport pathway coupled to ATP synthesis [52]. Additionally, V-ATPase uses energy from ATP hydrolysis to transport protons across membranes, which is important for various cellular processes, as well as acidification-independent processes (e.g., secretion and membrane fusion) [53]. The observed changes to NADH dehydrogenase and V-type proton ATPase subunits indicated that oxidative phosphorylation and ATP generation in pitaya were affected by drought. An earlier investigation proved that the excessive production of PUMP1, which inhibits ROS production under stress conditions by uncoupling the electrochemical gradient from ATP synthesis, induced a hypoxic response [54]. The increased accumulation of PUMP1 detected in this study implied that the relatively flexible energy metabolism of pitaya enables adaptations to drought stress [55]. The NADP-dependent malic enzyme and PPC contents increased in both comparisons. Their responses to drought stress were confirmed in a previous study [56] (Table 2). Moreover, NADP-dependent malic enzyme is considered to have multiple functions that enhance drought resistance. For example, it stabilizes the cytoplasmic pH, controls the stomatal aperture, and provides the essential reductive coenzyme NADPH during flavonoid and lignin biosynthesis. Furthermore, NADPH is crucial for ROS metabolism by the AsA–GSH pathway and NADPH-dependent thioredoxin reductase [57]. The abundance of LHCB7, which is the major antenna protein of photosystem II, increased in stressed pitaya at the 6 h time-point, but there was no difference between the control and stressed samples at 3 days after starting the stress treatment (Table 2). The changes to the accumulation of PSAE-1 and PSAH (i.e., two photosystem I reaction center subunits) were similar to those of LHCB7. The harvesting of light is the first step of photosynthesis. Consequently, the light-harvesting antenna must be regulated in response to the physiological status of plants and environmental signals [58]. A recent study indicated that PSAH expression is upregulated by high salinity and drought [59]. The observed increases in LHCB7, PSAE-1, and PSAH levels may reflect the temporary increase in the light-capturing ability and ion partitioning of pitaya plants under drought conditions. In contrast, the decrease in DEGP2 accumulation detected in this study may inhibit the primary cleavage of the photodamaged D1 protein in the central oxygen-evolving photosystem II reaction center, which has been reported for Arabidopsis plants exposed to salt and desiccation stresses [60]. Overall, drought stress enhances energy metabolism in pitaya, which may provide the energy necessary for initiating other metabolic pathways responsible for short-term adaptations to drought stress.

#### 4.4. Amino Acid Metabolism

Amino acid metabolism plays an important role in signal transduction and osmoregulation [32]. Glutamine synthetase catalyzes the critical incorporation of inorganic ammonium into glutamine in an ATP-dependent manner [61]. A previous study confirmed that the abundance of glutamine synthetase increases in chickpea (*Cicer reticulatum* L.) grown under drought conditions [62]. However, another investigation indicated that the overexpression of genes that encode glutamine synthetase can increase the sensitivity of plants to drought stress [63]. Moreover, glutamine synthetase might influence stress-induced proline production [64]. The opposite changes to the accumulation of GLN2 and  $\delta$ -OAT in the current study were indicative of a negative correlation between these enzymes (Table 3). In addition, the proline content changes lagged behind the changes to the abundance of  $\delta$ -OAT (Figure 1c, Table 3), which is involved in proline synthesis, revealing an inconsistency between some physiological indices and changes in protein levels. Glutamate decarboxylase (GAD) is an enzyme that catalyzes the conversion of L-glutamate into  $\gamma$ -aminobutyric acid (GABA) [65], which accumulates in many plant species in response to environmental stresses [66]. The increased accumulation of GAD and GABA-TP3 in the current study suggested both enzymes may be critical for pitaya adaptations to drought stress. Furthermore, the contents of Asp3 increased substantially in pitaya subjected to drought stress, suggesting ASP3 may be important for the conversion and accumulation of amino acids that protect pitaya plants from the detrimental effects

of drought stress. Spermine and spermidine are useful osmotic solutes and important regulatory signals [67]. NOA1 and SPMS, detected as significantly downregulated DAPs only in the OS6H vs. NS6H comparison, may modulate the early drought stress responses of pitaya (Table 3). Additionally, ADT6, which catalyzes the final step in the biosynthesis of phenylalanine (i.e., the precursor for flavonoid biosynthesis) [68], was a downregulated DAP in the OS3D vs. NS3D comparison (Table 3). A similar change in protein abundance was observed for RT (Table 5). Taken together, drought stress significantly altered the balance between the conversion and accumulation of amino acids in pitaya.

