Research Article

Physiological Responses and Proteomic Analysis on the Cold Stress Responses of Annual Pitaya (*Hylocereus* spp.) Branches

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Received 17 October 2021; Revised 28 October 2021; Accepted 30 October 2021; Published 22 November 2021

Academic Editor: Wenneng Wu

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In this study, the physiological response of the annual branches of three varieties of pitaya (Xianmi, Fulong, and Zihonglong) in cold stress was investigated using a multivariate statistical method. Physiological change results showed that cold stress could decrease the moisture and chlorophyll contents, on the contrary, increase the relative electric conductivity, the contents of malonadehyde, soluble protein, soluble sugar, and free proline, and enhance the enzyme activities of peroxidase, superoxide dismutase, and catalase. Meanwhile, a comparative proteomic approach was also conducted to clarify the cold resistance-related proteins and pathways in annual pitaya branches. Proteomics results concluded that the cold tolerance of annual pitaya branches could be improved by modulating autophagy. Therefore, we hypothesized that an increased autophagy ability may be an important characteristic of the annual pitaya branches in response to cold stress conditions. Our results provide a good understanding of the physiological responses and molecular mechanisms of the annual pitaya branches in response to cold stress.

1. Introduction

Pitaya (*Hylocereus* spp.), a member of the family Cactaceae, is a perennial climbing cactus plant which is rich in anthocyanins, betanin, and plant albumin [1, 2]. Because of the ability of pitaya to resist prolonged drought, it is considered to have a high potential for agricultural development, especially in the drought areas [3, 4]. Therefore, pitaya has been a thriving fruit and large-scale commercial cultivation in the karst regions of southwest China, such as Guangxi, Yunnan, and Guizhou provinces, which are frequently exposed to severe drought stress. However, low temperature is also found to be the most important environmental factor which can limit the development of pitaya production [5]. Literature has reported that most pitaya cultivars can tolerate 0°C, which may lead the pitaya fruits and its young buds, shoots, and even some mature branches to death [6]. The lower the temperature with the longer the duration, the more serious the effect on the pitaya yield and quality [7].

Meanwhile, literature has reported that cold stress (classified as chilling (0 to 15°C) or freezing (<0°C) stress)

can affect agricultural production [8]. When exposed to low temperature, to adapt to the cold stress, plants require physiological response and cold resistance to survive, which is known as ‘cold acclimation’ [9]. In the past few years, significant progress in many plants, such as pitaya fruit [7], cassava [10, 11], alfalfa [12], petunia seedlings [13], castor seeds [14], rice [15], and grape [16], has been made in understanding the molecular mechanisms under cold stress.

In recent years, literatures reported that the moisture content, chlorophyll content, relative electric conductivity (REC), malonadehyde (MDA) content, soluble sugar content, soluble protein content, free proline content, catalase (CAT) activity, peroxidase (POD) activity, and superoxide dismutase (SOD) activity can be used as the indices to identify the cold resistance [11, 17–21]. Different pitaya varieties have different adaptabilities to the cold resistance, and the cold resistance among different pitaya varieties could be determined by the multi-index comprehensive evaluation [22]. Meanwhile, in the past few years, the comparative proteomic approach has been become a promising tool that is crucial for plants’ stress response

[23–27]. However, to date, our understanding of cold stress mechanisms in pitaya branch is limited.

In this study, the annual branches of three varieties of pitaya (Xianmi (XM), Fulong (FL), and Zihonglong (ZHL)) were selected, and a multivariate statistical method and a comparative proteomic approach were used to investigate the tolerance to cold stress of the annual pitaya branches.

2. Materials and Methods

2.1. Plant Material, Growth Condition, and Cold Treatments. The annual branches of three varieties of pitaya (XM, FL, and ZHL) with basically the same cultivation management measures and similar growth potential (approximately 50 cm length and 10 cm width without fruits and flowers) were selected from the pitaya demonstration garden of Luodian experimental station of Guizhou Fruit Institute in Dec. 2020. The branches are required to be complete and smooth, with no obvious disease spots on the surface and no obvious mechanical injury or freezing damage. The annual branches of three varieties of pitayas were transferred to a chamber for pretreatment for 1 day at 25°C. After that, the temperature of the chamber was dropped to 0°C with the gradient of 5 °C/h for cold treatment with light/dark cycles of 16/8 h. The annual branches were exposed to 0°C for 0, 1, 3, 5, and 7 days, respectively, and then frozen at −80°C. The branches in the chamber maintained at 25°C for 7 days were used as negative controls (CK).

