Drought Stress Induces Morpho-physiological and Proteome Changes of *Pandanus amaryllifolius*

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Abstract

Drought is one of the significant threats to the agricultural sector. However, there is limited knowledge on the plant response to drought stress and post-drought recovery. *Pandanus amaryllifolius*, a moderate drought-tolerant plant, is well known for its ability to survive in low-level soil moisture conditions. Understanding the molecular regulation of drought stress signaling in this plant could help guide the rational design of crop plants to counter this environmental challenge. This study aimed to determine the morpho-physiological, biochemical and protein changes of *P. amaryllifolius* in response to drought stress and during recovery. Drought significantly reduced leaf relative water content of *P. amaryllifolius*. In contrast, relative electrolyte leakage, proline and malondialdehyde contents, and the activities of antioxidant enzymes in the drought-treated and recovered samples were relatively higher than the well-watered sample. The protein changes between drought-stressed, well-watered, and recovered plants were evaluated using tandem mass tags (TMT)-based quantitative proteomics. Of the 1,415 differentially abundant proteins, 74 were significantly altered. The majority of proteins differing between them were related to carbon metabolism, photosynthesis, stress response, and antioxidant activity. This is the first study that reports the protein changes in response to drought stress in *Pandanus*. The data generated provide an insight into the drought-responsive mechanisms in *P. amaryllifolius*.

Keywords

Antioxidant enzymes; drought stress; *Pandanus amaryllifolius*; proteomics; stress-responsive proteins; TMT-labelled LCMS/MS

1. Introduction

Drought stress is a significant threat to agricultural productivity worldwide, causing 83% of agricultural economic losses. About USD29 billion was lost from all combined agriculture damages due to natural disasters [1]. Drought has affected the rice grain yield and caused about US\$840 million losses in several rice-producing regions in Thailand [2]. In Malaysia, a 12-51% reduction in rice yield due to

drought stress was reported from 2007 to 2011 [3]. Moreover, an expanding world population increases pressure on agriculture to use water more efficiently. Hence, it is indispensable to understand the drought response and adaptive mechanisms of plants as it could help to improve crop performance under drought stress conditions.

Drought stress disturbs physiological and biochemical processes in plants, including cell membrane, disrupting transportation of solutes, photosynthesis rate, nutrient uptake, translocation, and causes electron leakage and excessive accumulation of reactive oxygen species (ROS) [4,5]. The impacts of drought stress on plants rely on the severity and the growth period of plants [6]. To cope with these adverse effects, plants have developed intricate response and adaptive strategies. These include overproduction of compatible osmolytes, alteration of endogenous hormonal levels, and regulation of physiological and molecular changes [7]. Under moderate drought conditions, plants respond and adapt by altering root architecture [8] and stomatal closure [9], hoping to maintain a balance between stress tolerance and growth. However, if drought conditions become severe, plants tend to activate protection mechanisms against cellular damage, adjust *in vivo* antioxidant enzyme systems to eliminate excessive ROS, and accumulate proteins to maintain cell turgor, aiming to survive under such conditions [9].

Extensive efforts have been made to understand the plant responsive and adaptive mechanisms to drought stress using different “omics” techniques. Proteomics approaches have been used to determine plants' proteome responses under drought stress. For instance, Liu et al. determined the protein changes of mulberry in response to drought stress using tandem mass tags (TMT)-label LCMS/MS technique [10]. The authors found that proteins involved in photosynthesis, energy and sugar metabolisms, antioxidant production, hormones, and cell homeostasis were abundantly changed under drought conditions. Using the same technique, Xiao et al. identified 123 differentially changed proteins between 30-d drought-stressed cotton fine roots and control [11]. The number of proteins was increased to 1,273 when cotton was exposed to 45-d drought treatment. Goche et al. reported that 237 and 187 root proteins were significantly altered in drought-susceptible and drought-tolerant sorghum varieties, respectively [12]. Other proteomics drought studies have also been reported on chickpea [13], grapevine [14], wheat [15], and banana [16].

Pandanus amaryllifolius is a member of the screw pine family Pandanaceae. This plant is commonly known as fragrant screw pine, pandan (Malay), pandan mabango (Philippines), and toei hom (Thailand). *P. amaryllifolius* is widely cultivated in Southeast Asia, such as Thailand, Indonesia, and Malaysia. The pandan plants grow in clumps with a 40-80 cm tall and a width of about 4.5 cm. Its leaves are dark green with sharp spines on the margins and commonly used to flavor food items, as natural colorants and as herbal medicine. Besides its aromatic value, *P. amaryllifolius* leaves have also been found to contain phenolic compounds that possess health benefits. For instance, Ghasemzadeh and Jaafar reported that gallic acid and cinnamic acid isolated from *P. amaryllifolius* could inhibit 78% of breast cancer MCF-7 cell lines [17]. In addition, metabolic syndromes, such as weight gain, abdominal

adipose tissue deposition, and blood pressure, can be reduced after treating with leaf extract of *P. amaryllifolius* [18].