#### 4.5. GSH and AsA Metabolism

The ASA–GSH metabolism is an important antioxidative response pathway for plant responses to drought stress [38]. In the current study, the concentrations of only a few proteins related to this pathway decreased following the exposure to drought stress (Table 4). The increased abundance of most of the vital enzymes in this pathway (e.g., LGALDH, APX, GSTT1, and G6PD6) in the PEG-treated pitaya seedlings was consistent with the observed enhanced enzymatic activities (Figure 1g,i; Table 4). These results were in accordance with following the findings of other studies [69,70]. However, two UDP-glucose 6-dehydrogenases (UGD4 and UGD5) were revealed as downregulated DAPs in the OS6H vs. NS6H comparison, which was in contrast to the results of an earlier investigation [71]. In pitaya plants cultivated under drought conditions, these two enzymes may contribute to the formation of hemicellulose and pectin to strengthen cell walls [72]. Interestingly, the enhancer proteins identified as DAPs in this study (At5g05600) had rarely been reported as plant proteins responsive to drought stress. Hence, they are potentially interesting candidate proteins for future studies on drought-resistant germplasm.

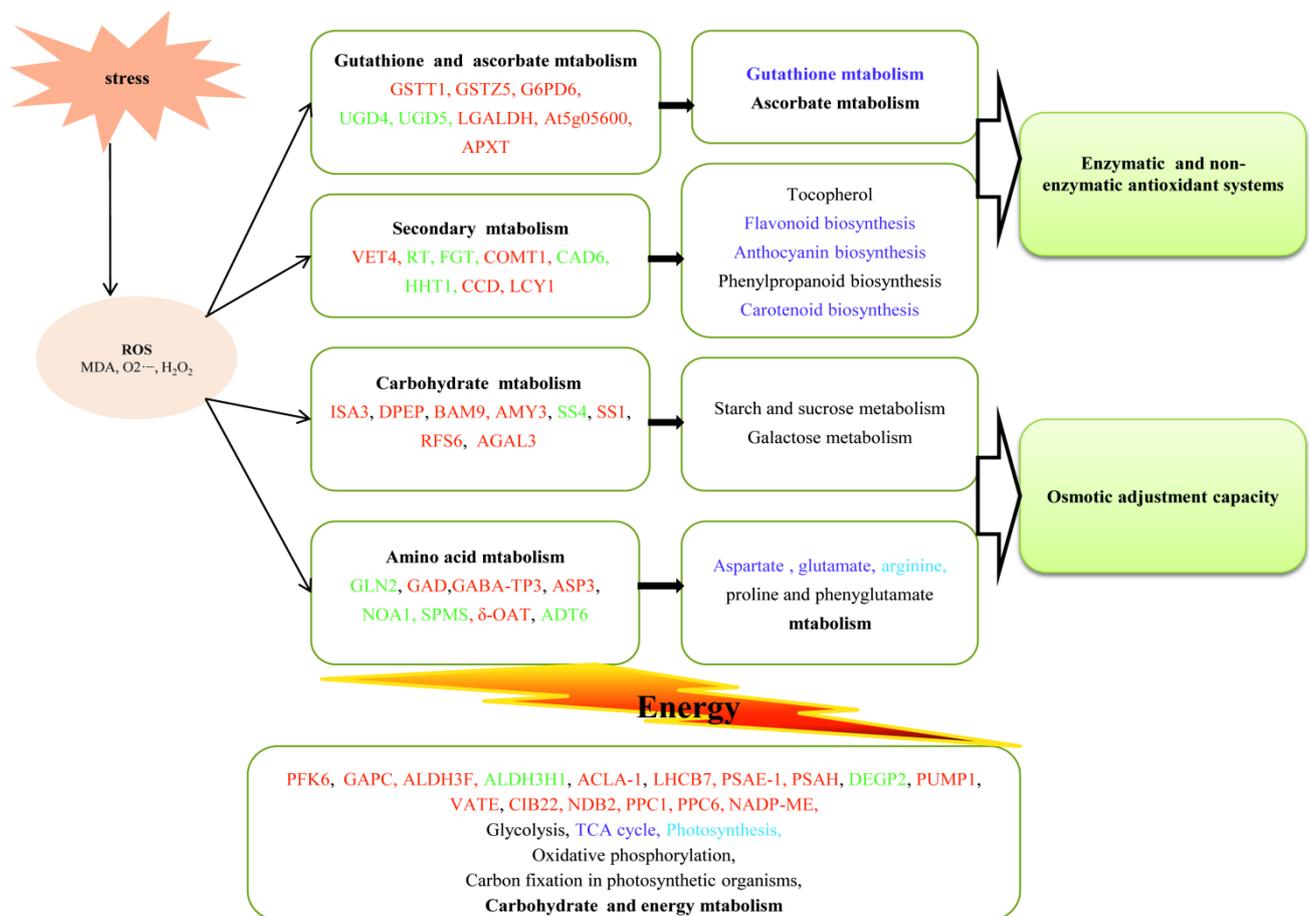
#### 4.6. Secondary Metabolism

Ten DAPs were classified as proteins affecting secondary metabolism (Table 5). Three proteins associated with lignin monomer synthesis accumulated differentially between the control and stressed plants. More specifically, COMT1 was an upregulated DAP in the two comparisons, whereas CAD6 and HHT1 were detected as downregulated DAPs in the OS3D vs. NS3D comparison. The downregulation of CAD6 and HHT1 may have been because lignin accumulation is restricted in pitaya