2.2. Physiological Response Analyses. To analyze the physiological responses of the annual pitaya branches under cold stress, the REC, the contents of moisture, chlorophyll, MDA, soluble protein, soluble sugar, and free proline, and the enzyme activities of POD, SOD, and CAT were measured in this study.

2.2.1. Moisture Content Determination. The moisture contents of the annual branches of the three varieties of pitayas were determined according to AOAC official method 934.06 and calculated using formula (1).

$$\text{Moisture content (\%)} = \frac{(m_a - m_b)}{m_a} \times 100. \quad (1)$$

In this formula, m_a and m_b are the qualities of samples before and after drying at 70°C, respectively [28].

2.2.2. Chlorophyll Content Determination. The chlorophyll contents of the annual branches of the three varieties of pitaya were determined by a reported method with some modifications [29]. Each test sample (50 mg) was placed in a 5 mL precooled mixture solution with 85% acetone and 85% ethanol (v/v = 1/1) to be homogenized and incubated in a chamber for 0.5 h at 25°C. Then, the supernatant was obtained by centrifuging at 6,500 g for 15 min. The OD₆₆₃ and OD₆₄₅ values of the supernatant were monitored by using a Multiskan Sky 1530 microplate reader (Thermo Scientific, Poland). The contents of chlorophyll a (C_a), chlorophyll b (C_b), and total chlorophyll (C_t) were calculated using the following equations:

$$C_a \text{ (mg/L)} = 0.0127\text{OD}_{663} - 0.00269\text{OD}_{645}, \quad (2)$$

$$C_b \text{ (mg/L)} = 0.0229\text{OD}_{645} - 0.00468\text{OD}_{663}, \quad (3)$$

$$C_t \text{ (mg/L)} = C_a + C_b. \quad (4)$$

2.2.3. REC Determination. The REC was determined according to Wang's method [30]. Each test sample (2 g) was placed in 30 mL distilled water at room temperature for 3 h to determine the value of electrical conductivity (EC_1). After that, the test sample was boiled for about 20 min and then quenched to room temperature to determine the EC_2 value. EC_0 value was the electrical conductivity value of the distilled water. REC was calculated using the following equation:

$$\text{REC (\%)} = \frac{(EC_1 - EC_0)}{(EC_2 - EC_0)} \times 100. \quad (5)$$

2.2.4. Determination of the Contents of Soluble Protein, Soluble Sugar, Free Proline, and MDA. The soluble sugar contents were identified according to Solarbio kits (Solarbio, Beijing, China). Coomassie brilliant blue G250 was used to measure the content of soluble protein [31]. The contents of free proline and MDA were measured using the proline and MDA content determination kits (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China).

2.2.5. Determination of the Enzyme Activities of CAT, SOD, and POD. The enzyme activities of CAT, SOD, and POD were measured with the corresponding enzyme assay reagent kits (Suzhou Comin Bioengineering Institute, Suzhou, China).

2.2.6. Fuzzy Synthetic Evaluation. The cold resistance abilities of the annual branches of the three varieties of pitaya were evaluated using the fuzzy mathematics method [32]. The positive subordinate function values, such as REC, the contents of MDA, soluble protein, soluble sugar, and free proline, and the activities of SOD, POD, and CAT, were calculated as equation (6); meanwhile, the negative subordinate function values, such as moisture and chlorophyll contents, were calculated as equation (7):

$$f(X_{ij}) = \frac{(X_{ij} - X_{\min})}{(X_{\max} - X_{\min})}, \quad (6)$$

$$f(X_{ij}) = 1 - \frac{(X_{ij} - X_{\min})}{(X_{\max} - X_{\min})}, \quad (7)$$

where $f(X_{ij})$ is the value of the i pitaya variety of the j item, X_{ij} is the value of the i pitaya variety of the j item, and $X_{\max}$ and $X_{\min}$ are the maximum and minimum values of the j item, respectively.

