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iTRAQ-based comparative proteomic analysis of two coconut varieties reveals aromatic coconut cold-sensitive in response to low temperature

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ABSTRACT

Coconut (*Cocos nucifera* L.) is an important economic fruit and oil crop largely cultivated in humid and sub-humid tropical coastal zones worldwide. To date proteomic profile analysis of coconut under cold stress yet not been conducted. In order to understand the cold stress tolerance in coconut, the iTRAQ approach was employed to dissect proteomic response of two coconut varieties Hainan Tall, BenDi (BD) and Aromatic coconut, XiangShui (XS) under cold stress. Under cold treatment at (8 °C) for 2 days, 193 up and 134 down-regulated in BD (Cn-DB-0_VS_Cn-DB-2) and 140 up and 155 down-regulated DEPs in XS (Cn-XS-0_VS_Cn-XS-2) were identified. The 5 days post cold treatment also identified increased abundance of up-regulated proteins in BD compared to XS. The 5 days post treatment (dpt) depicted 172-up/127-down and 108-up/134-down accumulated proteins for BD (Cn-DB-0_VS_Cn-DB-5) and XS (Cn-XS-0_VS_Cn-XS-5) respectively. A total of 22, 12 and 14 DEP categories were enriched in biological process, cellular component and molecular function respectively in Gene Ontology (GO) analysis of two coconut varieties. Metabolic and biosynthesis of secondary metabolites pathways were highly enriched in KEGG pathway analysis of DEPs between two varieties. Twenty-two different functional classes revealed differentially expressed proteins in two varieties. Among those, four major categories involved in metabolism, stress response, photosynthesis and respiration related DEPs increased abundance in two varieties. However, general function perdition only (GFPO) and stress-responsive proteins were greatly up-regulated in BD than XS. Increased abundance of stress response related proteins up-regulation under cold stress suggested that BD is cold-tolerant variety. Collectively, iTRAQ-based coconut leaf proteomic analysis showed that XS (aromatic) coconut variety is cold-sensitive compared to BD (Hainan Tall) variety. This study provided a basis for further functional analyses to understand the molecular mechanisms of tropical crops adapting to cold stress.

Significance: Leaf proteomic approach determines the role of differentially expressed proteins (DEPs) under cold stress in crops. However, cold stress could damage the coconut fruit lead to decrease in crop yield during winter in China. Here, we report the first ever iTRAQ-based proteomic analysis of two coconut varieties in response to cold stress. The study identified the proteins involved in biosynthesis of secondary metabolites, photosynthesis, respiration, biotic and abiotic stresses under cold stress in two coconut varieties. Moreover, the increased abundance of stress-responsive and general function proteins in BD under cold stress suggested that Hainan Tall is cold-tolerant compared to aromatic coconut variety. Inhibition abundance of photosynthesis related proteins may reduce photodamage owing to the over energized state of thylakoid membrane lead to ROS generation during oxidative stress. This could be the reason for adaption of BD to low temperature stress. Nonetheless, further research may insight the mechanism involved in cold tolerance/sensitive in coconut in response to low temperature.

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1. Introduction

Cold stress is a main abiotic stress which adversely affects the plant development, physiology, geographical distribution and crop yield [1,2]. Cold stress largely influences plant biochemical and physiological processes such as, respiration, photosynthesis, water circulation and antioxidant enzymatic activity [3]. To mitigate such adverse condition, plants could adopt several strategies including, raising levels of antioxidants and chaperons, activation of primary metabolites, and change in gene transcription under cold stress [4–6]. However, numerous crops including, barley, wheat and rye are low temperature tolerant, on the contrary several crops such as maize, rice and soybeans, are cold stress sensitive [1,7]. Transcriptome analysis revealed various cold induced genes in different plants [8–11]. The gene expression profile at transcription and post-transcription level is usually poor correlated with their corresponding transcripts compared to protein expressions [12,13]. Hence proteins are directly involved in abiotic stress responses to decipher plant proteome posttranscriptional events and posttranslational modifications under cold stress [14,15]. Previously, combined proteomics and cold-resistant mutation dissected the role of cold-induced genes which paved a way to understand the possible molecular mechanisms and signaling pathways involved in plants under cold stress [16]. Furthermore, proteomics approach unveiled complex regulatory network under cold stress in number of plant species including, *Oryza sativa* [17,18], *Populus euphratica* [19], *Arabidopsis* [20]. Nevertheless, the iTRAQ (isobaric tags for relative and absolute quantification) approach is high throughput quantitative proteomics technique used for identification and quantification of cellular metabolic changes in proteome [21]. This technique has been widely used in plants to identify proteins under abiotic stresses. For instance, drought stress responsive-proteins were identified in tobacco leaves [22], new metabolic pathway was revealed in wheat crop under hydrogen peroxide stress via iTRAQ technique [23]. Nonetheless, nutrient stress in tomato root brought change in proteome through iTRAQ method [24].

Particularly, iTRAQ-based technique also demonstrated the role of plant protein responses to cold stress. Differentially expressed proteins (DEPs) were identified in maize crop under cold stress [2]. Recently, iTRAQ-based proteomic analyses conducted in wheat [6], *Anabasis aphylla* [3], *Brassica* [25,26], watermelon [14], tea [27] and Castor [28] under cold stress. Moreover, differentially expressed proteins (DEPs) were determined at low temperature in tea leaves via iTRAQ-based proteomic analysis [29]. Additionally, Zheng et al. [30] applied iTRAQ proteomic approach and unraveled differentially expressed proteins (DEPs) associated with leaf senescence in cotton under cold stress. Whereas, one study identified cold-responsive proteins in wheat crop using iTRAQ and virus-induced gene silencing (VIGS) analyses [6].

Coconut (*Cocos nucifera* L.) is tropical fruit and oil crop cultivated in tropical zone at mean annual temperature 29 °C (27–32 °C) worldwide [31,32]. Notwithstanding, low temperature (below 13 °C) could damage coconut flowering and fruiting (wrinkled kernel inside the nuts) in China. Though, Hainan Tall variety is reported to be cold tolerant in China [31]. Low temperature treatment causes the leaf injury and anatomical structure change and Hainan Tall shows more low temperature tolerance than aromatic coconut [33]. Generally, low temperature reduces nut production and abnormal fruit development in coconut crop grown in Hainan province, China. As mentioned earlier the Hainan Tall variety is cold resistant, and we hypothesized that Hainan Tall variety may play a role in terms of iTRAQ-based proteomics analysis under cold stress compared to other coconut varieties. Nonetheless, the aromatic coconut is predominant variety in coconut-producing area of province. This prompted us to investigate iTRAQ-based quantitative proteomic analysis of these two coconut varieties under cold stress. The leaves of two coconut varieties namely, Hainan Tall coconut (BD) and aromatic coconut (XS) were brought under low temperature (8 °C) for two and five days for proteomic analysis. We found the aromatic coconut (XS) was cold sensitive as it down regulated more number of proteins in iTRAQ data compared to Hainan Tall coconut (BD). Our study would provide a basis for molecular mechanism understanding the adaptation and coconut productivity under low temperature in China.

2. Materials and methods

2.1. Plant materials and cold stress treatment

Seedlings of two coconut (*Cocos nucifera* L) varieties Hainan Tall coconut (BD) and aromatic coconut (XS) were grown in nursery at Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences, Wenchang, Hainan, P.R. China before cold stress treatment. Seedlings germinated in same nursery were selected for cold treatments. For control seedlings of both varieties were grown in growth chamber with 16 h/ 8 h (light/dark) at 28 °C for 2 or 5 days separately. For cold treatment seedlings of both Hainan Tall coconut (BD) and aromatic coconut (XS) varieties were kept at 8 °C for 2 (samples names Cn-BD-2 and Cn-XS-2) and 5 days (samples names Cn-BD-5 and Cn-XS-5) in separate growth chambers. The ten individual coconut seedlings were selected for each treatment i.e., Cn-BD-0, Cn-BD-2, CnBD-5 for Hainan Tall and Cn-XS-0 Cn-XS-2 and Cn-XS5 for aromatic coconut variety (the total 60 seedlings). For each seedling, three leaves from cold treated (8 °C) (2 and 5 days post cold treatment for both varieties) and control conditions (28 °C) (samples names Cn-BD-0 and Cn-XS-0) were harvested and quickly frozen in liquid nitrogen individually. Simultaneously, samples were stored at −80 °C for protein extraction. There were three biological replicates per treatment for iTARQ-based proteomic analysis.

2.2. Protein extraction and iTRAQ labeling

TRAQ-based proteomic analysis was carried out in Beijing Genomics Institute (BGI), Shenzhen, China. Total proteins of leaves from each sample were extracted as described by Xu et al. [26]. Each sample contained two biological replicates. However, protein quality and concertation were determined following (SDS-PAGE) and Bradford assay [34] respectively. Total 100 μg of protein from each sample was digested with trypsin (Promega, Madison, WI, USA) with protein/enzyme ratio at 20:1 for 8 h at 37 degrees Celsius. Peptides reconstitutions were combined with 0.5 M (TEAB) and processed using 8-plex iTRAQ reagent (Applied Biosystems) in accordance with manufacture's protocol. Subsequently, leaf samples of BD were labeled as 113/114 for Cn-DB-0_VS_Cn-DB-2, 113/115 for Cn-DB-0_VS_Cn-DB-5 and 114/115 for Cn-DB-2_VS_Cn-DB-5. The XS was labeled as 116/117 for Cn-XS-0_VS_Cn-XS-2, 116/118 for Cn-XS-0_VS_Cn-XS-5 and 117/118 for Cn-XS-2_VS_Cn-XS-5. Comparatively labelling for both varieties was as 113/16 for Cn-DB-0_VS_Cn-XS-0, 114/117 for Cn-DB-2_VS_Cn-XS-2 and 115/118 for Cn-DB-5_VS_Cn-XS-5. The labeled peptides were then mixed and dried through vacuum centrifugation.