under drought-stress conditions [73,74]. The accumulation of COMT1 generally decreases in response to drought stress. The observed increase in COMT1 abundance in this study is suggestive of additional roles for this protein in the melatonin biosynthesis pathway [75]. Although RT has been identified in *Petunia hybrida* (J. D. Hooker) Vilmorin [76], the effects of drought stress on this protein have not been elucidated. Apocarotenoid compounds, including hormones, pigments, and volatiles, have diverse communication-related functions in plants. Apocarotenoids are produced via the cleavage of carotenoids by carotenoid cleavage dioxygenase (CCD). An earlier study revealed CsCCD4c expression is upregulated by wounding, heat, and drought stress [77]. In tobacco, LCY1 is likely a cyclization-catalyzing enzyme involved in carotenoid accumulation, but it also mediates drought-stress tolerance [78]. The identification of CCD4 and LCY1 as upregulated DAPs in the OS3D vs. NS3D comparison provided additional evidence that these types of enzymes protect plants from drought stress. Additionally, VET4 is reportedly a potential biomarker of oxidative damage [79]. The drought-induced increase in the abundance of VET4 observed in the current study may be involved in mitigating damages due to ROS and lipid peroxidation.

### 5. Conclusions

In summary, in pitaya, drought stress increased osmolyte accumulation, lipid peroxidation, and antioxidant enzyme activities. Proteomic data revealed several pathways were activated in pitaya plants, including carbohydrate and energy metabolism at 6 h and 3 days after starting a simulated drought treatment. Other metabolic activities, including pathways related to aspartate, glutamate, GSH, and secondary metabolites, were affected

