iTRAQ-based quantitative proteomics analysis of cantaloupe (*Cucumis melo* var. *saccharinus*) after cold storage

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Abstract

Background: During the low temperature storage, cantaloupe is susceptible to the cold stress, resulting in the loss of edible and commercial quality. To ascertain the molecular mechanisms of cold tolerance in cantaloupe, cold-sensitive cultivar Goldqueen-308 (GE) and cold-tolerant cultivar Jiashi-310 (JS) were used for quantitative proteomic analysis with iTRAQ in parallel.

Results: In this work, two commercial cultivars were treated at 0.5°C for 0, 12 and 24 days. Phenotypes assays showed that GE suffered a more severe damage as the cold treatment time extended. Proteomic analysis revealed that the number of differentially expressed proteins (DEPs) changed significantly over time in cold-exposed cantaloupe. Comparing with GE, JS responded in a prompter manner in terms of expressing cold-responding proteins during the similarly cold treatment. Furthermore, much more different groups of proteins were mobilized in response to the cold treatment in JS comparing with GE. Metabolic analysis indicated that more amino acids were up-regulated in JS during the early phases of cold stress. This study also identified some DEPs since they were up-regulated in JS or down-regulated in GE in terms of molecular mechanisms, which were mainly related to carbohydrate and energy metabolism, structure proteins, ROS scavenging, amino acid metabolic and signaling transduction. Moreover, iTRAQ analysis was confirmed to be reliable via the results of phenotypes assays, metabolic analysis and q-PCR validation.

Conclusion: By proteomics information, we found that the prompt response and the significant mobilization of proteins in JS maintained a higher level of cold tolerance, and the delay of cold response in GE could be a critical reason for the severe chilling injury. The candidate proteins we found will be the basis of future studies for further investigations and our findings may help to better understand the novel mechanisms of cold tolerance in cantaloupe.

Background

The cantaloupe (*Cucumis melo* var. *saccharinus*) is rich in various nutrients and is a characteristic fruit of Xinjiang, China, being one of the main economic crops, which plays a significant role in promoting local economic development [1]. Because of the high sugar content and susceptible to infection by pathogenic microorganisms [2], refrigerated storage is considered to be the most effective method for preserving the quality of cantaloupe, allowing long-distance transport [3]. During the low temperature storage, however, cantaloupe is susceptible to the cold stress, resulting in peel pitting, softening, and weight loss [4]. Longer storage time, more cold-sensitive cultivar and lower temperature are the major factors that easily cause the cold damage.

Cold stress is regarded as a major environmental stress affecting plant growth, which triggers a series of changes in physiological and molecular processes [5] and the results in electrolyte leakage, accumulation of reactive oxygen species (ROS), hydrogen peroxide (H₂O₂) and malondialdehyde (MDA), as well as changes in the levels of endogenous abscisic acid [6, 7], ethylene [8, 9] and soluble sugars [10] in plant

cells. The accumulation of ROS could result in oxidative stress, which damages the plant plasma membrane, decreases enzyme activity, and inhibits the rate of photosynthesis, protein processing [11, 12]. After a long period of evolution, plants have developed complex regulatory mechanisms to cope with chilling stress. On the one hand, stress-responsive signaling pathways regulate the expression of several upstream stress-related genes in response to cold stresses [13]. On the other hand, non-enzymatic and enzymatic antioxidant systems participate in ROS scavenging to protect plant cells from oxidative damage [14].

To investigate the mechanisms of plants coping with abiotic stress, the physiological changes and the transcriptomics approaches have been employed in previous researches [4, 15]. Though transcriptome sequencing is a powerful method for identifying the novel transcripts and analyzing the gene expression at the transcriptional level, the changes of mRNA determined by transcriptomics cannot invariably correlate with the changes of corresponding proteins [16]. In addition, the proteomics expression is still poorly reported in cantaloupe response to cold. Consequently, the proteomics technology is used as a more applied tool to directly visualize the proteins being expressed in our study.

To better understand the mechanisms of cold responses in cantaloupe at the level of temporal changes, two different cultivars Goldqueen–308 (GE) and Jiashi–310 (JS) were investigated at 0.5°C in parallel based on iTRAQ coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS). In the present work, we found that at 12 days, the chilling injury began to appear, and at 24 days, the chilling injury was more obvious and severe. Alternatively, the findings of this study will lay a novel foundation for the further investigation of chilling tolerance mechanisms in cantaloupe, surely improving the breeding programs and commercial storage of this fruit.