Pandanus spp. is a moderate drought-tolerant plant [19]. For example, *P. tectorius* can survive on drought conditions for more than 6-months, whereas *P. odoratissimus* can survive under an area with rainfall of less than 2,000 mm annually [20]. However, there is insufficient information on the extent of drought tolerance of *P. amaryllifolius* despite its medicinal value. Understanding the plant's strategy to adapt and survive under drought stress may refine our understanding of drought stress responses in plants and help to develop drought-tolerant crops. In this study, we examined the growth morphology, antioxidant enzymatic activities, and protein changes between drought-stressed, well-watered, and water-recovered *P. amaryllifolius* plants.

2. Results

2.1. Morphological Changes of Drought-Stressed *Pandanus amaryllifolius*

The morphological changes of *Pandanus* plants in response to drought stress were determined (Figure 1A). The percentage of leaf relative water content (LRWC) for drought-stressed samples was significantly reduced after 4 days of drought treatment, whereas the percentage of relative electrolyte leakage (REL) for drought-stressed samples was significantly increased after 10 days of drought treatment (Figures 1B and 1C). The fresh and dry weights of shoots and roots in well-watered plants were generally higher than drought-stressed *Pandanus* (Figures 1D-1G). Surprisingly, the root dry weight of the 7-day drought-stressed plants was significantly higher compared to well-watered plants (Figure 1G). Similarly, the root-to-shoot ratio of the 7-day drought-stress samples was higher than well-watered plants (Figure 1H). After rewatering, only 7-day drought-stressed *Pandanus* plants were able to recover (Figure 2A). Hence, day 7 of drought treatment was selected for the subsequent experiments.

Figure 1. Responses of drought-stressed and well-watered *Pandanus amaryllifolius*. *P. amaryllifolius* plants were subjected to drought stress by withholding water for 4, 7, 10, and 14 days. Well-watered *P. amaryllifolius* was served as control. (A) Photographs of *Pandanus* plants were taken at 4, 7, 10, and 14 days; (B) The percentage of LRWC of *P. amaryllifolius* leaves on different time points; (C) The

percentage of REL for each sample on different harvest point; **(D)** Shoot fresh weight of *Pandanus*; **(E)** Root fresh weight of *Pandanus*; **(F)** Shoot dry weight of *Pandanus*; **(G)** Root fresh weight of *Pandanus*; **(H)** Root-to-shoot ratio of *Pandanus* dry weight. Means labelled with asterisk were significantly different based on the Student's t-test when its p -value < 0.01 .

To understand the drought-responsive mechanism of *Pandanus* plants, a new set of experiments comprising well-watered, 7-day drought-stressed, as well as 7-day drought-stressed and rewatered plants was conducted. The LRWC in *Pandanus* plants was decreased with the decreasing soil moisture content (Figure 2, Figure S1). In particular, the drought-stressed *Pandanus* plants showed a 20% reduction compared to well-watered plants but recorded comparable LRWC with well-watered plants after rewatering (Figure 2B). The REL of the drought-stressed samples was higher than the well-watered and recovered plants (Figure 2C). The mass of both shoots and roots for each treatment were not significantly different (Figures 2D and 2E). However, the root-to-shoot ratio in drought-stressed *Pandanus* was significantly higher than other treatments (Figure 2F).

The *Pandanus* leaves showed slight wilting and clamping after 7 days of drought treatment but recovered after rewatering. However, there was a browning effect observed on the tips of mature leaves (Figure S2A). The yellow-green leaf color pigment pixel percentage in drought-stressed and recovered plants was slightly higher than well-watered plants (Figure S2B).

Figure 2. Comparison of well-watered, drought-stressed, and water-recovered *Pandanus amaryllifolius* plants. (A) *Pandanus* plants were subjected to drought stress by withholding water for 7 days, whereas the water-recovered plants were rewatered after 7-day drought treatment and rewatered for 7 days. Well-watered *P. amaryllifolius* was served as control. The line bar indicates the scale of the plant = 10 cm; (B) The percentage of leaf relative water content of *P. amaryllifolius* leaves; (C) The percentage of relative electrolyte leakage for each sample; (D) Shoot mass of *P. amaryllifolius*; (E) Root mass of *P. amaryllifolius*; (F) Root-to-shoot mass ratio of *P. amaryllifolius*. Means labelled with alphabet were significantly different based on the ANOVA followed by post hoc when its *p*-value < 0.01.

2.2. Changes of Proline and Malondialdehyde Contents in *Pandanus* Plants under Drought Stress

The proline content of the drought-stressed *Pandanus* plants ($1.6 \mu\text{M g}^{-1}$ FW) was significantly higher than well-watered ($1.3 \mu\text{M g}^{-1}$ FW) and recovered plants ($0.8 \mu\text{M g}^{-1}$ FW) (Figure 3A). Similarly, the malondialdehyde (MDA) content of the drought-stressed *Pandanus* plants was the highest (9.2 nM g^{-1} FW) (Figure 3B). The well-watered and recovered samples recorded the same MDA content (7.7 nM g^{-1} FW).