2.3. LC-ESI-MS/MS analysis based on triple TOF 5600

iTRAQ-labeled peptide mixtures were fractionated on a LC-20 CE nano-HPLC (Shimadzu, Kyoto, Japan) as described by Yang et al. [35]. Peptides then were eluted on a 10 cm analytical C18 column (inner diameter 75 μm) packed in-house following methods described by Zeng et al. [25]. LC-ESI-MS/MS analysis was performed with TripleTOF 5600 System (AB SCIEX, Concord, ON, Canada) and data was acquired utilizing an ion spray voltage of 2.5 kV following Bradford [34]. Information-based data acquisition was performed as previously described by Wang et al. [2].

2.4. Protein identification and quantification

iTRAQ Proteins were identified using Mascot search engine (Matrix

Science, London, UK; version 2.3.02) using BLAST search against *Cocos nucifera* (https://www.ncbi.nlm.nih.gov/protein/?term=txid13894) and oil palm species (for paralogous) search at (NCBI) (https://www.ncbi.nlm.nih.gov/protein/?term=txid51953) database containing 44,450 sequences. The detailed parameters and factors identifying the proteins were followed as previously described by Wang et al. [2]. Ratios for quantitative protein was determined by median ration in Mascot with *P*-values < .05 and fold changes of > 1.2 (up-regulated) and < 0.833 (down-regulated) were considered as significant [14].

2.5. Bioinformatics analysis

Functional annotations of the differentially expressed proteins were performed utilizing Blast2GO program against the non-redundant protein database (NR; NCBI). Further the detailed functional information was analyzed and confirmed at UniProt data base (https://www.uniprot.org/). Classification of DEPs were conducted at COG database (http://www. ncbi.nlm.nih.gov/COG/). However, functional categorization of DEPs was carried out at Gene Ontology (http://www.geneontology.org). The metabolic pathways were determined using Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) database. The significant threshold for GO and KEGG pathways enrichment was at *P*-value ≤.05.

3. Results

3.1. Primary data analysis and protein detection in two coconut varieties under cold stress

The iTRAQ experiment generated a total of 215,400 spectra number using nine samples of coconut leaves for 2 day and 5 days at low temperature (8 °C). In detail, the total of 24,658 spectra matched to known spectra, 20,053 matched known unique spectra, 8599 peptides, 7581 unique peptides and 2975 protein were detected by Mascot software (2.3.02) version (Fig. 1A). The unique peptide number distribution determined the total of 1376 proteins covered by single peptide. Although, 2–6 peptides, 7–10 peptides and > 10 peptides were 1413, 131 and 55 respectively (Fig. 1B). The peptide sequence distribution showed proportion of proteins with different coverage, of which 59.1% of proteins covered less than 10% peptide sequences. However, 10–15% and 15–20% constituted 15% and 9% respectively (Fig. 1C).

3.2. Differentially expressed proteins (DEPs) identification in two coconut varieties

The total 2468 DEPs were identified in nine leaves samples comparisons, based on fold changes > 1.2 (Up-regulated) and < 0.833 (down-regulated) at *P*-value < .05. Subsequently, Cn-DB-0_VS_Cn-DB-2 (193-up/134-down), Cn-DB-0_VS_Cn-DB-5 (172-up/127-down) and Cn-DB-2_VS_Cn-DB-5 (107-up/92-down) DEPs were identified in BD coconut variety for 2 and 5 days post cold treatment (Fig. 2A). In case of XS variety the Cn-XS-0_VS_Cn-XS-2 (140-up/155-down), Cn-XS-0_VS_Cn-XS-5 (108-up/134-down) and Cn-XS-2_VS_Cn-XS-5 (104-up/87-down) DEPs were identified in 2 and 5 days post cold treatment (Fig. 2B). Comparatively, Cn-DB-0_VS_Cn-XS-0 at (28 °C) temperature as control was with (148-up/120-down) DEPs. However, Cn-DB-2_VS_Cn-XS-2 (140-up/208-down) and Cn-DB-5_VS_Cn-XS-5 (129-up/161-down) proteins were detected in both varieties for control, 2 and 5 days post cold treatment (Fig. 2C).

3.3. Gene Ontology (GO) and functional classification analysis of two coconut varieties under cold stress

The GO analysis was conducted to assess the functions of DEPs. A total of 22, 12 and 14 categories were enriched in biological process, cellular component and molecular function respectively (Fig. 3). Different processes (metabolic, cellular, single/multicellur-organism, response to stimulus and biological regulation) were on the top enriched proteins in biological process. The cell, cell part, organelle, organelle part, membrane, membrane part and macromolecular complex were dominated in GO cellular component cluster frequency. Furthermore, binding and activity functions including; binding, catalytic, transporter, structural molecule, electron carrier and enzyme regulator activities were most of the differential expressed proteins in molecular function (Fig. 3).

Moreover, Orthologous Groups of proteins (COG) functional classification of differential expressed proteins could categorize into 23 different classes (Fig. 4). The most number of protein abundance was in general function prediction only (GFPO) followed by posttranslational modification, protein turnover, chaperones (PTMPTC), carbohydrate transport and metabolism (CTM), translation ribosomal structure and biogenesis (TRSB), energy production and conversion (CPC) and amino acid transport and metabolism (AaTM). Nevertheless, the detailed analysis for both BD and XS classified into 22 categories and only important differential expressed proteins accumulated under cold stress for 2 and 5 days post treatment described in Table S1–4. Consequently, Cn-DB-0_VS_Cn-DB-2 anticipated 157 up-regulated (out of 193) and 126 down-regulated (out of 134) important DEPs respectively (Table S1). Similarly, Cn-DB-0_VS_Cn-DB-5 displayed 144 (out of 172) up-regulated and 111 (out of 127) down-regulated proteins in different categories (Table S2). The Cn-XS-0_VS_Cn-XS-2 accumulated 127 up-regulated (out of 140) and 141 (out of 155) were down-regulated in XS (Table S3). Cn-XS-0_VS_Cn-XS-5 resulted 105 (out of 108) up-regulated and 122 (out of 134) down-regulated the important Differential expressed proteins in different categories (Table S4). The general function prediction only (GFPO) and function unknown (FU) DEPs were the most up and down accumulated proteins in BD. Total 44 up-regulated and 19 down-regulated DEPs were of (GFPO) category. Twenty up-regulated and fourteen down-regulated proteins were found function unknown (FU) in Cn-DB-0_VS_Cn-DB-2. Translation ribosomal structure and biogenesis (TRSB) and posttranslational modification, protein turnover, chaperones (PTMPTC) were more down-regulated proteins compared to up-accumulated (Fig. 5A). Lipid transport and metabolism (LTM) related DEPs were accumulated more compared to down-regulated proteins. Total of eleven DEPs were up-accumulated and 10, 14, 13 and 7 down-regulated in carbohydrate transport and metabolism (CTM), amino acid transport and metabolism (AaTM), energy production and conversion (EPC) and inorganic ion transport and metabolism (IITM) respectively in Cn-DB-2 (Fig. 5A). Remaining categories regulated less than 7 or no any DEPs (Table S1; Fig. 5A). Thirty four number of proteins up-regulated and twenty three down-regulated in (GFPO) and same results prevailed in function unknown (FU) class which expressed more DEPs compared to down-regulated in Cn-DB-0_VS_Cn-DB-5 (Fig. 5B). Additionally, the more number of DEPs were up-regulated in (CTM), (IITM), (AaTM) and (LTM), nonetheless, (EPC) and (TRSB) were down-regulated with more number of DEPs. The fifteen number of proteins were up-regulated and thirteen down-regulated in (PTMPTC). The rest of the categories were regulated less than 8 or no any proteins accumulated in Cn-DB-5 (Table S2; Fig. 5B).

In contrast, the Cn-XS-0_VS_Cn-XS-2 (XS) down-regulated more number of DEPs (40) and 20 up-regulated proteins in (GFPO) category. Posttranslational modification, protein turnover, chaperones (PTMPTC) also found with more number of down-regulated proteins with thirty-one and only nine DEPs were up-accumulated (Fig. 5C). While (CTM), (FU), (LTM) and AaTM Up-regulated more number of proteins compared to down-regulated DEPs. Interestingly, translation ribosomal structure and biogenesis (TRSB), transcript and Signal transduction mechanisms (TSTM) and replication, recombination and repair (RRR) were up and down regulated equal number of DEPs with 9, 4 and 2 respectively. In (EPC) category the 12 and 15 proteins were up and down regulated respectively in Cn-XS-2. Three categories found with no any proteins either up or down regulated (Table S3; Fig. 5C). Finally,