Results

Contrasting phenotypes changes of GE and JS under cold treatment

Comparing with JS, changes in GE were evident after 12 days of storage. At 24 days, GE was suffering a more severe chilling injury including rotting and bacterial infections, while JS exhibited a better quality and acquired fewer injuries (Fig. 1a). Cold stress resulted in an increase in REL during the early phase of storage, which was higher in GE than in JS, and a decrease at the late phase of cold storage (Fig. 1b). The similar tendencies were also identified among the changes of MDA content and H₂O₂ content (Fig. 1b). By and large, our evaluations confirmed that cold-sensitive GE suffered a more severe chilling injury and expressing higher levels of electrolyte leakage, lipid peroxidation, and H₂O₂ content than JS during the early phase of storage.

Identification and quantitation of DEPs by iTRAQ

Differentially expressed proteins (DEPs) were analyzed after cold treatment and control (0 d) condition. By using iTRAQ labeling LC-MS/MS analysis, a total of 10,742 (GE: 5450; JS: 5291) proteins were specifically identified from 639,980 LC-MS/MS spectra and 60,756 peptides (Additional file 1: Table S1).

With the cut-off of ratio fold change of >1.200 or <0.833 in expression ($P < 0.05$), a total of 807 DEPs (12 days: 360; 24 days: 447) and 722 DEPs (12 days: 391; 24 days: 331) were identified in GE and JS under chilling stress treatment, respectively (Additional file 2: Fig. S1; Additional file 3: Table S2). After 12 days cold treatment, we identified 360 DEPs, 142 of which were up-regulated and 218 of which were down-regulated in GE (Fig. 2a). In JS, there were 391 DEPs, from which 251 were up-regulated and 140 were down-regulated (Fig. 2a). At 24 days, there were 447 DEPs, 160 of which were up-regulated and 287 were down-regulated in GE (Fig. 2a). Similarly, in JS, 331 DEPs were found, from which 138 were up-regulated and 193 were down-regulated (Fig. 2a). A higher number of DEPs were identified in JS than in GE at the early stage of cold treatment, and during the late phases of treatment, the number of DEPs was increased in GE (Fig. 2a). According to these observations, we assumed that cold-tolerant cultivar JS responded faster in terms of expressing cold-responding proteins at the similarly cold treatment, which was significant for cold resistance to ensure the expression of self-regulation in response to the chilling stress and protect the plant cells and part of organelles from the damages induced by ROS, and the delay of cold response in GE could be a critical reason for the severe chilling injury [17]. Meanwhile, among the 807 DEPs in GE, 181 (22.42%) and 268 (33.20%) were specifically identified during the periods of 12 days and 24 days, respectively. 179 (22.18%) were shared by both stages (Fig. 2b). However, in JS, only 114 out of 722 DEPs (15.79%) were common to both stages, while 277 (38.37%) and 217 (30.06%) were specifically identified in two stages, respectively (Fig. 2b). The notion was firmly convinced that different groups of proteins were mobilized in response to two treatment stages in chilling-tolerant JS, which was in line with the findings of Weijian Cen [12].

In order to identify the proteins more likely to be related to the acquisition of chilling tolerance in cantaloupe, a careful analysis of common identified DEPs was carried out. The selected proteins were those reflecting a differential expression pattern and common expressed during the whole treatment period in both cultivars. A hierarchical cluster analysis (HCL) was performed for analyzing the correlation of the common expressed DEPs after cold exposure. Notably, the changes among 24 common DEPs were significant and their abundance can be illustrated as 8 clusters, revealing that two cultivars mobilized numerous proteins and differentially regulated their abundance to cope with low temperature stress. The first cluster illustrated 8 proteins and most of which were down-regulated in both cultivars. The second cluster represented 4 proteins that were dramatically up-regulated in JS at two time points. The third cluster showed 2 proteins with down-regulation in all cold treatments in both cultivars. The fourth cluster exhibited 2 proteins that were up-regulated more obviously in JS. The fifth cluster was composed of 2 proteins with significant down-regulation in GE. The sixth cluster showed 3 proteins that were up-regulated significantly in JS, while the seventh and the eighth clusters were comprised of 3 proteins with down-regulation in all conditions (Fig. 2c; Additional file 3: Table S2). Furthermore, the principal component analysis (PCA) we performed over the expression data above indicated that in all conditions, both cultivars presented a different protein expression pattern. The changes between JS chilled at 12 and 24 days were smaller, clustering close together with little separation in either axes (Fig. 2d; Additional file 3: Table S2). In contrast, the changes between 12 and 24 days stored samples in GE clustered away from each other. Consequently we speculated that comparing with JS, there was a greater impact on the

expressions of proteins in GE under cold stress with the time prolonging, which could explain the severe damage to GE during the similar treatment [18].

Primary functional classification of DEPs

From Clusters of Orthologous Groups (COG) database, we found that the largest group was 'posttranslational modification, protein turnover, chaperones' (119 DEPs), followed by 'general function prediction only' (94 DEPs) and 'translation, ribosomal structure and biogenesis' (83 DEPs) (Fig. 3a; Additional files 4: Table S3). The further analysis will be discussed below.