Figure 3. Osmolyte and lipid peroxidation changes of the drought-stressed and well-watered *Pandanus amaryllifolius*. (A) Proline content of *P. amaryllifolius* leaves as quantified in $\mu\text{M g}^{-1}$ fresh weight (FW); (B) Malondialdehyde (MDA) content of *P. amaryllifolius* leaves as quantified in $\mu\text{M g}^{-1}$ FW. Means labelled with alphabet were significantly different based on the ANOVA followed by post hoc when its p -value < 0.01 .

2.3. Antioxidant Enzyme Changes in *Pandanus* Plants in Response to Drought Stress

Drought stress generally increases the activity of antioxidant enzymes. The highest hydrogen peroxide (H_2O_2) ($30.0 \mu\text{M min}^{-1} \text{g}^{-1}$) and superoxide dismutase (SOD) ($462.1 \text{ g}^{-1} \text{FW}$) levels were recorded in water-recovered plants (Figures 4A and 4B). The catalase (CAT) and peroxidase (POD) for both well-watered and recovered plants showed significantly higher activity than drought-stressed plants (Figures 4C and 4D). The highest activity of ascorbate peroxidase (APX) ($10.4 \text{ M min}^{-1} \text{g}^{-1}$) and glutathione reductase (GR) ($126.0 \text{ M min}^{-1} \text{g}^{-1}$) was found in drought-stressed samples (Figures 4E and 4F).

Figure 4. Activity of (A) hydrogen peroxide (H₂O₂) and antioxidant enzymes (B) superoxide dismutase (SOD), (C) catalase (CAT), (D) peroxidase (POD), (E) ascorbate peroxidase (APX), and (F) glutathione reductase (GR) in *Pandanus amaryllifolius* leaves in response to drought stress and water recovery. Absorbance was measured through a spectrophotometer. H₂O₂ accumulation was shown in $\mu\text{M min}^{-1} \text{g}^{-1}$ fresh weight (FW), whereas SOD was shown as $\text{U g}^{-1} \text{FW}$ based on NBT coloration and inhibition. CAT, POD, APX and GR were shown in $\text{M min}^{-1} \text{g}^{-1} \text{FW}$. Means labelled with alphabet were significantly different based on the ANOVA followed by post hoc when its p -value < 0.01 .

2.4. Proteins Changes in Well-Watered, Drought-Stressed, and Recovered *Pandanus* Plants

To identify the protein changes of *Pandanus* plants under drought stress, total protein from the well-watered, drought and water-recovered plants were extracted for nano-LC-MS/MS analysis. Of the 1,415 identified proteins, 74 proteins were found to be significantly altered (Table S1). These proteins were visualized with hierarchical clustering (Figure S3A) and clustered into 4 groups based on the log ratio expression between treatments (Figure S3B). Cluster 1 showed 12 proteins in the well-watered samples were decreased in abundance when exposed to drought stress (Figure S3B). In contrast, cluster 4 indicated that 9 proteins in the recovered samples were increased in abundance when compared to drought-stressed samples (Figure S3B).

Among the 74 identified proteins, carbohydrate- and stress-related proteins were the largest differentially changed protein group in this study (Figures 5A and 5B). Of these, 39 unique proteins were found in comparison 1 (Drought vs Well-watered), 40 proteins in comparison 2 (Recovery vs Well-watered), and 57 proteins in comparison 3 (Recovery vs Drought) (Figure 6A).

Figure 5. Heat map of the differentially changed proteins classes identified between well-watered, drought-stressed and water-recovered *Pandanus amaryllifolius*. **(A)** Carbon-related proteins identified between treatments; **(B)** Stressed-related proteins identified between treatments. The intensity scale indicates the range of upregulation (purple) or downregulation (yellow) of proteins between treatments.

To classify the function of the 74 differentially changed proteins, KEGG pathway enrichment analysis was performed (Figure 6). The results showed that 15 of differentially changed proteins were involved in carbohydrate metabolism and another 8 proteins are involved in genetic information processing and cofactors and vitamin metabolism (Figure 6B). Based on the gene ontology (GO) functional

classification, most of the differentially changed proteins were involved in photosynthesis processes and stress responses (Figure 6C).

Figure 6. Functional categorization and enrichment of differentially changed proteins between well-watered, drought and recovery samples. **(A)** The Venn diagram represented the comparison of proteins identified in the leaves of *Pandanus* plants treated with drought stress, well-watered, and recovery; **(B)** KEGG enrichment of differentially changed proteins based on functional category; **(C)** Gene ontology enrichment based on KEGG pathway according to biological processes, molecular functions, and cellular components.

3. Discussion

3.1. Drought Stress Affected the Growth and Antioxidant Enzyme Activities of *Pandanus* plants

Drought stress has significantly affected the growth of *Pandanus* plants, such as LRWC, root-to-shoot ratio, shoot and root biomass, and REL, and led to an accumulation of ROS that damage cell membranes. A decline in leaf water content could reduce the cell turgor pressure and affect the strength and mechanical structure of the plants [21]. This was observed in the leaf morphological responses in drought-stressed *Pandanus* plants. Moreover, the percentage of REL was significantly increased in drought-stressed *P. amaryllifolius*, suggesting that cell membrane integrity is likely to be affected due to oxidative stress. In contrast, the rewatered *Pandanus* showed reduced REL. In sugarcane, an increase of 62% REL was found in the drought-stressed samples compared to control [22] but reduced after rewatering [23]. Similarly, Oraee and Tefranifar found that drought treatment increased the REL in pansy plants, while the REL in the rewatered drought-stressed pansy was reduced [24].