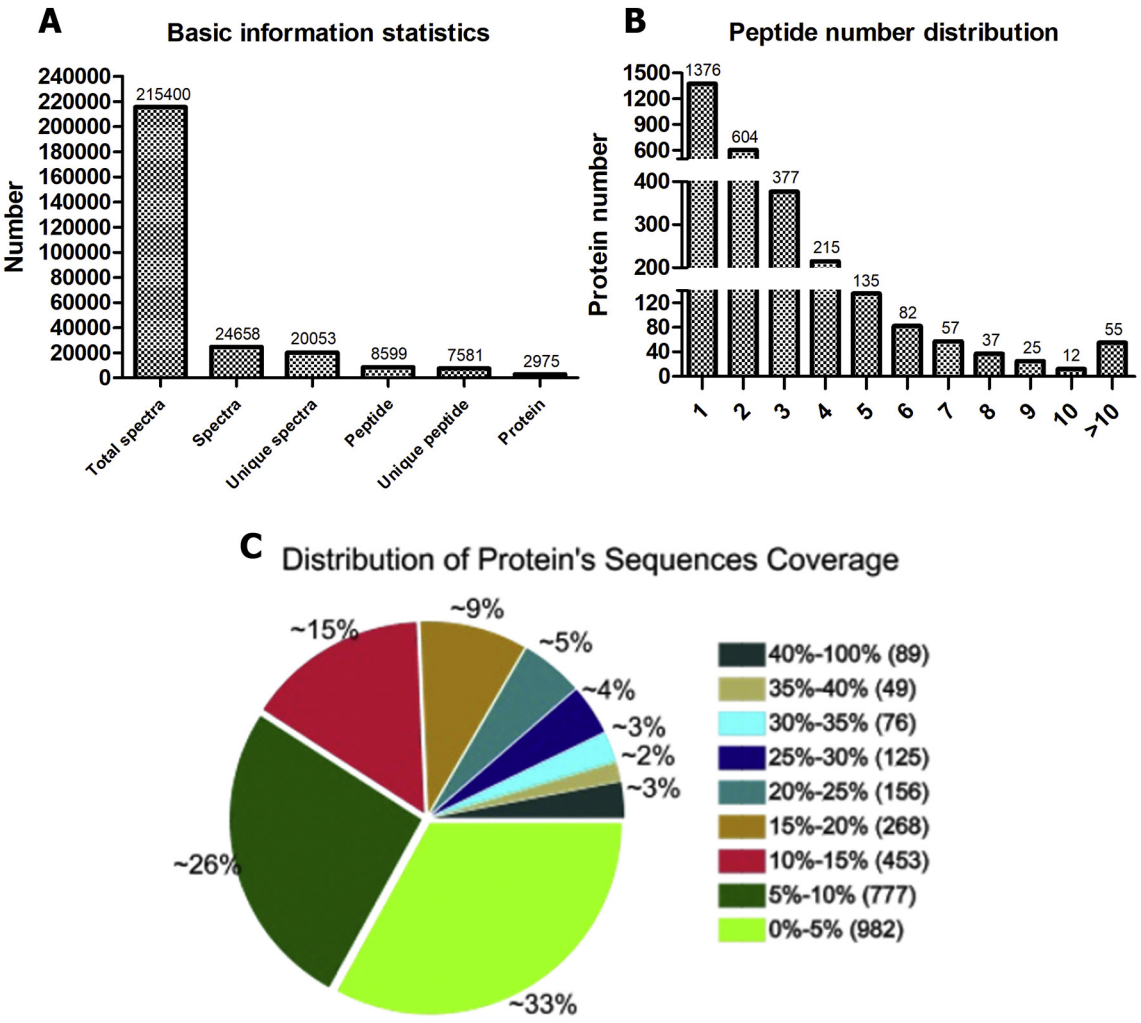

Fig. 1. General information of iTRAQ data output. (A) Basic information statistics. (B) Number of unique peptide matched to identified proteins. (C) Peptide sequence coverage for identified proteins.

the (GFPO), (FU), (EPC) and (PTMPTC) categories found more number of proteins down-regulated with 29, 18, 13 and 12 DEPs respectively compared to up-accumulated (14, 13, 9 and 9) in Cn-XS-0_VS_Cn-XS-5 (Fig. 5D). Three classes namely; (CTM), (LTM) and (TRSB) up-regulated more number of DEPs compared to its counterpart. The (IITM) and cell wall/membrane/envelope biogenesis (CWMEB) distributed with equal number of up and down regulated DEPs with 5 and 2 respectively. Four categories demonstrated only down-regulated proteins either two or one but no any protein up-accumulated in Cn-XS-5 (Table S4; Fig. 5B).

Numerous proteins characterizing different classes frequently down-regulated in XS (CnXS-2) compared to BD (CnBD-2) (Fig. 6A). Intriguingly, several DEPs belonging to similar categories decreased their expressions in aromatic coconut the XS variety (CnXS-5). Concurrently, same proteins were up-regulated in Hainan tall variety BD (CnBD-5)

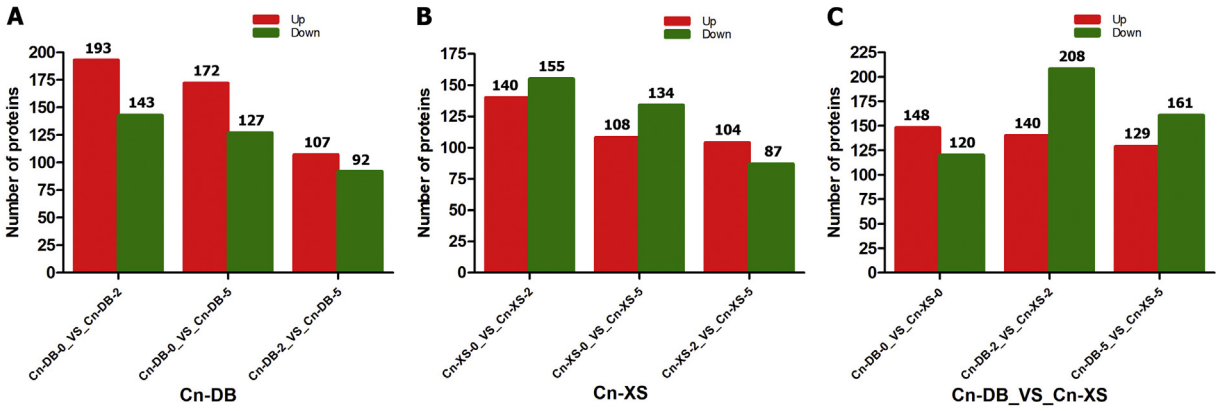

Fig. 2. Differentially expressed proteins (DEPs) identified in coconut varieties under cold stress. (A) Represents up and down-regulated proteins in BD. (B) Indicates up and down-regulated protein in XS. (C) A comparative up and down-accumulated proteins in BD and XS.

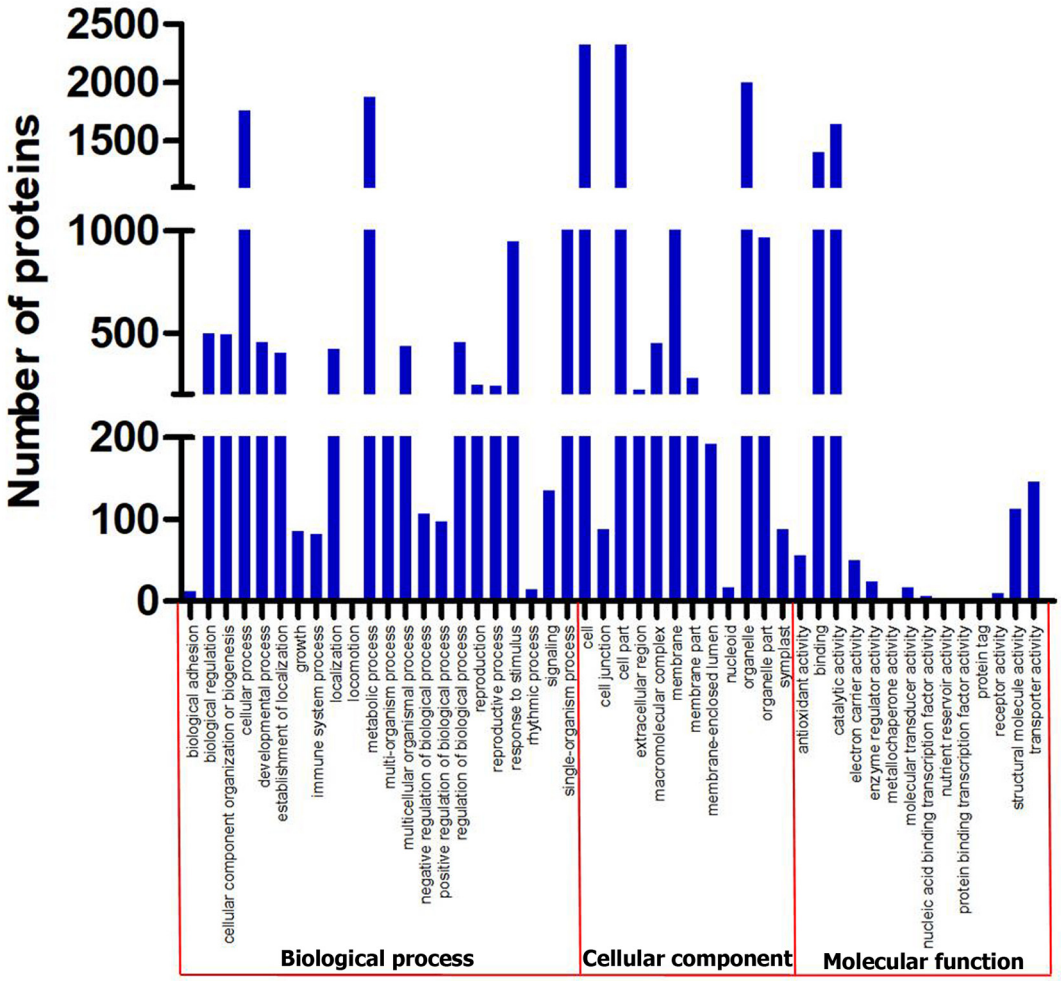

Fig. 3. Gene Ontology (GO) analysis of the differentially expressed proteins in BD and XS under cold stress. The vertical red lines are indicating the biological process, cellular component and molecular function at x-axis.

under cold stress (Fig. 6B).

These results suggested that XS variety (aromatic coconut) is more sensitive under cold stress as it down-accumulated more number of DEPs in both 2 and 5 day post treatment compared to BD (Hainan tall) variety (Fig. 6A and B).