DEPs in response to early phases of the cold stress

By using gene ontology (GO) analysis, DEPs were classified based on three categories, including cellular component (CC), molecular function (MF) and biological process (BP) [19]. During the early phases of cold stress in both cultivars, the most highly represented categories were 'cell', 'cell part', 'intracellular', 'intracellular part' and 'cytoplasm' in CC (Fig. 3b); 'catalytic activity', 'binding' and 'heterocyclic compound binding' in MF (Fig. 3b); 'metabolic process', 'organic substance metabolic process' and 'cellular process' in BP (Fig. 3b). The results indicated that the majority of DEPs were involved in metabolic process, cellular process, cell and catalytic activity, suggesting that cold stress mainly affected the physiological metabolism and cell differentiation in cantaloupe (Additional files 5: Table S4). More intriguingly, all three categories were at a higher level in JS comparing with GE, revealing the fact that under cold stress, the proteome pattern of cold tolerant JS showed a more sensitive and faster change than cold sensitive GE.

To further identify the functions of the DEPs, KEGG pathway analysis was also performed. Only significantly enriched categories with their P -value < 0.05 were selected. We found that cold stress could affect ribosome, phagosome and phenylpropanoid biosynthesis in both cultivars while protein processing in endoplasmic reticulum, photosynthesis-antenna proteins, plant-pathogen interaction, were highly enriched in GE, whereas other KEGG terms, photosynthesis, galactose metabolism, cyanoamino acid metabolism, were considerably enriched in JS (Table 1; Fig. 4a, c; Additional files 6: Table S5). According to Fig. 5a, c, obviously we can see the connection between each functions and a conspicuously protein-protein interaction among ribosome and other functions ($P < 0.05$).

DEPs in response to late phases of the cold stress

Similarly, during the late phases of the cold treatment, proteins were characterized by 'cell', 'cell part', 'intracellular', 'intracellular part', 'cytoplasm' in CC (Fig. 3c); 'catalytic activity', 'binding', 'heterocyclic compound binding' in MF (Fig. 3c); 'metabolic process', 'cellular process', 'organic substance metabolic process' in BP (Fig. 3c). Comparing with them at 12 days, all three categories increased significantly in GE than in JS, indicating that there was a greater impact on GE under cold stress as the cold treatment time prolonged (Additional files 5: Table S4). The tardiness of cold response in GE could be a critical reason for its severe chilling injury.

KEGG pathway analysis indicated that protein processing in endoplasmic reticulum and galactose metabolism could be affected in both cultivars after low temperature stress. Ribosome, carbon fixation in photosynthetic organisms, plant-pathogen interaction, one carbon pool by folate, and phenylpropanoid biosynthesis were highly enriched in GE while photosynthesis-antenna proteins, phagosome, amino sugar and nucleotide sugar metabolism, fructose and mannose metabolism, pentose and glucuronate interconversions, and linoleic acid metabolism were enriched in JS (Table 1; Fig. 4b, d; Additional files 6: Table S5). Like the hallmark of the protein-protein interaction at 12 days, there was still a significant interaction among ribosome in GE, while dramatic changes of cluster functions happened in JS, elaborating the fact that various proteins were mobilized and there was a positive relationship between the diversity of proteins and the cold resistance (Fig. 5b, d).

Interaction networks among cold induced proteins

Protein interaction networks constructed with differentially expressed proteins from JS based on GO analysis [20] (Additional files 7: Fig. S2). In terms of cellular component, membrane, cell part, cell, and organelles were significantly regulated in JS. In molecular function, catalytic activity and binding were most positively regulated. In biological processes, cellular process, metabolic process and organic substance metabolic process were the highly regulated.

Analysis of metabolites respond to cold stress

A limit of a 1.000-fold change coupled with a Student's *t*-test ($P < 0.05$) was used to identify the differentially expressed metabolites. Metabolic data indicated that, during the chilling response, the accumulation of amino acids, such as proline, 3-hydroxy-L-proline 3 and 3-cyanoalanine were up-regulated during the whole periods of cold treatment in JS comparing with GE (Table 2; Additional files 8: Table S6).

Validation of gene expression of DEPs

To validate the expression patterns of proteins obtained from the iTRAQ analysis, we randomly selected 8 genes for q-PCR analysis using specific primers (Fig. 6; Additional files 9: Table S7). The results indicated that the expression patterns of 5 of 8 genes (62.50%) in both GE and JS were consistent with the iTRAQ data, suggesting that these independent evaluations revealed a reliability of the iTRAQ data [21].