Drought stress generally leads to the accumulation of MDA and proline [25]. MDA is an index of oxidative injury, whereby the MDA production correlates with ROS scavenging ability [26]. Proline is an essential adaptive response to drought stress in plants. It acts as a ROS scavenger, osmoprotectant, redox balancer, detoxification activator, and source for nitrogen and carbon [27]. The results clearly showed that drought stress increased the MDA and proline contents in *P. amaryllifolius*. Rewatering reduced the MDA and proline contents, indicating the capacity of *P. amaryllifolius* to maintain the membrane integrity and restore its functions. These findings agree with the previous studies, where MDA and proline are elevated under drought stress in various plants [28,29].

ROS, such as superoxide ($O_2^{\cdot-}$), free radical ($\cdot OH$), and H_2O_2 , is generated and accumulates in plants in response to drought stress [30]. Antioxidant enzyme system protects plants against abiotic stress via ROS scavenging mechanism. In this study, the activity of antioxidant enzymes, such as CAT, APX, POD and GR, in drought-stressed plants was generally higher than well-watered plants. The higher antioxidant activities have contributed to the low level of H_2O_2 . While SOD constitutes the first line of defense against ROS, its activity in drought-stressed *Pandanus* plants was comparable to the well-watered plants, probably due to the detoxification of ROS by other antioxidant enzymes. Taken together, the enhanced antioxidant enzyme system helps *Pandanus* plants to cope with an accumulated ROS under drought conditions.

3.2. Stress and Defense Protein Abundance under Drought Stress

Plants respond and adapt to drought stress conditions by changing regulatory circuits in transcription and protein expression, reorganizing metabolic pathways and physiological processes [16]. This is the first study to determine protein changes in response to drought stress in *Pandanus* using a TMT-labelled proteomics approach. Proteins involved in stress-responsive and carbohydrate metabolism were identified as the largest differentially changed protein group in this study, suggesting their important roles in response to drought stress conditions.

In the present study, the abundance of heat shock proteins 70 (HSP70) and DnaJ homolog proteins in drought-stressed samples was increased compared to other treatments. HSPs are molecular chaperones that protect plants by maintaining proper folding and preventing the aggregation of proteins [31]. Proper protein folding and disaggregation are vital for cell survival under stress conditions. To date, several members of the *HSP70* gene family have been identified in various plant species. Of these, some HSP70 members have been reported to be involved in the drought stress response. For instance, HSP70 in peanut was increased in abundance in response to drought [32]. DnaJ homolog proteins are essential components in the chaperone machine and could be induced by cold [33], heat [34], and drought stresses [35,36]. Other studies showed that several HSPs, co-chaperone DnaJ, and Grp chaperonin 60b were differentially altered under drought conditions [37,38]. Emerging evidence indicates that the accumulation of HSPs is correlated with stress tolerance in plants, as these proteins are only induced upon stress treatment [39]. These studies have revealed the importance of HSP70 and DnaJ-like proteins in conferring abiotic stress tolerance in plants.

Polycystin, Lipxygenase, Alpha-toxin, and Triacylglycerol lipase (PLAT) domain proteins are induced by abscisic acid and involved in abiotic stress response. These proteins mediate the abscisic acid signaling pathway after binding to bZIP transcription factors AREB/ABFs. PLAT protein was found to be abundantly accumulated in this study. The amount of PLAT was also increased in salinity-stressed quinoa [40]. Hyun et al. investigated the function of the Arabidopsis PLAT, *AtPLAT1* in tobacco [41]. The authors found that expressing *AtPLAT1* in tobacco promoted growth and increased stress tolerance towards cold, drought and salt stresses. However, the role of PLAT proteins in drought stress response is still lacking and need further exploration.

3.3. Cell Membrane-Related Proteins Increased under Drought Stress

Networked (NET) actin-binding proteins increased in drought-stressed *Pandanus* plants compared to well-watered plants, probably due to the need of maintaining plant cell integrity. NET proteins associate with different membrane compartments in plant cells and facilitate membrane-actin interaction [42]. At the transcript level, Li et al. found that *NET1A* gene in cotton was induced by drought stress, and its expression was significantly different between control and drought treatments [43]. Although the role of NET proteins in stress response is still unclear, it was proposed that mechanical, gravitational, and osmotic stresses could directly induce the accumulation of NET proteins [44].

Alpha-L-arabinofuranosidase (ASD) is a cell wall protein, catalyzing the hydrolysis of L-arabinofuranosidic bonds in L-arabinose-containing hemicelluloses [45]. In this study, ASD1 protein was increased when exposed to drought stress. The role of ASD in drought stress has been shown in maize [46] and maritime pine [47].