3.4. KEGG pathway enrichment analysis of DEPs between two coconut varieties

The KEGG pathways analysis revealed two predominant the metabolic pathways [ko01100] and biosynthesis of secondary metabolites [ko01110] with 34.95% and 21.01% respectively. Among top twenty KEGG enriched pathways, the ribosome, spliceosome, glycolysis/gluconeogenesis, RNA transport, protein processing in endoplasmic reticulum, pyruvate metabolism, plant-pathogen interaction and carbon fixation in photosynthetic organism pathways were found with large number of DEPs in coconut under cold stress (Fig. 7A). Specifically with *P*-value < .05 as a cutoff, the total of 9, 6, 11 and 5 significantly enriched pathways were identified in CnBD-0_VS_CnBD-2, CnBD-0_VS_CnBD-5, CnXS-0_VS_CnXS-2 and CnXS-0_VS_CnXS-5 respectively (Fig. 7B; Table S4). The DEPs for BD variety the metabolic pathways [ko01100], phenylpropanoid biosynthesis [ko00940] and tyrosine metabolism [ko00350] were observed at three top significantly enriched pathways at 2 days post treatment of low temperature (Cn-BD-2). Whereas two inositol phosphate metabolism [ko00562] and beta-alanine metabolism [ko00410] enriched pathways were only found in same variety (Cn-BD-5). In addition, the glyoxylate and dicarboxylate metabolism [ko00630], pyruvate metabolism [ko00620] and glycolysis/gluconeogenesis were among the top three enriched pathways which only assigned to (Cn-XS-2) and ribosome [ko03010] solely found in (Cn-XS-5) of XS variety. The five KEGG pathways namely; photosynthesis [ko00195], propanoate metabolism [ko00640], photosynthesis-antenna proteins [ko00196], purine metabolism [ko00230] and phenylalanine metabolism [ko00360] significantly found in both varieties (Table S5; Fig. 7B).

3.5. DEPs related to metabolism under cold stress in two coconut varieties

Total of 189 DEPs were involved in metabolism related differential accumulated proteins in both varieties of coconut. Out of total, 104 DEPs were only expressed, however, 71 found solely down-regulated proteins. 14 DEPs showed both expression up/down-regulations in both BD and XS varieties (Table S6; Fig. 8). Based on fold changes > 2.0 as a cutoff for up-regulated total of five DEPs including; glucosinolate-specific transporter, glycerophosphodiester phosphodiesterase GDPDL1, monocopper oxidase-like protein SKU5, cysteine protease (CPRF), flavanone 3-hydroxylase were highly expressed in BD variety. GDPDL1 was also expressed in XS but showed fold lower than 1.9 (Table S1–4 and Table S6). The three DEPs, i.e., isoflavone reductase, bifunctional chitinase/lysozyme and momilactone A synthase were expressed in XS. The isoflavone reductase also up-regulated in BD (CnBD5) at fold changes lower than 2 under cold stress. Fourteen metabolisms related

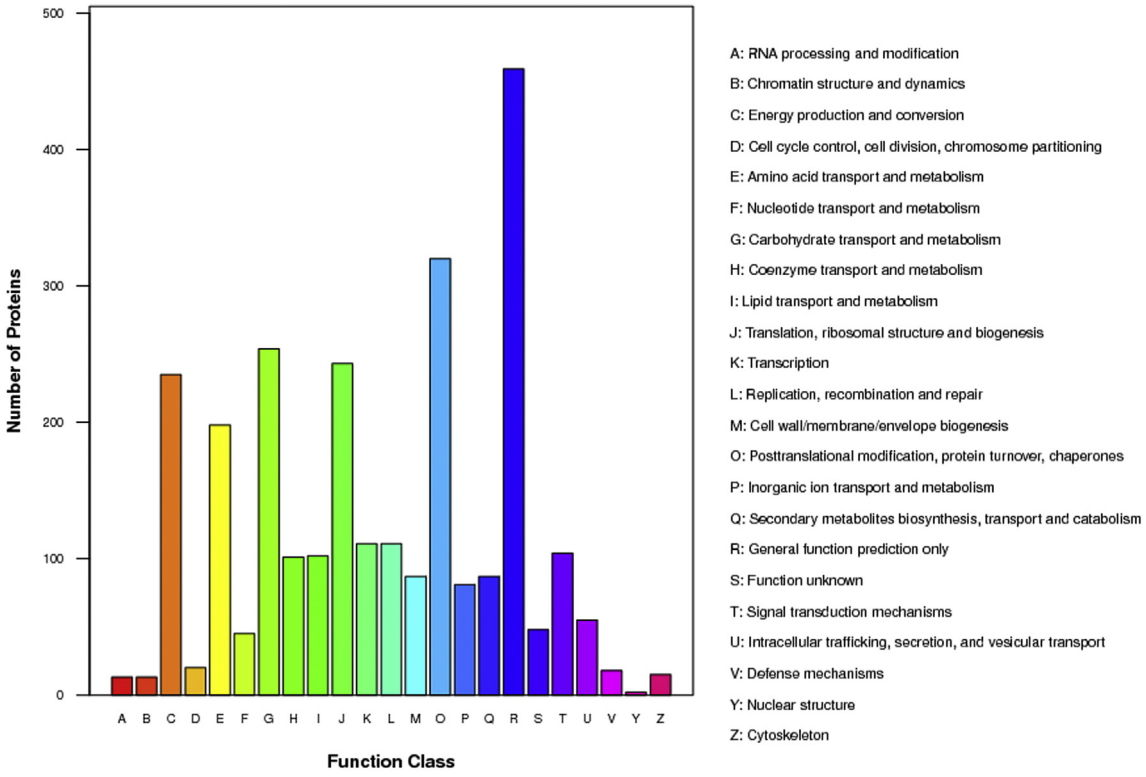

Fig. 4. Clusters of Orthologous Groups of proteins (COG) classification of differentially expressed proteins that were detected in coconut seedlings under cold stress. The different colors represent the each category of functional classes.

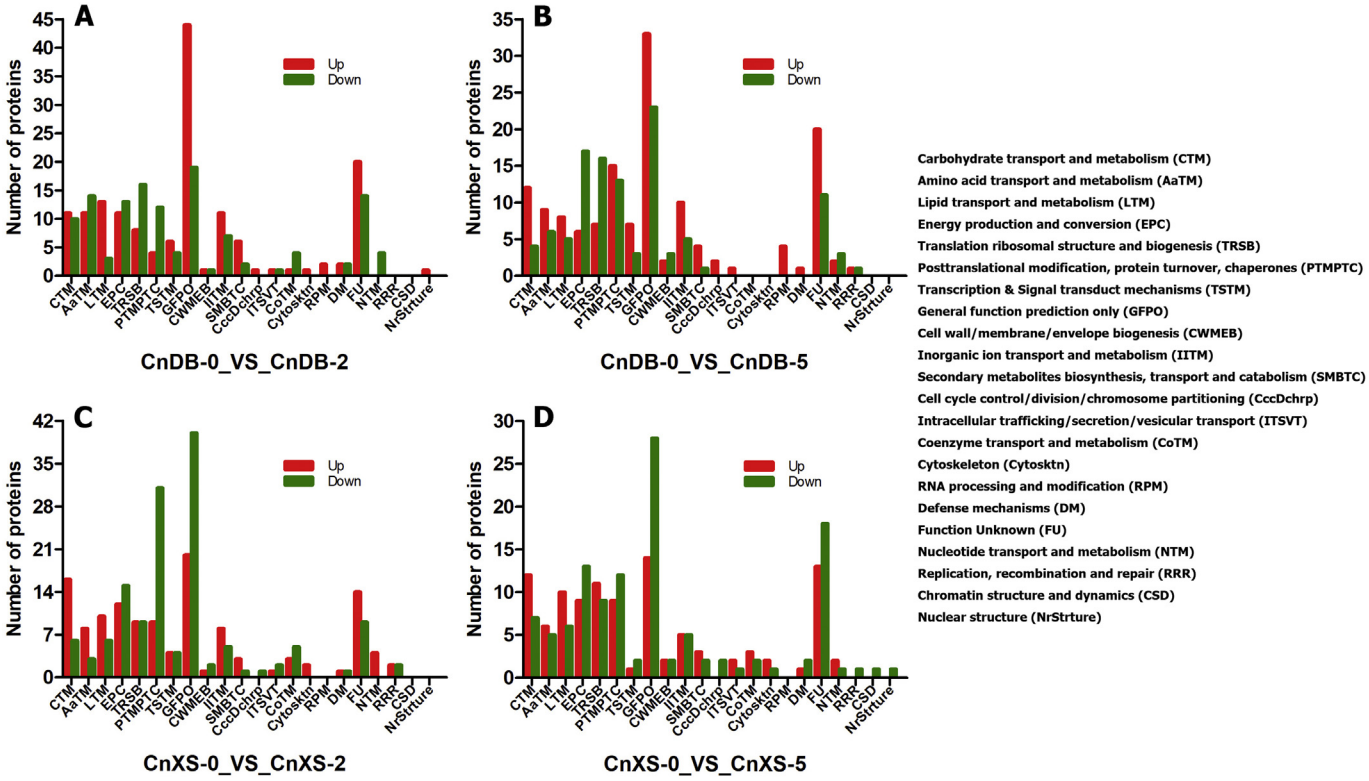

Fig. 5. Functional classification of DEPs analyzed in two coconut varieties under cold stress. (A) and (B) denote the up and down-regulated proteins observed in BD for 2 and 5 dpt. (C) and (D) symbolized DEPs for XS in both 2 and 5 dpt.