Putative candidate proteins for cold tolerance in cantaloupe

In an attempt to identify proteins that may be involved in the cold tolerance mechanisms in cantaloupe, a series of proteins that showed differential expression among two cultivars were selected. The selected candidate proteins were grouped according to the phenotypes analysis and bioinformatics analysis above as follows: carbohydrate and energy metabolism, stress response, structure proteins, amino acid metabolic and signaling transduction (Additional files 10: Table S8). Their expression patterns and possible roles will be discussed below.

Discussion

Carbohydrate and energy metabolism related proteins

Metabolism is tightly associated with physiological adaptation in plant during the response of stress [22]. Photosynthesis is a fundamental metabolic process for plant growth development, being very sensitive to cold stress [23]. The chloroplast is an important organelle for photosynthesis in plant cells and recent studies showed that the chloroplast also participates in the response to environmental stresses [24, 25]. Easily affected by various environmental stresses, chlorophyll a/b-binding proteins are associated primarily with chlorophylls and xanthophylls, which serve as antenna complexes that could absorb sunlight and transfer the excitation energy to the core complexes of PS II to drive photosynthetic electron transport [26, 27]. Our study indicated that the expression of chlorophyll a/b-binding proteins were inhibited by cold stress, however, much more were down-regulated in GE than JS. During 12 days, three chlorophyll a/b-binding proteins were down-regulated in GE (XP_008462084.1, XP_008459494.1, XP_008439659.2), whereas only 2 of 13 were down-regulated in JS. During 24 days, 2 of 3 chlorophyll a/b-binding proteins were down-regulated in GE, while 9 of 13 were up-regulated in JS. Xianfeng Shi et al. [28] identified 4 chlorophyll a/b-binding proteins (Cla019105, Cla022963, Cla011748, and Cla013826) were increased in cold tolerant RG, whereas no change was observed in cold sensitive SG. Our results confirmed that JS had a higher photosynthetic rate than GE under low temperature. As a major contributor to cold acclimation, carbohydrate metabolism acts as cryoprotectants and ROS scavengers along with their direct relationships with photosynthesis [29]. Numerous studies have shown a positive correlation between carbohydrate metabolism and the degree of cold tolerance in plants [30, 31]. Based on the COG analysis mentioned above, 7 out of 37 carbohydrate metabolism related proteins were up-regulated and 12 were down-regulated in GE at early phases of cold treatment. However in JS, 10 of 35 were up-regulated and 10 were down-regulated in the meanwhile (Additional file 10: Table S8). Comparing with GE, much more proteins were up-regulated in JS, suggesting that carbohydrate metabolism related proteins may be essential for cold stress adaptation, and overexpression of carbohydrate metabolism at early phases could provide sufficient energy production in JS to overcome cold stress.

Being postulated as a significant factor in plants cold tolerance, the plasma membrane plays a central role in plant nutrition and growth as the major ion pump. In all living organisms, lipid bilayer membranes constitute chemical barriers to the environment and, in eukaryotes, between organelles [32]. Under cold stress, the plasma membrane is the primary site for freezing injury in plants, and the physiological metabolic functions such as metal ions, metabolites, nutrient exchange and regulation of cellular processes are disturbed, which in turn affects the normal growth and metabolism of plants [33]. At the early phases of cold treatment, a total of 42 (21up-regulated–21down-regulated) and 38 (10up-regulated–28down-regulated) membrane related DEPs were identified in GE and JS, respectively (Additional files 10: Table S8). During the late phases, 52 (37up-regulated–15down-regulated) and 31 (9 up-regulated–22down-regulated) DEPs were identified in GE and JS, respectively (Additional files 10: Table S8). The results revealed that both GE and JS were affected in the level of membrane after cold treatment. However, much more membranes were affected in GE under chilling stress, causing the severe

damage in GE. Interestingly, we identified three DEPs (XP_008459785.1, XP_008451480.1, XP_016900197.1) that were specifically up-regulated in JS.

Among these membrane proteins, ATP synthase, which could produce ATP from ADP in the presence of a proton gradient across the membrane and has a positive role in cold resistance, was identified [34, 35]. V-type ATPase is one of the key proteins in maintaining the ion homeostasis inside plant cells [36]. The V-type proton atpase subunit D (XP_008449506.1) and V-type proton ATPase subunit F (XP_008450453.1) remained unchanged in JS, while they were down-regulated in GE. Moreover, 2 ATPase related proteins (XP_008442454.1, XP_008446865.1) were up-regulated in JS but remained unchanged in GE at 12 days. Consequently, it has been speculated that JS has a greater ability to produce energy than GE, which could help plants to cope with low temperature.