2.3. Recovery Growth of Annual Pitaya Branches after Cold Treatment. In order to investigate the recovery growth of the annual pitaya branches after cold treatment at 0°C for 7 days, we transplanted the precooling treated annual pitaya branches into the greenhouse with the temperature increased from 0 to 25°C by 5°C/h, light/dark cycles of 16/8 h, and 90% relative humidity. The survival rate and germination rate were counted after 60 days.

2.4. Proteomics Analysis

2.4.1. Protein Extraction and LC-MS/MS Analysis. The annual branches of the three varieties of pitaya, preexposed to 0°C for 7 days, were used as the test samples for proteomics analysis. In each experiment, about 1.5 g fine powder samples were suspended in a 10 mL precooling acetone solution containing 0.15% trichloroacetic acid, 10% polyvinylpyrrolidone, and 0.07% β -mercaptoethanol. The solution was stored at -20°C overnight and centrifuged at 8,000 g and 4°C for 30 min. The precipitate was washed using 10 mL precooled acetone three times and then dissolved in 5 mL of precooled protein extraction buffer (40 mM dithiothreitol, 0.1 M KCl, 0.7 M sucrose, 50 mM EDTA, and 0.5 M Tris-HCl (pH 7.5)). The protein solution was collected by centrifugation at 12,000 g and 4°C for 15 min. The polypeptide, digested to polypeptide with trypsin at 37°C overnight, was dissolved in 50 μ L of HPLC-grade H₂O containing 0.1% formic acid and detected using an AB SCIEX Triple TOF 5600 mass spectrometer (Foster City, CA, USA). The parameters of MS were set according to the manufacturer's recommendations.

2.4.2. Sequence Database Searching and Bioinformatics Analysis. MS/MS spectra (Wiff. files) were analyzed and quantified using the MaxQuant software and searched against the *Cactaceae* proteome by the reported methods [33, 34]. The raw data were uploaded in iProX (<http://www.iprox.org>) with the accession number of IPX0001296003. The differentially expressed proteins (DEPs) (the expression level > 2-fold and p value < 0.01) at the Gene Ontology (GO) term, named cellular components (CC), biological processes (BP), and molecular function (MF), were calculated. Then, the pathway enrichment of DEPs was identified from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

2.5. Statistical Analysis. GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) was used to analyze the significant difference at $p < 0.05$.

3. Results and Discussion

3.1. Physiological Changes with Cold Treatment. Low temperature can affect the growth and development of many plants, especially originating from tropical and subtropical origins. Due to the time and region of domestication, different varieties of pitaya have different cold resistance. Therefore, in this study, the annual branches of the three

varieties of pitaya were selected and the physiological responses on the cold resistance abilities of annual pitaya branches were investigated. The dynamic changes results of moisture and chlorophyll contents in cold stress are listed in Figure 1. Figure 1 shows that compared with the control group (0 day), the moisture content (Figure 1(a)) and chlorophyll content (Figure 1(b)) of the annual branches of the three varieties of pitaya have a tendency to decrease from 1 to 7 days under the cold treatment. In the previous study, the relationship between plant moisture content and cold resistance under low temperature was studied, and the results revealed that the cold resistance was positively correlated with the moisture content [35]. In this study, we found that the cold resistance could decrease the moisture content in annual pitaya branches which are in accord with the previous report [35]. Meanwhile, previous studies showed that the chlorophyll content, which is closely related to photosynthesis, has been proved to be an effective parameter for evaluating plant cold resistance [36–39]. In this study, our results showed that cold stress could significantly decrease the chlorophyll contents in the annual branches of the three varieties of pitaya, which are in accord with the previous reports [40, 41].