Fig. 6. Abundant number of up-regulated proteins observed in BD and simultaneously these down-regulated in XS under cold stress. (A) Showing the up-regulated and down-regulated proteins related to different classes at 2 dpt for BD and XS respectively. (B) Represents a comparison of up and down-regulated proteins of different categories at 2 and 5 dpt in BD and XS respectively. Red and green colors are indicating the up and down-regulated proteins.

DEPs were down-regulated at less than 0.6 expression values in both varieties including 8 in XS and 6 in BD. Furthermore, fold changes at > 1.2 (up-regulation) or < 0.833 (down-regulation), total twelve metabolic proteins showed both up and down-regulations of expression in both varieties (Table S1–4 and Table S6).

3.6. DEPs related to biotic, abiotic and oxidative stresses under cold stress in two coconut varieties

Seventy four total DEPs were related to various stimuli accumulated in both varieties of coconut (Table 1; Fig. 8). Thirty-seven DEPs involved in abiotic stress related stimuli including; 20 heat shock, 5 drought, 4 metal ion and hormonal stress, 2 ROS and, 3 for cold and salt stress. Biotic and oxidative stress-related DEPs were 19 and 10 respectively in both varieties. Though, eight multi-stimuli responsive (oxidative, pathogens, ROS and wounding) proteins including; peroxidase 3/4/5/12/17/30/52 and putative 52 were observed in both varieties under cold stress (Table 1 and Table S1–4). Fold changes at > 2.0 cutoff for diverse stimuli related proteins such as glutathione S-transferase F10, rRNA N-glycosidase, PNC1, putative peroxidase 52, peroxidase 12, 52, cold shock proteins, SODCP, PI206 were significantly up-regulated under cold stress in BD variety. Simultaneously, last four proteins were significantly down-regulated in XS. Following the same criterion, the XS was found with four highly expressed stress related DEPs including; HSP101, peroxisomal acyl-coenzyme A oxidase 1, DnaJ protein homolog and glucan endo-1,3-beta-glucosidase under cold stress (Table S1–4). Each of variety down-accumulated three DEPs including; soluble inorganic pyrophosphatase 6, peroxisome type ascorbate peroxidase and protein CutA 1 for BD; and non-specific lipid-transfer protein, CPN20 and high mobility group B protein 1 for XS at expression values < 0.5 fold. Only one DEP the peroxiredoxin Q suppressed in both varieties. The more number of DEPs were down-regulated in XS compared to BD (Table 1 and Table S1–4).

3.7. DEPs related to photosynthesis under cold stress in two coconut varieties

Total of 52 photosynthesis related DEPs were found in both varieties. Out of total, only thirteen DEPs were up-regulated, and three proteins shown both up-and-down-accumulation regulations, although, rests of DEPs were down-regulated in two BD and XS coconut varieties (Table 2; Fig. 8). The three DEPs including; photosystem I reaction center subunit VI, fructose-2,6-bisphosphatase and photosystem I P700 chlorophyll *a* apoprotein A2 were found up-regulated in XS at > 1.6 fold changes cutoff (Table S3 and 4). The two proteins the ferredoxin and light-regulated protein, Chloroplastic (Ferredoxin-NADP + oxidoreductase) were up-accumulated in BD at the same criterion (Table S1 and 2). Concurrently, the last protein was down-regulated in XS. The ten and eight photosynthesis related DEPs were significantly reduced their expression at < 0.6 fold changes in XS and BD respectively (Table 2 and Table S1–4). Notably, expression of 73% DEPs was decreased in both of varieties. Fig. 9 showing that both of varieties were sensitive to various photosynthesis related proteins which consistently decreased their expression in both BD (CnBD-2 and 5) and XS (CnXS-2 and 5) under cold stress.

3.8. DEPs related to respiration under cold stress in two coconut varieties

Total of twenty-six DEPs were involved in respiration related proteins in both of varieties (Table 3; Fig. 8). Of total, 17 proteins were up-regulated, 8 down-regulated and only one protein showed both up-and-down-regulations of expressions in both varieties. The four DEPs, pyruvate kinase (cytosolic isozyme), succinyl-CoA ligase [ADP-forming] subunit alpha-1 (mitochondrial), pyruvate kinase and aconitate hydratase in BD; and two citrate synthase (mitochondrial) and ATP synthase subunit beta (mitochondrial) were highly expressed in XS at > 1.7 fold changes. Six and four DEPs decreased their expression in BD and XS respectively at < 0.6 fold changes under cold stress (Table 3 and Table S1–4).

4. Discussion

4.1. iTRAQ-based proteomics analysis revealed XS a cold-sensitive aromatic coconut variety

Previously, various proteomic studies characterized and determined differentially expressed proteins (DEPs) under cold stress in several plant species [3,6,14,25]. This study identified that Hainan Tall variety up-accumulated large number of DEPs compared to aromatic coconut under cold stress (Fig. 2). Generally, (GFPO) and (PTMPTC) categories up-regulated the more number of proteins in BD compared to XS (Fig. 6 A-D). The leaf proteomic analysis of two coconut varieties revealed multiple-stress including abiotic, biotic and oxidative stresses in response to low temperature. Mainly abiotic stress related proteins were involved followed biotic and oxidative stresses in both coconut varieties (Table 1). The heat shock proteins were found abundantly in this study. These results are not only consistent with those of previously studies on watermelon and wheat under cold stress [14,36] but also cucumber cultivars under salinity stress [37]. However, a few numbers of drought and cold induced proteins were also found in response to low

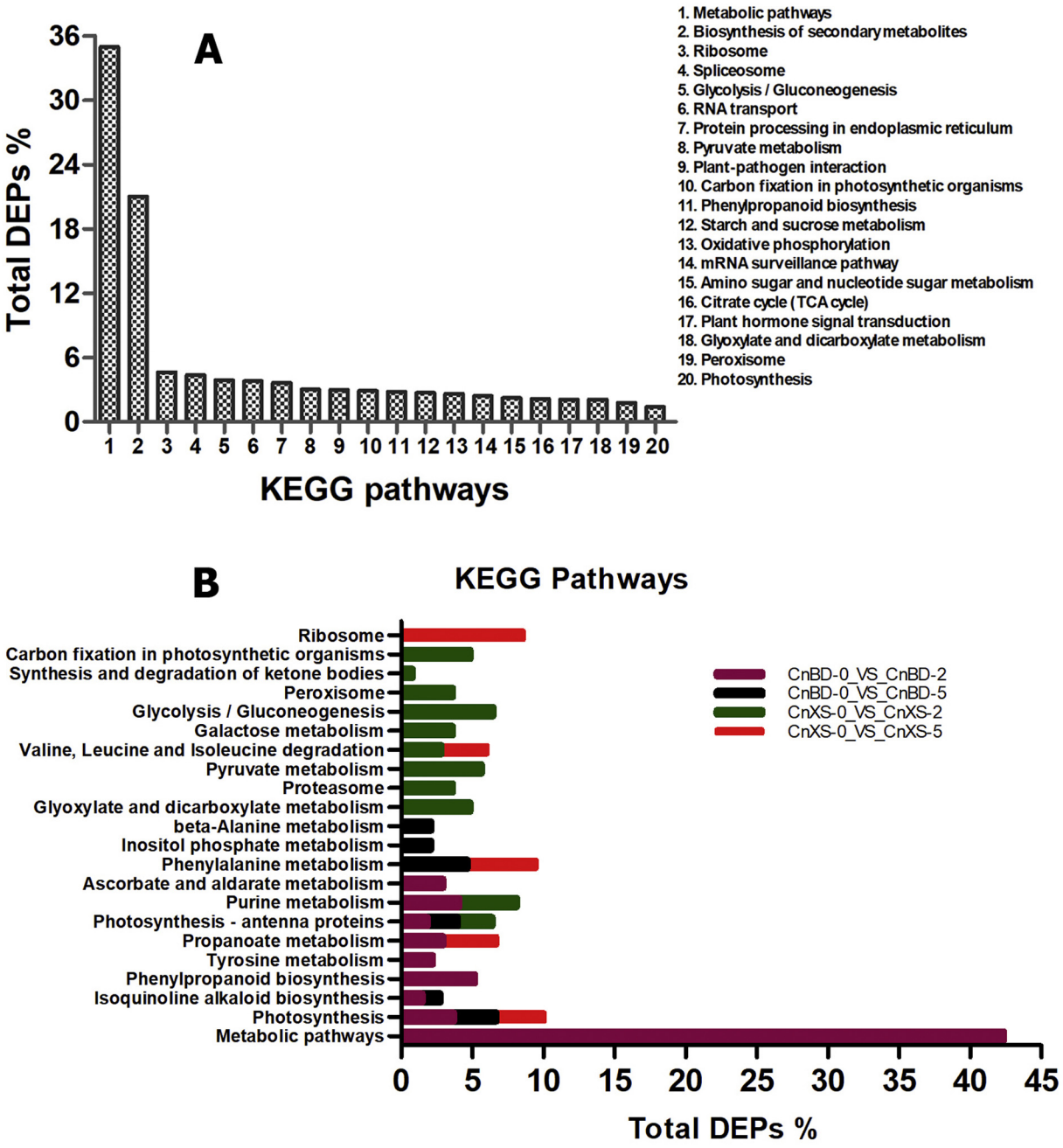

Fig. 7. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEPs involved in coconut seedlings under cold stress. (A) Top 20 KEGG pathways enrichment of DEPs along with percentage (%) of coconut seedlings under cold stress. (B) The twenty-two different KEGG pathways enrichment of DEPs (%) involved in BD and XS under cold stress at 2 and 5 days cold treatment.

temperature in coconut varieties. Similar results were found in salt-sensitive and salt-tolerant cucumber cultivars previously [37].