Stress response related proteins

As one of the earliest responses of plant cells to various environmental stresses, oxidative burst, which is caused by the generation of large quantities of ROS, may cause cell death in plants. Playing important roles in oxidative stress tolerance and cellular detoxification, glutathione S-transferase (GST) involved in antioxidant defense, and its stress-response mechanisms were investigated in sweet potato [37]. A total of 27 GSTs related DEPs were identified in GE and JS, being Tau and Phi classes the most representative in cantaloupe. Among these GSTs, more up-regulated DEPs were found in JS than in GE, though most of which were up-regulated in both cultivars. Especially, glutathione S-transferase zeta class-like (XP_008460863.1) and glutathione S-transferase-like (XP_008451606.1) were up-regulated during the whole periods in JS. Thus, we speculated that the continuous up-regulation of GSTs were involved in the response to the homeostasis of ROS scavenging and increased the tolerance to low temperature. It could be confirmed that peroxiredoxin contributes the stability of macromolecules under cold stress [38]. The results exhibited that two specifically up-regulated thioredoxins (XP_008445916.1, XP_016902770.1) in JS at 12 days. We also identified three specifically up-regulated proteins (XP_008448277.1, XP_008457297.1, XP_016901582.1) in JS at the early phases. The above results may partly explain why JS has a better cold-tolerance than GE, as confirmed by the data analysis of phenotypes shown in Fig. 1b.

Structure proteins

The functional analysis of DEPs in cantaloupe after low temperature storage revealed that ribosome and protein processing in endoplasmic reticulum were highly enriched in both cultivars under cold treatment. Numerous studies indicated that ribosomal proteins [39], endoplasmic reticulum related proteins [40], molecular chaperones involved in protein folding [41] are essential for protein synthesis, and have been speculated to play a central role in regulating stress tolerance of plants. In this study, 38 ribosome related proteins were found in GE at 12 days, from which 21 were down-regulated and 3 (XP_008447825.1, XP_008447383.1, XP_008453374.1) were up-regulated. In contrast, 1 out of 42 were down-regulated in JS while 31 out of 42 were up-regulated. During 24 days, 10 out of 38 ribosome related proteins showed a stable level while 27 out of 38 proteins were down-regulated in GE. However, in JS only 3 out of 42 were

down-regulated, while two (XP_008445497.1, XP_008458587.1) were up-regulated. It can be hypothesized that the high expression of ribosome related proteins at early phases of cold treatment could make JS synthesize and translate the relative proteins immediately to cope with chilling stress, being related to the superior cold tolerance of JS.

By regulating protein folding, stability, solubility, biogenesis and enzymatic activity, and preventing proteins from proteolytic degradation, endoplasmic reticulum could mitigate the damage caused by accumulation of misfolded proteins under environmental stresses such as cold [42]. We identified 24 down-regulated proteins processing in endoplasmic reticulum in GE, but in JS, 21 out of them remained unchanged, and 2 (XP_008457228.1, XP_008463154.1) were up-regulated at the early period. At the late period, 30 out of 32 DEPs were down-regulated while 2 of which were up-regulated in GE. In JS, however, only 3 were down-regulated while 18 DEPs were up-regulated. This observation denoted that the up-regulated expression of endoplasmic reticulum related proteins enhanced the tolerance of JS as the cold exposure time extended.

As molecular chaperones, proteins related to protein folding are involved in various stress responses [43]. Widely known to be involved in the plant response to oxidative stress, heat shock proteins (HSPs) have been shown to facilitate plant adaptation to environmental changes [44]. In our proteomic study, 15 of 20 HSPs were down-regulated in GE at 12 days, whereas 4 (XP_008448853.1, XP_008448861.1, XP_008463154.1, XP_008463443.1) out of 25 HSPs remained up-regulated in JS. At 24 days, 19 HSPs were down-regulated in GE while 19 HSPs were up-regulated in JS, which revealed the fact that the continuous high level of HSPs, acting as molecular chaperones, may help plants adapt to the stress stimulus [45]. DnaJ protein and CHAperonin were also reported to protect plants against biotic and abiotic stress [46, 47]. Herein, Chaperone protein dnaJ 10 isoform X2 (XP_008453046.1) was specifically up-regulated in JS at 12 days. Moreover protein GrpE (XP_008455767.1) was specifically up-regulated in JS. This result showed that the stabilization of molecular chaperones could mobilize the process of proteins so that enhance the cold tolerance of JS under cold stress.