3.4. Carbohydrate Metabolism-Related Protein Abundance under Drought Stress

Proteins involved in carbohydrate metabolism were increased in abundance, most probably due to energy demand caused by drought stress. One of the prominent proteins in this category is sucrose phosphate synthase (SPS). When plants experienced drought stress, SPS catalyzed the conversion of uridine diphosphate-glucose and fructose 6-phosphate into sucrose. Nemati et al. found that SPS changed in abundance after being exposed to drought stress in wheat seedlings [48]. Other studies showed that drought treatment markedly increased SPS activity [49,50]. The high abundance of SPS shown by other studies in different drought-stressed plant species might be due to the need of cells for extra energy to cope with stress.

4. Materials and Methods

4.1. Plant Material and Drought Treatment

Three-month-old disease-free *P. amaryllifolius* purchased from the I Green Nursery Sdn. Bhd., Muar, Johor, Malaysia, were transferred to polybags (20 × 11 cm) containing 600 g clay silt loam soil. The plants were acclimatized in a growth room at Universiti Malaya, Malaysia, and maintained at 28 ± 2 °C with a light intensity of 1,500 lux under a 12:12 h photoperiod cycle and 80 ± 5 % relative humidity for 2 weeks. All plants received 20 mL water once a day and foliar fertilizers once per week prior to treatment. The drought treatment was conducted by withholding water for 4, 7, 10, and 14 days, and rewatered for 7 days after drought treatment. Well-watered plants were watered daily throughout the experimental period. Based on the morphological results, an appropriate time point was then selected for biochemical and proteomics analysis. The soil moisture content in all pots was monitored every day using a soil moisture meter (HH2 moisture meter, Delta-T, UK). The experiments were carried out in a completely randomized block design with 8 biological replicates for each treatment.

4.2. Determination of Leaf Relative Water Content

Leaf relative water content (LRWC) was measured according to Turner [51]. Briefly, the harvested leaf samples were cleaned with 70% ethanol, cut into small pieces (2 cm²), and weighed for their fresh weight (FW) before transferred to a Petri dish. The leaf samples were submerged in 20 mL of distilled water for 6 h at room temperature. The saturated weight (SW) of the samples was measured before being oven-dried for 2 days. The dried samples were then measured for their dry weight (DW). LRWC was calculated using the following formula:

$$\text{LRWC (\%)} = [(FW-DW)/(SW-DW)] \times 100$$

4.3. Measurement of Relative Electrolyte Leakage

The relative electrolyte leakage (REL) was measured according to Quan et al. [52]. About 100 mg leaves were immersed in 10 mL deionized water and incubated at room temperature for 6 h under shaking condition of 150 rpm. After measuring initial electrical conductivity (C_i) using a conductivity

meter (Cyberscan CON 11, Eutech Instrument, Thermofisher, Singapore), the leaf samples were boiled for 20 min and measured for the conductivity of lysed cells ($C_{\max}$). REL was calculated as:

$$\text{REL (\%)} = C_i / C_{\max} \times 100$$

4.4. Measurement of Plant Weight

The fresh and dry weights of the leaves and roots were measured separately. The fresh weight of the samples was measured immediately after harvesting. The samples were then stored in a paper bag prior to drying in an oven for 7 days or until a constant weight was achieved to measure their dry weight. The dry weight of the roots and leaves was used to calculate the root-to-shoot ratio.

4.5. Automated Colorimetric Assay

Leaf color was evaluated by harvesting 6 leaf tips (10-12 cm in length), wiped with 70% ethanol, and arranged on a white background. Leaves were photographed by using Nikon D5100, processed by Image J, and analyzed by automated colorimetric assay [53]. The pixels were grouped into four categories, namely green, green-yellow, yellow, and brown.

4.6. Determination of Malondialdehyde Content

Malondialdehyde (MDA) content was measured as described by Heath and Packer [54]. Fresh leaf samples (100 mg) were homogenized in liquid nitrogen, then extracted with 1.5 mL 0.1% (w/v) trichloroacetic acid (TCA), and centrifuged at 13,000 rpm for 10 min at 4°C. About 300 µL supernatant was added into a 1 mL reaction mixture containing 0.5% (v/v) thiobarbituric acid (TBA) in 20% (w/v) TCA. The mixture was heated at 95°C for 30 min, cooled on ice, and centrifuged at 10,000 rpm for 10 min. The supernatant was measured using a spectrophotometer at wavelengths 532 and 600 nm and calculated using the following formula:

$$\text{MDA} = [(A_{532} - A_{600}) \times V_{\text{Tr}} \times 1000] / (\text{Extinction coefficient MDA} \times 1 \text{ cm} \times V_{\text{e}}) \div \text{g FW}$$

$A_{532} - A_{600}$ = Absorbance of MDA - TBA

V_{Tr} = Volume of reaction (mL)

V_{e} = Volume of enzyme extract (mL)

Extinction coefficient of this MDA-TBA adduct at 532 nm is $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

FW = Fresh weight of the sample

4.7. Measurement of Proline Content

Proline content was measured according to Bates et al. [55] with modifications. About 200 mg leaf samples were ground with 2 mL 70% (v/v) ethanol and the mixture was centrifuged at 13,000 rpm for