more after 3 days than after 6 h. In contrast, photosynthesis and arginine metabolism were modulated solely at the 6 h time-point. Moreover, some key proteins identified in this study, including mitochondrial uncoupling protein 1 (PUMP1), 2-oxoglutarate-dependent dioxygenase (At5g05600), anthocyanidin-3-O-glucoside rhamnosyltransferase (RT), and omega-hydroxypalmitate O-feruloyl transferase (HHT1), have rarely been reported as responsive to drought stress. The possible functions of these proteins influencing drought resistance will need to be experimentally verified. A model for the comparative physiological and proteomic analysis of pitaya responses to PEG-induced drought stress is presented in Figure 6. The high drought tolerance of pitaya was attributed to its high osmotic adjustment capacity and great ability to orchestrate its enzymatic and nonenzymatic antioxidant systems, thus avoiding significant oxidative damage.



**Figure 6.** Model of pitaya responses to PEG-induced drought stress developed according to physiological and proteomic changes. Abbreviated enzyme names are listed in Table S6. Enzymes in green and red represent downregulated and upregulated proteins, respectively. Metabolic pathways in light blue and purple were mainly affected at 6 h and 3 days after initiating a drought-stress treatment, respectively.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/agriculture11070632/s1>, Figure S1: The drought experiment design for pitaya. Pitaya seedlings exposed to drought stress for 6 h and 3 days were designated as OS6H and OS3D, respectively, with the corresponding controls designated as NS6H and NS3D, respectively; Figure S2: Distribution of the number of peptides per protein; Table S1: Detailed information for all identified proteins; Table S2: DAPs in pitaya at 6 h after starting the drought-stress treatment; Table S3: DAPs in pitaya at 3 days after starting the drought-stress treatment; Table S4: Peptide results for 21 DAPs selected for the PRM analysis of pitaya plants exposed to PEG-induced drought stress for 6 h; Table S5: Protein results for 21 DAPs selected for the PRM analysis of pitaya plants exposed to PEG-induced drought stress for 6 h; Table S6: Abbreviated enzyme names in the model.

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## Abbreviations

|                               |  |
|-------------------------------|--|
| PEG                           | Polyethylene glycol                              |
| MDA                           | Malondialdehyde                                  |
| O <sub>2</sub> <sup>-</sup>   | Superoxide anion                                 |
| H <sub>2</sub> O <sub>2</sub> | Hydrogen peroxide                                |
| SOD                           | Superoxide dismutase                             |
| ASA                           | Ascorbate  |
| GSH                           | Glutathione                                      |
| APX                           | Ascorbic peroxidase                              |
| ROS                           | Reactive oxygen species                          |
| GO                            | Gene ontology                                    |
| DAPs                          | Differentially accumulated proteins              |
| PUMP1                         | Mitochondrial uncoupling protein 1               |
| LHCB7                         | Chlorophyll a-b binding protein 7                |
| PSAE-1                        | Photosystem I reaction center subunit IV         |
| PSAH                          | Photosystem I reaction center subunit VI         |
| GAPC                          | Glyceraldehyde-3-phosphate dehydrogenase         |
| SS                            | Starch synthase                                  |
| KEGG                          | Kyoto Encyclopedia of Genes and Genomes          |
| GAD                           | Glutamate decarboxylase                          |
| ASP3                          | Aspartate aminotransferase 3                     |
| OAT                           | Ornithine aminotransferase                       |
| NOA1                          | NO-associated protein 1                          |
| SPMS                          | Spermine synthase                                |
| PRM                           | Parallel reaction monitoring                     |
| G6PD6                         | Glucose-6-phosphate 1-dehydrogenase 6            |
| At5g05600                     | Probable 2-oxoglutarate-dependent dioxygenase    |
| GST                           | Glutathione S-transferase                        |
| LGALDH                        | L-galactose dehydrogenase                        |
| VET4                          | Probable tocopherol O-methyltransferase          |
| CCD                           | Probable carotenoid cleavage dioxygenase         |
| RT                            | Anthocyanidin-3-O-glucoside rhamnosyltransferase |
| COMT1                         | Caffeic acid 3-O-methyltransferase               |
| HHT1                          | Omega-hydroxypalmitate O-feruloyl transferase    |

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