Amino acid metabolic related proteins

Metabolic adaptation is crucial for abiotic stress resistance in plants, and the accumulation of specific amino acids as well as secondary metabolites derived from amino acid metabolism has been implicated in increased tolerance to adverse environmental conditions [48, 49]. As a compatible osmolytes, proline has been shown to exert several adaptive functions under stress, which could stabilize membranes and proteins, acts as a radical scavenger or provides carbon, nitrogen and energy for recovery upon relief under stress [50]. In addition, enhanced proline biosynthesis under stress has been proposed to balance the redox status of cell by maintaining a favorable NADP⁺ /NADPH ratio, since proline synthesis is a reductive pathway that requires NADPH and generates NADP⁺, respectively [51]. In this work, one proline related protein (XP_008454439.1) was up-regulated in JS while it remained unchanged in GE. Our study also suggested that three cyanoamino acid relevant proteins (NP_001303611.1, XP_008452081.1, XP_016899729.1) were up-regulated in JS, which was in accordance to Yue's report [52](Table 2).

Signaling transduction related proteins

Having been validated to regulate many biological processes, such as metabolism, light and hormone signaling, 14-3-3 proteins (14-3-3s) are highly conserved regulatory proteins, being deeply involved in interactions of proteins, mediating diverse signaling pathways [53]. Herein, 14-3-3 protein 7-like (XP_008444837.1) was up-regulated in JS, while it remained down-regulated in GE at 12 days, suggesting that the up-regulation of 14-3-3s play vital roles in cantaloupe to resist cold stress.

Ca²⁺ signaling-related protein is an important regulator of transcription, posttranscriptional processes and different metabolic functions in dicotyledonous species [54]. In our study, we identified four up-regulated Ca²⁺ signaling-related proteins (XP_008444340.1, XP_008448907.1, XP_008457834.1, XP_008453475.1) in JS at the early phases. The results indicated that these Ca²⁺ signaling-related protein may act as positive regulators to improve the cold tolerance in JS. Our studies also identified two serine/threonine-protein kinases were down-regulated (XP_008458707.1, XP_008441406.1) in GE at early phases, while two serine/threonine-protein kinases (XP_008442585.1, XP_008451160.1) remained up-regulated in JS. Interestingly, we found uncharacterized protein C167.05 (XP_008442491.1), probable protein phosphatase 2C 39 (XP_008463673.1), protein BOLA2 (XP_008460912.1), uncharacterized protein LOC103500783 isoform X2 (XP_016902839.1) and universal stress protein A-like protein (XP_008463151.1) were specifically up-regulated in JS, and their regulation mechanisms remain to be further studied in the future.

Conclusion

iTRAQ-based proteomic analysis was utilized to compare the cold tolerance JS with its cold sensitive recurrent parent, GE, under low temperature at two time points. Physiological data indicated that both GE and JS began to accumulate ROS at an early time point in the chilling treatment. Based on the proteomics data, the protein expression level of several significant categories, including carbohydrate and energy metabolism, stress response related proteins, structure proteins, amino acid metabolism, and signaling transduction were found to be changed under cold stress in two cultivars. Metabolic analysis indicated that more factors were up-regulated in JS during 12 days comparing with GE. Comparing with GE, both bioinformatics analysis and physiological results revealed that under the similar cold treatment, the prompt response and the dramatic proteomic motivation in JS maintained a higher level of energy metabolism to enhance the synthesis and degradation of proteins, increased the ROS scavenging capacity and obtained a higher level of cold tolerance. In addition, some candidate proteins like glutathione S-transferase, ribosomal protein, heat shock protein, proline iminopeptidase and calyculin-binding protein acted as positive roles in cold tolerance. The identified candidate proteins related to cold tolerance could be further studied in future, and proteomics combined with metabolomics and physiological analysis could illuminate and deepen the understanding of the mechanisms that underlie chilling stress tolerance in cantaloupe.

Methods

Plant materials and treatment conditions

The commercial cantaloupes (*Cucumis melo* var. *saccharinus*) Goldqueen-308 (GE) and Jiashi-310 (JS) were collected from No. 121 Regiment farm in Shihezi, Xinjiang, China. They were identified by Processing and Storage of Fruits & Vegetables Institute, Shihezi University, Xinjiang, China. No other permissions were necessary to select samples. After harvest, the fruits with uniform sizes were stored in chambers at 0.5°C ($0 \pm 0.5^\circ\text{C}$) for 0 (control), 12 and 24 days, respectively. The fruits were divided into three replicates per cultivar, each consisting in 6 fruits of similar size [18]. After storage, electrolyte leakage, H₂O₂ content and lipid peroxidation were measured, and the exocarp tissue of each replicate was frozen in liquid nitrogen and stored at -80°C. Three biological replicates were performed at each time point.

The determination of chilling stress induced physiological changes

Determined as malondialdehyde (MDA) content, lipid peroxidation and H₂O₂ content were determined according to F. Carvajal [18]. Relative electrolyte leakage (REL) was measured as described [55].