Besides, the biotic and oxidative stress responsive proteins were detected more in number, enhanced their expression in Hainan tall than aromatic coconut under cold stress condition. Nevertheless, these proteins are not cold-induced specific but widely determined in pepper against pests and in banana in response to salt stress using iTRAQ technique [38,39]. Proteomic analysis of subcutaneous adipose tissue of cows under negative energy balance during peripartum identified heat shock protein beta-1 (HSPB1) [40], suggesting that abiotic stress induces general stress responses in eukaryotic system.

4.2. Increased abundance of metabolism and respiratory-related proteins under cold stress

Carbohydrate and energy metabolism is an indispensable survival strategy adopted by plants against abiotic stresses including cold stress [17,18,20]. This study found with most number of DEPs involved in metabolism assigned to carbohydrate/amino acid/lipid/coenzyme transport and metabolism; energy production and conversion; secondary metabolites biosynthesis, transport and catabolism; and some of posttranslational modification, protein turnover, chaperones (Table S1–4 and Table S6). Previously, iTRAQ-based leaf proteomic analysis reduced expression of DEPs involved in metabolism in grapevine under heat stress [41]. We have identified 62% of proteins increased their expression involved in metabolism. The energy production and

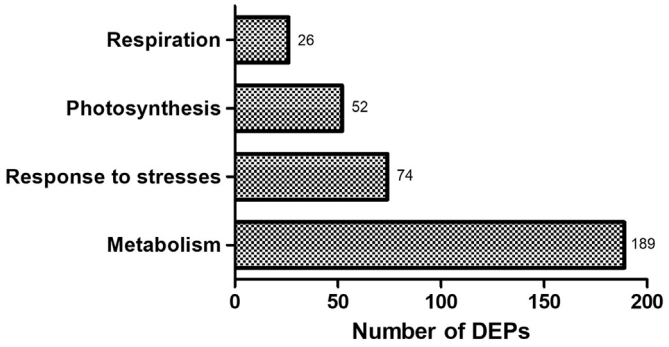

Fig. 8. DEPs related to metabolism, stress-responsive, photosynthesis and respiration between two coconut varieties under cold stress.

conversion; lipid transport metabolism; secondary metabolites biosynthesis, transport and catabolism and carbohydrate transport and metabolism related proteins were expressed abundantly in metabolic activities in both of XS and BD (Fig. 8; Table S6). The V-type proton ATPase proteins which are essential components of vacuolar proton pump generating electrochemical gradient across tonoplast [42]. The ATPase proteins abundance was decreased in root proteomic analysis of *Brassica rapa* under cold stress [25]. This study found one putative V-type H + -ATPase catalytic subunit protein down-regulated in BD variety. However, another protein V-type proton ATPase 16 kDa proteolipid subunit was up-regulated in same variety. Though, V-type ATPase increased abundance in *Arabidopsis* during cold acclimation [43]. The 6-phosphofructokinase of carbohydrate metabolism category down-regulated in BD variety and this is in consensus with [25]. Whereas, phosphoglucomutase, chloroplastic, (PGMP) up-accumulated in XS variety (Table S6), nevertheless, same protein was down-regulated in Longyou7 a grapevine variety under cold stress [25].

Besides, respiration is a center of energy metabolism and, it generates chemical energy and material for several other organic components in plant system [26]. This study identified 9 (Q42954, B9MYJ3, H6TNP0, Q9SXU6, Q43117, Q2KNB9, B9T6R6, B3TLL4, Q41141) proteins involved in glycolysis and entire expressed except fructose-2, 6-bisphosphatase (FBP) which showed 0.714 fold in BD under low temperature (Table 3). Contradictory, the (FBPs) were increased under cold stress in *A. aphylla* leaf proteomic analysis [3]. Whereas the expression pattern of pyruvate kinases and triose phosphate isomerase cytosolic isoform (B3TLL4) of both coconut varieties was in agreement with results of *A. aphylla* [3] and maize leaves proteome under cold stress [2]. Of 7, the six proteins were observed up-regulated in citrate cycle (TCA) related proteins, except isocitrate dehydrogenase [NADP] (Q7XMA0) in BD (Table 3). The isocitrate dehydrogenase [NADP] was suppressed in cold stress leaves of Longyou 7, but not in Tianyou 4 the varieties of *Brassica* [26]. Aconitate hydratase and succinate-CoA ligase were down-regulated in cold stressed brassica leaves [26], but we have found those up-regulated in both coconut varieties (Table 4; Table S1–4). This could be owing to temperature and species differences. The six electron transport chains (mitochondrial respiratory chain) related proteins were significantly down-regulated except NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6 (Q9LHI0) and gamma carbonic anhydrase 1, mitochondrial (Q9FWR5) which were expressed in XS variety (Table 3). Though, those proteins were down-regulated in root proteomic analysis of *Brassica rapa* under cold stress [25]. The two proteins of ATPase activity class the ATP synthase subunit alpha mitochondrial and beta (P12862 and P19023) were found up-regulated in XS (Table 3 and Table S1–4). Those proteins were expressed in *A. aphylla* plant under cold stress [3] and cell culture suspension of *Halogeton glomeratus* under salt proteomic analyses [44]. These results suggest that two coconut varieties differed in molecular mechanisms in response to cold stress in terms of iTRAQ proteomic analysis. Whereas, major proteins involved in metabolic pathways through accumulating differentially expressed proteins under cold stress.

4.3. Increased abundance of stress-responsive proteins in BD under cold stress

Various studies identified DEPs related to stress and redox in response to abiotic stressful conditions including cold stress in plants [14,41,45]. We identified larger number of proteins expressed in BD than XS under cold stress (Table 1). Molecular chaperones, including chaperonins, chaperone DnaJ protein and heat shock proteins (HSPs) are important protective proteins involved in response to stress stimuli though mediating protein folding [46]. DnaJ proteins are co-chaperones of HSP70s, playing a vital role in response to stressful conditions [47]. In this study, we found that five heat shock proteins, namely, heat shock 70 kDa protein 17 ((HSP-17) a stromal 70 kDa heat shock-related protein), 66 kDa stress protein, 82 kDa heat shock protein, chloroplast chaperonin 21 (cpn21) and chaperone protein DnaJ were up-regulated in BD. While four HSPs including, 101 kDa heat shock protein (HSP101), chaperonin GroEL (HSP60 family) (D7TT48), heat shock protein 83 (HSP83 A) and DnaJ protein homolog in XS variety (Table 2). The seven HSPs, i.e., heat shock cognate 70 kDa protein 2 (HSP-2), 20 kDa chaperonin (CPN20), heat shock protein (C5YLG9), 17.9 kDa class II heat shock protein, heat protein 90 (B9G449), chaperonin GroEL (HSP60 family) (P28769) and HSP70 (Q43532) were significantly decreased the protein expression under cold stress in XS. Only three, the classes II small heat shock protein Le-HSP17.6, endoplasmin homolog (HSP90) and CCT5 (O04450) were down-regulated in BD. Prior to this, the HSP70 and chaperone protein DnaJ were up-regulated in watermelon seedling in response to low temperature [14]. The DnaJ protein increased expression in grafted watermelon seedlings in response to chilling stress [48]. Similarly, HSP70 was also up-regulated in wheat [36] and peach [49] under cold stress. Here we found that not only HSP70 but also several other HSP proteins were down-regulated in XS, compared to BD. Moreover, cold could induce the accumulation of several HSPs which lead to cope with stressful situations in organisms [50]. Nonetheless, under cold stress condition the HSPs may induce and translocate into different cell organelles to protect plants from low-temperature stress. However, theses molecular chaperones such as HSPs are not only confined to plants [50] but also found in variety of eukaryotic organisms including fungi [51]. Recently, one proteomic study investigated the effect of yeast (*Candida intermedia*) 253 volatilome and 2-phenylethanol (2-PE) on metabolic asset of *Aspergillus carbonarius* MPVA566. They also found that molecular chaperones including HSP70 superfamily-related proteins expressed abundantly in control compared to treatments [52]. These results suggest that HSPs are generally conserved in eukaryotic system including fungi.

Cold stress may trigger overproduction of ROS which may disturb cellular redox lead to oxidative damage in plant system. However, plants have developed an effective antioxidant system to cope with oxidative stress caused by low temperature [53,54]. Both thioredoxin and peroxiredoxin (Prx) as antioxidant agents play an important role in redox signaling [53,55]. We have found one thioredoxin-like protein (CDSP32) down-regulated in BD. This protein was up-regulated in rice leaf proteomic analysis under cold stress [56]. Furthermore, three peroxiredoxin Q (PRXQ) (Q9MB35, Q6QPJ6 and Q69TY4) proteins were down-regulated in both varieties (Table 1). Glutathione S-transferase (GST) reduces oxidative damage by ROS scavenging system [57]. Our results showed that one glutathione S-transferase F10 (GSTF10) was highly up-regulated in BD. This is consistent with previous findings in grafted watermelon seedlings under cold stress [14]. Additionally, three antioxidant enzymes were found including; one superoxide dismutase [Cu−Zn] (SODCP) expressed in BD and two CAT2 up-regulated and CAT1 down-regulated in XS (Table 1). Besides, twelve proteins belonging to peroxidase family were found in this study. Of total, the

Table 1
Proteins involved in abiotic, biotic and oxidative stress under cold stress in two coconut varieties.