20 min. The supernatant was added to a reaction mixture containing 500 µL glacial acetic acid:500 µL freshly prepared acid-ninhydrin reagent:500 µL sample extract or proline standards. The mixture was mixed and boiled at 100°C in a heat block for 1 h. After placing on ice for 30 min, the mixture was extracted with 1 mL toluene. The toluene phase was carefully collected into a test tube. The absorbance of the fraction was measured at 520 nm with toluene as blank. The proline standard curve was constructed at concentrations of 1, 5, 10, 25, 50, 100, and 200 µM. The proline content calculated based on the following formula:

$$\text{Proline } (\mu\text{Mg}^{-1} \text{FW}) = [(\mu\text{g proline/mL} \times \text{mL toluene}) \div \mu\text{g 115.5}/\mu\text{mole}]/(\text{g FW}/5)$$

4.8. Antioxidant enzymatic assays

About 200 mg leaf samples were mixed with 2 mL cold extraction buffer (100 mM phosphate buffer, pH 7.0, containing 0.1 mM disodium ethylenediaminetetraacetic acid, and 0.1 g polyvinylpyrrolidone). The mixture was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was used for the subsequent antioxidant enzymatic assays. The enzyme activity for each assay was calculated using the following formula:

$$\text{Enzyme activity (M min}^{-1} \text{g}^{-1} \text{FW)} = (\Delta A \times V_{\text{Tr}})/(\epsilon \times \Delta t \times 1 \text{ cm} \times V_{\text{e}} \times \text{g FW}) \times 1000$$

ΔA = Difference in absorbance

V_{Tr} = Volume of reaction (mL)

V_{e} = Volume of enzyme extract (mL)

Δt = Difference in time of absorbance (min)

For CAT, $\epsilon(\text{Hydrogen peroxide}) = 36.0 \text{ mol}^{-1} \text{ cm}^{-1}$

For APX, $\epsilon(\text{Ascorbic acid}) = 2.8 \text{ mmol}^{-1} \text{ cm}^{-1}$

For POD, $\epsilon(\text{Guaiacol}) = 26.6 \text{ mol}^{-1} \text{ cm}^{-1}$

For GR, $\epsilon(\text{NADPH}) = 6220 \text{ mol}^{-1} \text{ cm}^{-1}$

4.8.1 Superoxide Dismutase

SOD activity was determined according to Dhindsa et al. [56]. Briefly, 3 mL of reaction mixture containing 50 mM phosphate buffer (pH 7.0), 9.9 mM L-methionine, 55 µM nitro blue tetrazolium (NBT), 0.025% (v/v) Triton X-100, 100 µL enzyme extract, and 4.8 µM riboflavin was prepared in a test tube and covered by aluminum foil. The riboflavin was added last to initiate the reaction. The mixture was shaken and incubated at 30°C for 10 min under a white light source (35 W) placed at 20 cm height above the test tubes. The mixture was measured at 560 nm. The blank was prepared by replacing the sample with extraction buffer. The SOD activity was calculated based on the formula below:

SOD (Unit g⁻¹ FW) = [(Blank- Sample)A_{560nm}]/(Blank A_{560nm}) × (Volume reaction)/(Volume enzyme)
× 100 × 1/50 ÷ 0.1 g FW

4.8.2. Catalase

CAT assay was performed as described by Aebi [57] with modifications. The 3 mL reaction mixture containing 50 mM phosphate buffer (pH 7.0), freshly prepared 8.33 mM H₂O₂, and 100 µL enzyme extract. The enzyme extract was added last to initiate the reaction. The CAT activity was measured at 240 nm.

4.8.3. Ascorbate Peroxidase

APX activity was measured according to Chen and Asada [58]. The 1 mL reaction mixture contained 50 mM phosphate buffer (pH 7.0), 200 µL enzyme extract, 0.5 mM ascorbic acid, and 0.42 mM H₂O₂. The H₂O₂ was added last to initiate the reaction. The APX activity was measured at 290 nm wavelength.

4.8.4. Peroxidase

POD assay was carried out according to Maehly and Chance [59] with modifications. The 1 mL reaction mixture contained 100 mM phosphate buffer (pH 7.0), 0.5 mM guaiacol, 0.0833 mM H₂O₂, and 100 µL enzyme extract. The H₂O₂ was added last to initiate the reaction. The POD activity was measured at 470 nm wavelength.

4.8.5. Glutathione Reductase

GR activity was determined as described by Mannervik [60]. The 1 mL reaction mixture contained 500 µL assay buffer (0.2 M potassium phosphate buffer, pH 7.0, 0.2 mM EDTA, 50 µL 20 mM freshly prepared oxidized glutathione, 50 µL 2 mM NADPH solution, and 300 µL enzyme extract) and distilled water. The decrease in absorbance at 340 nm was monitored for 1 min.