Protein extraction

1 to 2 g of the sample was weighed and placed in a mortar with 10% PVPP, and then was ground into powder with liquid nitrogen. After grinding, the sample was transferred to a 50 ml round bottom centrifuge tube with 5 times volume of Lysis Buffer 3, final concentration of 1 mM PMSF and 2 mM EDTA. Being well mixed, the sample was placed on ice for 5 minutes, adding a final concentration of 10 mM DTT, and soaked for 5 minutes in ice bath (2 sec/3 sec). After centrifugation, appropriate amount of Lysis Buffer 3 was added to the supernatant, and then soaked for 5 minutes (2sec/3sec). Centrifuged at 25,000 g and 4 °C in ice bath for 20 minutes [12]. Took the supernatant and the protein solution was prepared.

Protein quantification and digestion

Bradford protein assay was used for the protein quantification. Standard proteins (0.2 µg / µl BSA) were sequentially added to the 96-well microtiter plates A1 to A10 followed by the addition of pure water, then 180 µl of Coomassie Brilliant Blue G-250 Quantitative Working Solution was added to each well. After calculation of sample protein concentration, SDS-PAGE was carried out according to a previous study [56]. After electrophoresis, Coomassie brilliant blue was used for staining for 2 hours, then appropriate amount of decoloring solution (40% ethanol 10% acetic acid) was added to shaker decolorization solution. After digestion, the enzymatic peptide was subjected to desalting using a Strata X column and vacuum-dried.

Peptide labeling and separation

Each tube of IBT (2mg) reagent was dissolved in 80 µl of isopropanol and shaken for more than 1 minute to fully dissolve. The label was placed and allowed to stand at room temperature for 2 hours to make it fully labeled. The Shimadzu LC-20AB liquid phase system was used for the protein separation, and the separation column was a 5µm 4.6x250 mm Gemini C18 column for liquid phase separation of the sample [12]. The elution peak was monitored at a wavelength of 214 nm and one component was collected per minute, and the samples were combined with a chromatographic elution peak map to obtain 20 components, which were then freeze-dried.

LC-MS/MS analysis and mass spectrometry

The dried peptide samples were reconstituted with mobile phase A (2% ACN, 0.1% FA), centrifuged at 20,000 g for 10 minutes, and the supernatant was taken for injection. Separation was carried out by a Shimadzu LC-20AD model nanoliter liquid chromatograph. The nanoliter liquid phase separation end was directly connected to the mass spectrometer. The peptides separated by liquid phase were ionized by a nanoESI source and then passed to a tandem mass spectrometer Q-Exactive (Thermo Fisher Scientific, San Jose, CA) for DDA (data dependent acquisition) mode detection.

Metabolite measurements

Gas chromatography/mass spectrometry (GC/MS) was optimized for amino acids according to Kind T and Dunn WB [57, 58]. Six biological replicates were collected for each cultivar at every time point and significant enrichment was detected with $P < 0.05$. Data analysis was carried out as described previously [49].

Quantitative real time reverse transcription PCR analysis

Total RNA extraction, DNase treatment and cDNA synthesis were performed as described [4]. The relative expression of each target gene was calculated by using the $2^{-\Delta\Delta C_t}$ method and the internal reference gene was used for the normalization of all C_t values. Values were presented as the mean of three independent analyses.

Bioinformatics analysis

The Clusters of Orthologous Groups (COG) (<http://www.ncbi.nlm.nih.gov/COG/>) database and GO (Gene Ontology) annotation were assigned according to those reported in the NCBI (<http://www.ncbi.nlm.nih.gov/gene>) and Swissprot database to functionally classify the DEPs. Pathway analyses of identified proteins were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/>) [16].

Abbreviations

GE: Goldqueen–308; JS: Jiashi–310; iTRAQ: Isobaric tags for relative and absolute quantification; DEPs: Differentially expressed proteins; ROS: Reactive oxygen species; H₂O₂: Hydrogen peroxide; MDA:

Malondialdehyde; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; REL: Relative electrolyte leakage; PMSF: Phenylmethanesulfonyl fluoride; EDTA: Ethylenediaminetetraacetic acid; DTT: DL-dithiothreitol; BSA: Albumin from bovine serum; SDS-PAGE: SDS-polyacrylamide gel electrophoresis; DDA: Data dependent acquisition; GC/MS: Gas Chromatography/mass spectrometry; HCL: Hierarchical cluster analysis; PCA: Principal component analysis; CC: Cellular component; MF: Molecular function; BP: Biological process; GST: Glutathione S-transferase; HSPs: Heat shock proteins; NADPH: Nicotinamide adenine dinucleotide phosphate; PAL: Phenylalanine ammonia-lyase; 14–3–3s: 14–3–3 Proteins.