Protein accession	Regulation		Score	Species	Description
	Up	Down			
Q9LS40	1.518[a]	–	117	*Arabidopsis thaliana*	Protein aspartic protease in guard cell 1
Q9LKJ1	1.398[a]	–	565	*Arabidopsis thaliana*	3-hydroxyisobutyryl-CoA hydrolase 1
Q9LXC9	–	0.273[a] 0.44[b] 0.568[c]	231	*Arabidopsis thaliana*	Soluble inorganic pyrophosphatase 6, chloroplastic
Q6B4V3	1.787[b]	0.775[a]	172	*Vitis vinifera*	Chloroplast chaperonin 21, cpn21
F4JMJ1	1.655[a] 1.724[b]	–	1222	*Arabidopsis thaliana*	Heat shock 70 kDa protein 17, HSP70–17 Stromal 70 kDa heat shock-related protein
Q9MB35	–	0.633[a]	278	*Sedum lineare*	Peroxiredoxin Q, chloroplastic, PRXQ,
Q336R9	–	0.721[a] 0.76[b]	349	*Oryza sativa*	Peptide methionine sulfoxide reductase A4, chloroplastic, Oxidative stress
B4UW51	–	0.789[a]	226	*Arachis hypogaea*	Class II small heat shock protein Le-HSP17.6
O04450	–	0.787[a]	910	*Arabidopsis thaliana*	T-complex protein 1 subunit epsilon, CCT5
Q84NN4	–	0.639[a]	65.5	*Oryza sativa*	Thioredoxin-like protein CDSP32, chloroplastic
Q94DM8	1.647[a] 2.14[b]	0.578[d]	124	*Oryza sativa*	Ubiquitin-fold modifier 1, Cold shock proteins
P90587	1.39[a]	–	83.6	*Physarum polycephalum*	66 kDa stress protein
B3TLP2	1.581[a]	–	152	*Elaeis guineensis*	Light-inducible protein ATLS1
P93407	1.689[a] 2.384[b]	0.373[c]	180	*Oryza sativa*	Superoxide dismutase [Cu−Zn], chloroplastic, SODCP
Q08655	1.417[a] 1.504[d]	–	–	*Solanum lycopersicum*	Abscisic stress-ripening protein 1, ASR1
Q9LSY7	1.515[a] 1.627[b]	0.728[d]	372	*Arabidopsis thaliana*	Peroxidase 30
A7QEU4	1.802[a]	–	273	*Vitis vinifera*	Peroxidase 5
Q96520	2.212[a] 2.212[b] 1.728[c] 1.293[d]	0.678[c]	436	*Arabidopsis thaliana*	Peroxidase 12
O23044	1.589[a] 1.376[b]	–	355	*Arabidopsis thaliana*	Peroxidase 3
A7NY33	1.286[a] 1.562[c] 1.261[b]	–	478	*Vitis vinifera*	Peroxidase 4
Q9FLC0	2.319[a]	0.797[d]	323	*Arabidopsis thaliana*	Peroxidase 52
B3TLT1	–	0.485[a]		*Elaeis guineensis*	Peroxisome type ascorbate peroxidase
Q9LZJ5	1.277[a] 1.56[d]	–	536	*Arabidopsis thaliana*	ABC transporter C family member 14, (ABCC14)
P13240	2.092[a] 2.23[b]	0.755[c] 0.534[d]	–	*Pisum sativum*	Disease resistance response protein 206, PI206
A7PQW3	1.246[a] 2.08[c] 1.598[d]	–	–	*Vitis vinifera*	Glucan endo-1,3-beta-glucosidase
P02879	1.648[a]	0.602[d]	–	*Ricinus communis*	Ricin
P42761	2.227[a] 3.802[b]	–	197	*Arabidopsis thaliana*	Glutathione S-transferase F10 (GSTF10)
D5LNF6	3.845[a]	–	–	*Camellia sinensis*	rRNA N-glycosidase
P83643	–	0.64[a]	–	*Oryza sativa*	ACT domain-containing protein DS12
P46518	–	0.533[a] 0.625[b] 0.626[d]	510	*Gossypium hirsutum*	Late embryogenesis abundant protein Lea14-A
Q9M158	–	0.809[a]	–	*Arabidopsis thaliana*	Rhodanese-like domain-containing protein 4, chloroplastic
P22195	1.929[a] 2.977[b]	–	363	*Arachis hypogaea*	Cationic peroxidase 1, PNC1
Q3I3Y4	–	0.682[a]	–	*Picea mariana*	Putative intracellular pathogenesis-related protein
Q9LUV2	–	0.517[a]	–	*Arabidopsis thaliana*	Stress-response A/B barrel domain-containing protein HS1
Q109R6	–	0.465[a]	249	*Oryza sativa*	Protein CutA 1, chloroplastic
H6TNP3	1.276[b]	–	–	*Elaeis guineensis*	Putative hypersensitive-induced response protein
P43177	–	0.647[b]	–	*Betula pendula*	Major pollen allergen Bet v 1-D/H
B9S4B6	2.463[b]	–	501	*Ricinus communis*	Peroxidase 52, putative
E0X6U7	1.885[b]	–	1099	*Oryza sativa*	82 kDa heat shock protein
F4YBC8	1.845[b]	–	591	*Solanum nigrum*	Chaperone protein, DNAJ, heat shock protein
P35016	–	0.604[b]	1155	*Catharanthus roseus*	Endoplasmin homolog, HSP90
Q6QPJ6	–	0.201[b] 0.392[d]	192	*Populus jackii*	Peroxiredoxin Q, chloroplastic
B9FLJ6	–	0.742[b]		*Oryza sativa*	Chaperonin GroEL (HSP60 family)
Q9SJZ2	1.83[b]	–	401	*Arabidopsis thaliana*	Peroxidase 17
P46573	1.457[c]	–	556	*Arabidopsis thaliana*	Probable serine/threonine-protein kinase PBL10
Q948T6	1.645[c] 1.522[d]	–	446	*Oryza sativa*	Lactoylglutathione lyase, GLYI-11
B3A0N2	–	0.69[c] 0.402[d]	–	*Lycium barbarum*	Non-specific lipid-transfer protein

(*continued on next page*)

Table 1 (*continued*)

Protein accession	Regulation		Score	Species	Description
	Up	Down			
O23264	–	0.717[c]	–	*Arabidopsis thaliana*	Selenium-binding protein 1
P0C0L1	–	0.723[c]	289	*Oryza sativa*	Probable L-ascorbate peroxidase 6, chloroplastic/mitochondrial
Q9LSV0	–	0.818[c]	268	*Arabidopsis thaliana*	Glyoxylate/succinic semialdehyde reductase 1
Q9SYS9	1.855[c] 2.15[d]	–	1550	*Zea mays*	101 kDa heat shock protein, HSP101
D7TT48	1.252[c]	–	921	*Vitis vinifera*	Chaperonin GroEL (HSP60 family)
P51819	1.604[c]	–		*Ipomoea nil*	Heat shock protein 83, HSP83A
P27322	–	0.747[c]	1179	*Solanum lycopersicum*	Heat shock cognate 70 kDa protein 2, HSC-2
O65282	–	0.41[c] 0.563[d]	172	*Arabidopsis thaliana*	20 kDa chaperonin, chloroplastic, CPN20
C5YLG9	–	0.698[c]	594	*Sorghum bicolor*	Heat shock protein
Q69TY4	–	0.607[c]	193	*Oryza sativa*	Peroxiredoxin-2E-1, chloroplastic
P05477	–	0.631[c]	226	*Glycine* max	17.9 kDa class II heat shock protein
B9G449	–	0.731[c]	1167	*Oryza sativa*	Heat shock protein 90
P28769	–	0.672[c]	793	*Arabidopsis thaliana*	Chaperonin GroEL (HSP60 family)
Q0PGJ6	–	0.557[c]	456	*Arabidopsis thaliana*	NADPH-dependent aldo-keto reductase, chloroplastic
Q9THX6	–	0.63[c] 0.698[d]	331	*Solanum lycopersicum*	Thylakoid lumenal 29 kDa protein, chloroplastic,
P55308	1.238[c]	–	895	*Hordeum vulgare*	Catalase isozyme 2, CAT2
P17598	–	0.521[c]	949	*Gossypium hirsutum*	Catalase isozyme 1, CAT1
Q42093	1.349[c]	–	685	*Arabidopsis thaliana*	ABC transporter C family member 2
O65202	2.278[d] 2.196[c]	–	1194	*Arabidopsis thaliana*	Peroxisomal acyl-coenzyme A oxidase 1
Q04960	2.196[d]	–	620	*Cucumis sativus*	DnaJ protein homolog, Heat Shock protein
Q43532	–	0.672[d]	869	*Lilium longiflorum*	heat shock protein 70 family, HSP70
Q39173	1.264[d]	–	439	*Arabidopsis thaliana*	NADP-dependent alkenal double bond reductase P2
Q6NKW9	–	0.751[d]	–	*Arabidopsis thaliana*	Glucan endo-1,3-beta-glucosidase 8
Q8LPK2	–	0.808[d]	2025	*Arabidopsis thaliana*	ABC transporter B family member 2
O49595	–	0.379[d]	149	*Arabidopsis thaliana*	High mobility group B protein 1
Q9LHE3	–	0.511[d]	97.1	*Arabidopsis thaliana*	Protein aspartic protease in guard cell 2
P84786	1.558[d]	–	–	*Drimia maritima*	Ribosome-inactivating protein charybdin

Superscription, [a, b, c and d] refers to CnDB-0_VS_CnDB-2, CnDB-0_VS_CnDB-5, CnXS-0_VS_CnXS-2 and CnXS-0_VS_CnXS-5 respectively.

nine proteins were largely expressed in BD, while, the XS only expressed two proteins and rest of proteins were significantly reduced (Table 1 and Table S1–4). These results suggest that BD is more cold tolerant and plays an important role in ROS homeostasis against oxidative stress in response to low temperature.