4.8.6. Hydrogen Peroxide

The H₂O₂ level in the leaf samples was determined according to Velikova et al. [61] with slight modifications. Briefly, 100 mg leaf powder was homogenized with 1.5 mL 0.1% (w/v) TCA in an ice bath. After centrifugation at 10,000 rpm for 15 min, about 250 µL supernatant was mixed with 1 mL reaction mixture containing 2.5 mM potassium phosphate buffer, pH 7.0, and 0.5 M potassium iodide. The H₂O₂ concentration was measured at 390 nm and calculated using a standard curve with concentrations of 2.5 to 100 µM.

4.9. Protein Extraction and Quantification

Total protein from five biological replicates from each treatment was extracted using TCA/acetone precipitation with phenol method [62] with slight modifications. The leaf samples (200 mg) were

extracted using 4 mL cold 10% (w/v) TCA/acetone containing 10 mM dithiothreitol (DTT) and 0.001% (v/v) protease inhibitor. The homogenate was centrifuged at 15,000 rpm for 5 min at 4°C. The pellet was resuspended with 1.5 mL 10% (w/v) TCA/acetone before centrifuged at 15,000 rpm for 5 min at 4°C. The pellet was then washed with 80% ice cold acetone containing 10 mM DTT. After centrifuged at 15,000 rpm for 5 min at 4°C, the pellet was resuspended with 1.5 mL sodium dodecyl sulfate (SDS) extraction buffer (1% (w/v) SDS, 0.15 M Tris-HCl, pH 8.8, 0.1 M DTT, 1 mM EDTA, and 0.001% (v/v) protease inhibitor). The mixture was incubated at 65°C for 1 h before being centrifuged at 15,000 rpm for 10 min at room temperature and the supernatant was collected in a new tube. An equal volume of Tris-buffered phenol (pH 8.0) was then added to the supernatant. The phenol phase at the bottom layer was extracted and mixed with washing buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.7 M sucrose). After centrifugation, the upper layer was mixed with 1.5 mL 0.1 M ammonium acetate in methanol. The mixture was incubated at -20°C for overnight and centrifuged at 15,000 rpm for 10 min at 4°C. The pellet was washed with 1.5 mL 0.1 M ammonium acetate in methanol, centrifuged at 15,000 rpm for 5 min at 4°C, and mixed with 1 mL 80% (v/v) ice cold acetone containing 20 mM DTT. After centrifugation, the pellet was vacuum-dried in a desiccator for 5 min and resuspended in either S-Traps lysis buffer (5% SDS, 100mM triethylammonium bicarbonate, TEAB, pH 7.55) (Protif, USA) for LCMS/MS analysis or resuspension buffer (7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris-HCl (pH 8.0), and 1% DTT) for protein content measurement. The protein content was determined using Bradford reagent [63], where bovine serum albumin was used as a protein standard.

4.10. Protein Preparation

The dried samples were sent to Australian Proteome Analysis Facility (APAF) for LC-MS/MS. About 140 µg total protein from each sample was resuspended in S-Trap lysis buffer and reduced by 10 mM DTT for 30 min at 56°C and alkylated by 25 mM iodoacetamide (IAA) for 30 min at room temperature in dark. The pH of the solution was adjusted by adding 12% aqueous phosphoric acid with 1:10 ratio for a final concentration of 1.2% and diluted by using S-Trap binding buffer (90% aqueous methanol containing 100 mM TEAB, pH 7.55). The S-Trap binding buffer was then added to the acidified lysis buffer. The mixture was transferred to a labelled S-Trap column before being centrifuged at 4,000 × g. S-Trap binding buffer was used to wash the column twice and the protein retained on the column was digested with 125 µL trypsin solution (~10 µg trypsin (Pierce, Thermo Scientific, USA) in 50 mM triethylammonium bicarbonate) at 47°C for 3 h. Next, 50 mM triethylammonium bicarbonate was added to the column following centrifugation to elute off the peptides, while the remaining peptides were eluted from the column by sequential centrifugation with 0.2% aqueous formic acid followed by 50% aqueous acetonitrile (ACN) containing 0.2% formic acid. The peptides were vacuum centrifuged and resuspended in 110 µL 200 mM HEPES (pH 8.8). Each 10 µL sample was then pooled into a tube. The

peptide concentration of the reconstituted solution was determined by using Pierce quantitative colorimetric peptide assay (Thermo Scientific, USA).

4.11. Protein TMT-Labeling and Fractionation

All samples were labelled in a 10-plex TMT label (Thermo Scientific, USA) and incubated at room temperature for 1 h with occasional vortexing. Next, 5% hydroxylamine was added to each sample, vortexed, and incubated at room temperature for 15 min to remove the excess TMT label. To ensure the number of peptides in each sample was equal, an equal amount of each TMT-labelled sample was mixed, vacuum dried, and resuspended in 2% ACN 0.1% formic acid to obtain the normalization factor. All TMT-labelled samples were pooled with a 1:1 ratio. The TMT-labelled peptides were vacuum dried, reconstituted in 0.1% trifluoroacetic acid and fractionated at three concentrations of 12.5%, 17.5%, and 50% ACN with 0.1% triethylamine from Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Scientific, USA).