Declarations

Ethics approval and consent to participate

All the available cantaloupes (*Cucumis melo* var. *saccharinus*) Goldqueen-308 (GE) and Jiashi-310 (JS) used in our study were procured from the collection of No. 121 Regiment Farm in Xinjiang, Processing and Storage of Fruits & Vegetables Institute, Shihezi University, China. In addition, samples collection complied with the institutional, national and international guidelines. Our project does not use transgenic technology hence it does not require ethical approval.

Consent for publication

No applicable.

Availability of data and materials

The datasets generated in this study are included in its additional files, and the raw data is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CHS, WS and FXT designed the experiments. WS, QZ and FKZ performed the experiments. WCC performed the HCL and PCA analysis; WS, MN and HT analyzed the data; WS wrote the manuscript. All authors approved the final version of manuscript.

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Tables

Table 1 KEGG pathway analysis of proteins in GE and JS during chilling stress at different time period

Pathway name	Pathway ID	Number of proteins			
		GE		JS	
		12 d	24 d	12 d	24 d
Ribosome	ko03010	34	42	38	-
Phenylpropanoid biosynthesis	ko00940	18	11	13	-
Protein processing in endoplasmic reticulum	ko04141	25	32	-	21
Photosynthesis - antenna proteins	ko00196	3	-	-	9
Plant-pathogen interaction	ko04626	12	11	-	-
Galactose metabolism	ko00052	-	10	9	10
Carbon fixation in photosynthetic organisms	ko00710	-	11	-	-
One carbon pool by folate	ko00670	-	4	-	-
Photosynthesis	ko00195	-	-	11	-
Phagosome	ko04145	8	-	11	12
Cyanoamino acid metabolism	ko00460	-	-	6	-
Amino sugar and nucleotide sugar metabolism	ko00520	-	-	-	11
Fructose and mannose metabolism	ko00051	-	-	-	8
Pentose and glucuronate interconversions	ko00040	-	-	-	7
Linoleic acid metabolism	ko00591	-	-	-	4

Table 2 Amino acids metabolites with changes in abundance (relative content) during chilling stress in GE and JS at different periods

Compound	GE		JS	
	12 d	24 d	12 d	24 d
Proline	3.005204	2.454753	1.400016	1.899369
3-Hydroxy-L-proline 3	1.272809	1.571988	0.682412	1.342197
3-Cyanoalanine	1.569883	1.087918	0.87722	1.185156

Figures

Figure 1

The phenotypic changes of GE and JS at different time points under cold treatment. a, Representative pictures of GE and JS for untreated samples (0d), exposed to 12d and 24d of cold treatment, respectively; b, The comparison of REL, MDA content and H₂O₂ content changes in GE and JS after cold treatment with control. Statistical analyses were performed by analysis of variance (ANOVA) and the letters at the top of bars indicate significance at $P < 0.05$. The data represents the mean value of three biological replicates

Figure 2

Identified differentially expressed proteins (DEPs) in GE and JS after 0, 12 and 24 days cold treatment. a, The number of DEPs in GE and JS at different time points; b, Venn diagram of common and specific expressed DEPs for GE and JS under cold treatment at different time periods; c, Hierarchical clustering analysis of common expressed DEPs from GE and JS (Up-regulated proteins in red, down-regulated proteins in green and no change in white); d, Principal component analysis of common expressed DEPs from GE and JS.

Figure 3

Function classification of all DEPs. a, Clusters of Orthologous Groups (COG) function classification of DEPs in GE and JS; b, Gene Ontology (GO) annotation of DEPs in GE and JS between cold stress and control conditions at early phases; c, Gene Ontology (GO) annotation of DEPs in GE and JS between cold stress and control conditions at late phases.

Figure 4

Statistics of KEGG pathway enrichment. a, Top 6 statistics of KEGG pathway enrichment in GE at 12 days; b, Top 7 statistics of KEGG pathway enrichment in GE at 24 days; c, Top 6 statistics of KEGG pathway enrichment in JS at 12 days; d, Top 8 statistics of KEGG pathway enrichment in JS at 24 days.

galactose metabolism in pink); d, PPI in JS at 24 days (Different nodes colors represent the types of enrichment in predicting the associations among proteins according to P-value: Photosynthesis-antenna proteins in red, phagosome in blue, galactose metabolism in green, protein processing in endoplasmic reticulum in yellow, amino sugar and nucleotide sugar metabolism in pink, fructose and mannose metabolism in dark green, linoleic acid metabolism in light blue). The DEPs were submitted to the STRING tool (<http://string.embl.de/>) to predict protein-protein interaction network

Figure 6

Relative quantification of expression of eight genes in GE and JS after cold treatment. Statistical analyses were performed by analysis of variance (ANOVA), and the letters at the top of bars indicate significance at $P < 0.05$. The data represents the mean value of three biological replicates

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