Compared with the control group (0 day), the REC (Figure 2(a)) and MDA content (Figure 2(b)) of the annual pitaya branches were increased under cold stress from 0 to 7 days. Overall, with the time extension of cold treatment from 0 to 7 days, the REC and MDA contents of ZHL were consistently lower than those of FL and XM. REC, which was used to research the cytoplasmic membrane damage, is a physiological parameter for evaluating plant cold resistance [42, 43]. In the previous study, Wang et al. [44] and Li et al. [45] found that the REC of corn seedling and tea leaf could increase under cold stress. In our study, we found that the REC of the annual pitaya branches increases under cold stress, proving that cold stress can increase the membrane permeability of the annual pitaya branches. MDA, an important physiological and biochemical index, can reduce the content of antioxidants to inhibit the activity of cell protective enzymes and accelerate the process of membrane lipid peroxidation [46]. Yin et al. [47] found that the low-temperature stress had a little effect on the content of MDA in cassava in the early period, but with the extension of stress time, large amounts of MDA were accumulated due to severe peroxidation of membrane lipids. Therefore, determination of MDA content and its dynamics in a plant can reflect the strength of cold resistance of the plant to low-temperature stress. This study results demonstrated that cold stress can increase the MDA content in annual pitaya branches. Similar results were also obtained in the previous studies about cassava [48], maize [49], peach [50], pineapple [51], tomato [52], and rapeseed [53].

Compared with the control group (0 day), the contents of soluble protein (Figure 3(a)), soluble sugar (Figure 3(b)), and free proline (Figure 3(c)) of the annual pitaya branches were enhanced with the extension of the cold stress time from 0 to 7 days. Soluble protein can increase the water content in the cell to reduce the cold injury [54]. In the present study, we demonstrated that the soluble protein

FIGURE 1: The changes of moisture content (a) and chlorophyll content (b) of the annual branches of the three varieties of pitaya with cold treatment. “*” indicates the changes of moisture content and chlorophyll content with a significant difference at $p < 0.05$ compared with the control group.

FIGURE 2: The changes of REC (a) and MDA content (b) of the annual branches of the three varieties of pitaya with cold treatment. “*” indicates the changes of moisture content and chlorophyll content with a significant difference at $p < 0.05$ compared with the control group.

content and cold resistance revealed a positive correlation, and similar results were also obtained by Wallis et al. [55]. Meanwhile, soluble sugar is an important osmotic substance in plant cells, and it can increase the content of intracellular solute. The increase of soluble sugar content is beneficial to the increase of osmotic pressure, thus enhancing the water retention ability of plant cells [55]. This study showed that the soluble sugar content in annual pitaya branches could increase to again be conducive to cold stress.

With the extension time of cold treatment from 0 to 7 days, the antioxidant enzyme activities, including SOD, POD, and CAT, exhibited an increasing trend under cold stress (Figure 4). In the rise trend, the SOD, POD, and CAT activities of ZHL were consistently higher than those of FL and XM. Antioxidant enzymes had an important role in protecting the membrane system for maintaining the normal physiological activities of plants [56]. SOD is a key factor in eliminating reactive oxygen species (ROS) to decrease the ability to minimize oxidative damage under cold stress [57].

Meanwhile, POD can induce the salicylic acid (SA) pathway to promote cell-wall reinforcement, thus activating the systemic acquired resistance (SAR) to cold stress [58, 59]. In addition, CAT can protect plant cells from oxidative damage under cold stress by catalyzing H_2O_2 to decompose to water and oxygen [60]. Therefore, our results demonstrated that cold stress may improve cold resistance of plants in the form of antioxidant enzymes.

The cold resistance abilities of the annual branches of the three varieties of pitaya were evaluated using the fuzzy mathematics method, and the results are shown in Table 1. The higher cold resistance abilities have higher value of synthetic evaluation values [61]. Table 1 shows that the synthetic evaluations of cold resistance indicator of the annual branches of XM, FL, and FHL were 0.495, 0.515, and 0.545, respectively, demonstrating that the cold resistance abilities of the annual branches of the three varieties of pitaya were ranked in the order of $ZHL > FL > XM$.