4.12. Liquid Chromatography-Tandem Mass Spectroscopy

1D data dependent acquisition (DDA) LC-MS/MS (Q-Exactive, Thermo Fisher, USA) was performed by injecting each TMT-labelled peptide fraction onto the peptide trap, followed by a loading buffer (99.9% water and 0.1% formic acid). The peptides were eluted from the trap onto the nano-LC column and separated with linear gradient of 1% mobile phase A (99.9% water and 0.1% formic acid) to 30% over 110 min at 300 nL/min, followed by 85% B (99.9% ACN and 0.1% formic acid) for 8 min. The ionization source was set as positive ion mode, whereby 350-1850 m/z peptide precursors were scanned at 70,000 resolutions. The 10 most intense ions in the survey scan were subjected to the fragmentation by HCD using a normalized collision energy of 35 with precursor isolation width of 0.7 m/z. The precursors with charges of +2 to +4 were subjected to MS/MS analysis under the parameters: minimum signal of 6,200 ions was required for MS2 triggering, an AGC target value of 2×10^5 for MS2 and maximum injection time of 250 ms with 70,000 MS/MS scan resolution, and 90 s of dynamic exclusion.

4.13. Proteomics Data Analysis

The raw data files generated were searched against Viridiplantae protein sequences downloaded from UniProt (Reviewed, 40,400 proteins sequences; 210312_uniprot-taxonomy_viridiplantae+reviewed.fasta) (Accessed on March 2021) using Proteome Discoverer 2.1.0.81 (Thermo Scientific, USA) and the Sequest HT search algorithm, allowing up to two missed cleavages per peptide and 20 ppm precursor mass tolerance. Carbonylation of cysteine was used as a fixed modification, whereas oxidation of methionine, deaminated (N, Q), Glu-> PyroGlu, Gln-> PyroGlu, N-Terminus acetylation, TMT6Plex (K), and TMT6Plex (N-Term) were used as variable modifications. Only high confidence peptides were selected using a percolator algorithm for the peptide-spectrum match (PSM) in database searches with false discovery rate (FDR) threshold set at 0.01 for protein identification. The identified proteins were

filtered based on their abundance ratio of comparisons; Comparison 1 (Drought vs Well-watered), Comparison 2 (Recovery vs Well-watered), and Comparison 3 (Recovery vs Drought), following p -value of less than 0.05 (Student's t -test) and in the range of less than 0.5-fold change and more than 1.2-fold change of abundance.

4.14. Functional Classification of Proteins

The significantly differentially changed proteins were visualized by hierarchical clustering using Perseus software (version 1.6.0.7) [64]. These differentially changed proteins were then subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.kegg.jp/>) for protein functional categorization, while enrichment pathway analysis was performed using DAVID functional annotation tools with *Arabidopsis thaliana* as background comparison.

4.15. Statistical Analysis

The morphological and antioxidant enzyme data were analyzed by analysis of variance (ANOVA) followed by a post hoc Tukey range using SPSS Statistics software (version 23.0; IBM). The analyzed data considered to be statistically significant when its p -value < 0.01 .

5. Conclusions

The imposed drought stress has affected the growth and development of *P. amaryllifolius*. The drought-stressed plants showed decreased LRWC and increased REL, MDA and proline contents. The accumulation of ROS in drought-stressed plants has enhanced their antioxidant enzyme activities. The drought-responsive mechanisms of *P. amaryllifolius* was determined at the proteome level. The majority of proteins differing between drought-stressed and well-watered plants were classified as heat shock proteins and photorespiration related proteins, while proteins involved in carbon metabolism, chlorophyll biosynthesis, and antioxidant were significantly altered in water recovered plants. Further research, such as interactions between proteins of interests, should be performed to better understand the drought-responsive mechanism in *P. amaryllifolius*.

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Data Availability Statement: The mass spectrometry proteomics data will be available in the ProteomeXchange Consortium [65] once the manuscript is accepted for publication.

Conflicts of Interest: The authors declare that they have no competing interests.

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Supplementary Materials

Figure S1. Soil moisture content was measured in well-watered, drought-stressed, and recovered samples throughout the 14 days of the experiment.

Figure S2. Comparison of *Pandanus amaryllifolius* leaves under different treatments. (A) The leaves were arranged according to their position from center (number 1) to the mature leaf (number 6) and the line bar = 5 cm. This layout was used for automated colorimetric assay (ACA); (B) The comparison of green and green-yellow pigments pixel percentage on each time point using ACA. Means labelled with alphabet was significantly different based on the ANOVA followed by post hoc when its *p*-value < 0.01.

Figure S3. Hierarchal clustering of average protein abundance and clustering between treatment comparisons for well-watered, drought-stressed, and recovered *Pandanus amaryllifolius*. (A) Heatmap of the significant protein abundance comparison between treatments according to its cluster group. The intensity scale indicates the range of upregulation (red) or downregulation (green) of proteins between treatments; (B) Significant differentially abundant proteins between treatments clustered into 4 groups according to the log-ratio expression.

Table S1. Differential abundance of proteins identified by TMT-labelled LCMS/MS in *Pandanus amaryllifolius* under drought stress and recovery treatments.