FIGURE 3: The changes of soluble protein content (a), soluble sugar content (b), and free proline content (c) of the annual branches of three varieties of pitaya with cold treatment. “*” indicates the changes of soluble protein content, soluble sugar content, and free proline content with a significant difference at $p < 0.05$ compared with the control group.

FIGURE 4: Continued.

FIGURE 4: The changes of enzyme activities of SOD (a), POD (b), and CAT (c) of the annual branches of the three varieties of pitaya with cold treatment. “*” indicates the enzyme activity changes of SOD, POD, and CAT with a significant difference at $p < 0.05$ compared with the control group.

3.2. Recovery Growth of Annual Pitaya Branches after Cold Treatment. After 60 days of recovery growth, the results, as shown in Table 2, showed that ZHL had the best survival rate (45.25%) and germination rate (39.16%), followed by FL (35.16% and 31.58%, respectively) and XM (26.62% and 21.49%, respectively), showing that the cold resistance abilities of the annual branches of the three varieties of pitaya were in the order of ZHL > FL > XM.

3.3. Label-Free Proteomics Analysis. The proteomics technique was used to analyze the cold stress responses of the annual branches of the three varieties of pitaya. A total of 2798 proteins were identified in the annual branches of the three varieties of pitaya, and the results are listed in Table S1, of which 1900, 2099, and 2023 proteins were identified in the annual branches of ZHL, FL, and XM, respectively. Meanwhile, the box plot (Figure 5(a)) and normal distribution (Figure 5(b)) of the protein expression of the three varieties of pitaya indicated that compared with FL and XM, ZHL had the highest protein expression abundance.

As shown in Figure 6(a) and Table S1, the numbers of identified proteins of ZHL vs. XM showing up- and downregulation were 699 and 450, respectively. Figure 6(b) and Table S1 show that the up- and down-regulated proteins of ZHL vs. FL were 494 and 784, respectively. Figure 6(c) and Table S1 also show that the up- and downregulated proteins of FL vs. XM were 492 and 390, respectively. Meanwhile, to clarify the cold resistance ability-related proteins in the annual branches of the three varieties of pitaya, the numbers of in turn up- (mode = 1) and downregulated (mode = -1) expression proteins were also investigated, and the results are listed in Figure 7 and Table S2. As shown in Figure 7 and Table S2, the numbers of in turn up- and downregulated

expression proteins in XM, FL, and ZHL were 479 and 261, respectively.

The functions of the in turn up- and downregulated expression proteins were annotated by GO analysis and further classified into the categories of MF, CC, and BP. GO term enrichment analysis of the in turn up- and downregulated expression proteins revealed that main CC involved ribosome (GO:0005840), cytoplasm (GO:0005737), membrane (GO:0016020), mitochondrion (GO:0005739), and chloroplast (GO:0009507); main BP involved translation (GO:0006412), carbohydrate metabolism (GO:0005975), small-molecule metabolism (GO:0044281), response to stress (GO:0006950), and transport (GO:0006810); and main MF involved structural molecule activity (GO:0005198), lyase activity (GO:0016829), rRNA binding (GO:0019843), oxidoreductase activity (GO:0016491), and ligase activity (GO:0016874). Meanwhile, KEGG analysis results, shown in Table S3, showed that the in turn up- and downregulated expression proteins identified in XM, FL, and ZHL were mainly related to autophagy (path:ko04138), carbon fixation pathways in prokaryotes (path:ko00720), fluid shear stress and atherosclerosis (path:ko05418), Epstein-Barr virus infection (path:ko05169), and biosynthesis of amino acids (path:ko01230). It is worth noting that the pathway with the highest enrichment was autophagy (path:ko04138). Autophagy, a major process of protein degradation, can recycle nutrient contents to remove the damaged proteins when exposed to the environmental stress conditions [62, 63]. Recent studies had divulged that autophagy is extremely important in environmental stress and plant development [64–69]. Therefore, our results demonstrated that autophagy may play a key role in response to cold stress in annual pitaya branches, and we concluded that the pitaya branches could be improve tolerance to cold stress by modulating autophagy to clean up the damaged cellular structures caused by cold stress conditions for recycling of nutrients.

TABLE 1: Synthetic evaluation of the cold resistance indicator of the annual branches of the three varieties of pitaya.

Pitaya variety	Moisture content	Chlorophyll content	REC	Subordinate function value ^a					Free proline content	SOD activity	POD activity	CAT activity	Synthetic evaluation ^a
				MDA content	Soluble protein content	Soluble sugar content							
XM	0.508 ± 0.05	0.532 ± 0.08	0.451 ± 0.11	0.510 ± 0.04	0.470 ± 0.05	0.408 ± 0.01			0.399 ± 0.02	0.521 ± 0.07	0.507 ± 0.16	0.646 ± 0.01	0.495 ± 0.04 A
FL	0.548 ± 0.04	0.520 ± 0.10	0.435 ± 0.04	0.526 ± 0.01	0.510 ± 0.11	0.485 ± 0.02			0.462 ± 0.02	0.519 ± 0.09	0.520 ± 0.05	0.625 ± 0.01	0.515 ± 0.08B
ZHL	0.542 ± 0.02	0.534 ± 0.62	0.493 ± 0.14	0.585 ± 0.02	0.528 ± 0.06	0.501 ± 0.04			0.532 ± 0.03	0.564 ± 0.04	0.528 ± 0.04	0.645 ± 0.03	0.545 ± 0.10 C

^aExperiments were repeated three times. Different uppercase letters indicate the values of synthetic evaluation with a significant difference among different pitaya varieties at $p < 0.05$.

TABLE 2: The survival rate and germination rate of the annual branches of the three varieties of pitaya.

Pitaya varieties	Survival rate (%) ^a	Germination rate (%) ^a
ZHL	45.25 ± 3.65 A	39.16 ± 2.65 A
FL	35.16 ± 2.95B	31.58 ± 4.16B
XM	26.62 ± 5.26 C	21.49 ± 1.98 C

^aExperiments were repeated three times. Different uppercase letters indicate the values of survival rate and germination rate with a significant difference among different pitaya varieties at $p < 0.05$.

FIGURE 5: The box plot (a) and normal distribution (b) of the protein expression of the annual branches of the three varieties of pitaya.

FIGURE 6: Continued.

FIGURE 6: Volcano plot of the relative protein abundance changes in the annual branches of the three varieties of pitaya. (a) ZHL vs. XM, (b) ZHL vs. FL, and (c) FL vs. XM. The green and red dots are the up- and downregulated DEPs, respectively.

FIGURE 7: The in turn up- (a) and downregulated (b) expression proteins in the annual branches of the three varieties of pitaya.

4. Conclusions

In conclusion, the physiological and proteome dynamic changes in the annual branches of the three varieties of pitaya were investigated using a multivariate statistical method and a comparative proteomic approach, respectively. Physiological response results showed that the contents of moisture and chlorophyll decreased, in contrast with the REC and the contents of MDA, soluble protein, soluble sugar, and free proline increased, and the enzyme activities of SOD, POD, and CAT enhanced in annual pitaya branches under cold stress. Meanwhile, proteomic analysis results concluded that the pitaya branches could improve tolerance to cold stress by modulating autophagy. In addition, the

physiological and proteome dynamic changes results demonstrated that the cold resistance of the annual branches of the three varieties of pitaya should be in the order of FHL > FL > XM which are in accord with the cold resistance ability in agricultural production under the natural environment condition. Our results provide a better understanding of how the annual pitaya branches respond and survive under low temperatures.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Junliang Zhou and Lijuan Wang contributed equally to this work.

Acknowledgments

This research was funded by the National Natural Science Foundation of China, grant no. 31560547; Guizhou Provincial Talent Base Project, grant no. [2018]3, and Special Funds for Innovation Capacity Building of Research Institutions, grant no. [2019]4002.

Supplementary Materials

Table S1: list of identified proteins in the annual branches of XM, FL, and FHL; Table S2: the in turn up- and down-regulated expression proteins in the annual branches of XM, FL, and FHL; Table S3: the KEGG analysis results of the in turn up- and downregulated expression proteins in the annual branches of XM, FL, and FHL. (*Supplementary Materials*)

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