Furthermore, five drought stress related DEPs were found and of total, two proteins (Q9LS40 and B3TLP2) found up-regulated in BD and two (P83643 and Q9LHE3) were down-regulated in XS, while one (P46518) reduced its expression in both varieties. The three cold stress responsive proteins found with two (Q9LKJ1 and Q94DM8) up-accumulated in BD and one (Q0PGJ6) down-regulated in XS. The six up-regulated and 3 down-regulated biotic stresses responsive were predicted in BD, in case of XS five up and six down-regulated proteins were found. Importantly two plant defense responsive proteins including; disease resistance response protein 206 (PI206) and ricin (P02879) were greatly expressed in BD but significantly down-accumulated in XS. Pathogenesis-related proteins ABC transporter C family member 14 (ABCC14) expressed and non-specific lipid-transfer protein (B3A0N2) a pathogenesis-related protein suppressed in both varieties (Table 1). The plant-pathogen interaction may lead to ROS generation to hypersensitive cell death of programmed cell death (PCD) [58]. We have observed one putative hypersensitive-induced response protein (H6TNP3) was only up-regulated in BD. The same protein was up-regulated in *Halogeton glomeratus* under salinity proteomic analysis [44]. Nevertheless, one study found the PCD related protein up-regulated in banana leaf proteomic analysis under salt stress [39]. The simultaneous response of plants to abiotic and biotic is possible [59] and coconut leaf proteomic analysis under cold stress may involve in multiple-stress including abiotic, biotic and oxidative stresses. Collectively, XS decreased abundance of stress-response proteins and seems sensitive in response to cold stress.

4.4. Decreased abundance of photosynthesis proteins between XS and BD under cold stress

Cold stress may significantly affect the various aspects of photosynthesis, for instance, cytosolic sucrose synthesis inhibition leads to accumulation of phosphorylated intermediates [60]. Our results showed that approximately 70% photosynthesis related proteins were significantly down-regulated in both varieties under cold stress (Fig. 9; Table 2 and Table S1–4). The most of DEPs involved in photosynthesis, light reaction or light harvesting were down-regulated in both varieties. Cytochrome b6-f complex iron-sulfur subunit 1, chloroplastic, Light-induced protein, chloroplastic, Chlorophyll a-b binding of LHCII type 1 protein and photosystem I reaction center subunit N, chloroplastic were down-regulated in both varieties at both 2 and 5 days post cold treatment (Table 2; Fig. 9).

The Chlorophyll a-b binding protein CP29.1, chloroplastic (Q07473) was expressed in XS. Previously, several chlorophyll-binding proteins were down-regulated in cold-stressed maize leaves proteomic study except chlorophyll *a*-b binding protein 4 [2], and this protein (P27521) was down-regulated in BD at 0.5 fold (Table 2). Chlorophyll a-b binding protein CP24 was up-regulated in proteomic expression profile in *Brassica* leaves under cold stress [26]; similarly, in our study we have observed up-regulation of same protein in BD. Entire proteins involved in photosystem I and II (PSI and PSII) were suppressed expect the two photosystem I P700 chlorophyll *a* apoprotein A2 (A6MMK6) and photosystem II 10 kDa polypeptide, chloroplastic (P10690) up-regulated in XS (Cn-XS-2). Nevertheless, photosystem I P700 apoprotein A2 was up-regulated in *A. aphylla* leaf proteomic analysis under cold stress [3]. Oxygen-evolving enhancer protein 3, chloroplastic (Q0D5P8) was down-regulated in BD both (Cn-BD-2 and 5) and XS (only Cn-XS-2), whereas, this protein was suppressed in maize [2]. The oxygen-evolving enhancer proteins were also expressed in *A. aphylla*

Table 2
Proteins involved in photosynthesis under cold stress in two coconut varieties.

Protein accession	Regulation		Score	Species	Description
	Up	Down			
Q7SIC9	1.208[a] 1.271[c]	–	1235	*Zea mays*	Transketolase, chloroplastic
P46225	–	0.668[a] 0.539[b] 0.596[d]	460	*Secale cereale*	Triosephosphate isomerase, chloroplastic
Q56YA5	1.549[c]	0.597[a]	257	*Arabidopsis thaliana*	Serine–glyoxylate aminotransferase
P13443	–	0.691[a]	553	*Cucumis sativus*	Glycerate dehydrogenase
P83527	1.258[a] 1.632[b]	–	194	*Capsicum annuum*	Ferredoxin
P20121	1.395[a] 1.851[c] 2.046[d]		172	*Pisum sativum*	Photosystem I reaction center subunit VI
Q6AVA8	–	0.694[a]	1416	*Oryza sativa*	Pyruvate, phosphate dikinase 1, chloroplastic
P13194	1.596[a]	–	120	*Hordeum vulgare*	Photosystem I reaction center subunit IV, chloroplastic
Q03200	1.6[a] 18.903[b]	0.556[c]	–	*Oryza sativa*	Light-regulated protein, Chloroplastic
A6N117	–	0.443[a] 0.501[b]	173	*Oryza sativa*	Ribulose bisphosphate carboxylase small chain
P32980	–	0.76[a]	233	*Nicotiana tabacum*	ATP synthase delta chain, chloroplastic
Q6Z2T6	–	0.687[a]	689	*Oryza sativa*	Geranylgeranyl diphosphate reductase, chloroplastic
Q2LGZ2	–	0.528[a] 0.529[b] 0.798[c]	566	*Vigna unguiculata*	ATP synthase subunit gamma, chloroplastic
P30361	–	0.761[a] 0.594[b] 0.564[c] 0.572[d]	248	*Nicotiana tabacum*	Cytochrome b6-f complex iron-sulfur subunit 1, chloroplastic
Q0D5P8		0.823[a] 0.661[b] 0.706[c]	246	*Oryza sativa*	Oxygen-evolving enhancer protein 3, chloroplastic
P05414	–	0.792[a] 0.782[d]	404	*Spinacia oleracea*	Peroxisomal (*S*)-2-hydroxy-acid oxidase
P80471	–	0.522[a] 0.454[b] 0.287[c] 0.287[d]	323	*Solanum tuberosum*	Light-induced protein, chloroplastic
P12355	–	0.595[a]	299	*Spinacia oleracea*	Photosystem I reaction center subunit III, chloroplastic
P14584	–	0.803[a] 0.793[b] 0.521[c] 0.619[d]	363	*Raphanus sativus*	Chlorophyll a-b binding of LHCII type 1 protein
P27522	–	0.78[a]	422	*Solanum lycopersicum*	Chlorophyll a-b binding protein 8, chloroplastic
P27521	–	0.563[a] 0.5[b]	452	*Arabidopsis thaliana*	Chlorophyll a-b binding protein 4, chloroplastic
Q7YJY8	–	0.816[a]	681	*Calycanthus floridus*	Photosystem II protein D1
O65107	–	0.487[a] 0.236[b] 0.325[c] 0.722[d]	190	*Zea mays*	Photosystem I reaction center subunit N, chloroplastic
P27524	–	0.751[a]	116	*Solanum lycopersicum*	Chlorophyll a-b binding protein CP24 10A, chloroplastic
G3MI94	1.476[b]	–	644	*Amblyomma maculatum*	Fructose-bisphosphate aldolase
Q944I4	–	0.484[b] 0.589[c]	560	*Arabidopsis thaliana*	D-glycerate 3-kinase, chloroplastic
Q9SCY3	–	0.555[b]	97.4	*Arabidopsis thaliana*	Photosynthetic NDH subunit of lumenal location 4, chloroplastic, PNSL4
Q39654	–	0.763[b]	313	*Cucumis sativus*	Photosystem I reaction center subunit XI, chloroplastic
Q9S713	–	0.643[b]	72	*Arabidopsis thaliana*	Serine/threonine-protein kinase STN7, chloroplastic
P85112	1.376[b] 1.3[c]	–	719	*Vitis vinifera*	Phosphoribulokinase, chloroplastic
G7JEF5	–	0.797[b]	–	*Medicago truncatula*	NDH-dependent cyclic electron flow protein
Q9XF89	1.287[b]	0.535[c] 0.671[d]	459	*Arabidopsis thaliana*	Chlorophyll a-b binding protein CP26, chloroplastic, LHCB5
P08222	–	0.723[b] 0.747[c]	504	*Cucumis sativus*	Chlorophyll a-b binding protein of LHCII type 1
P13869	–	0.627[b] 0.728[c]	296	*Petunia hybrida*	Chlorophyll a-b binding protein, chloroplastic
Q01517	1.208[c]	–	667	*Pisum sativum*	Fructose-bisphosphate aldolase 2, chloroplastic
P12782	1.346[c]	–	721	*Triticum aestivum*	Phosphoglycerate kinase, chloroplastic
I1ITE1	1.651[c] 1.561[d]	–	305	*Brachypodium distachyon*	Fructose-2,6-bisphosphatase
P21528	1.531[c]	–	662	*Pisum sativum*	Malate dehydrogenase [NADP], chloroplastic
Q677H7	–	0.693[c]	504	*Hyacinthus orientalis*	Chlorophyll a-b binding protein, chloroplastic

(*continued on next page*)

Table 2 (*continued*)

Protein accession	Regulation		Score	Species	Description
	Up	Down			
P27787	–	0.293[c] 0.462[d]	183	*Zea mays*	Ferredoxin-1, chloroplastic FDX1
P14936	–	0.403[c] 0.319[d]	228	*Raphanus sativus*	Ferredoxin, root R-B1
P00290	–	0.215[c] 0.425[d]	204	*Lactuca sativa*	Plastocyanin (Chain A, The Complex Of Cytochrome F And Plastocyanin)
Q42450	–	0.564[c] 0.723[d]	52.8	*Hordeum vulgare*	Ribulose bisphosphate carboxylase/oxygenase activase B, chloroplastic
P93527		0.524[c]		*Sorghum bicolor*	Phytochrome B
A6MMK6	1.846[c]	–	150	*Dioscorea elephantipes*	Photosystem I P700 chlorophyll *a* apoprotein A2
P10690	1.349[c]	–	173	*Spinacia oleracea*	Photosystem II 10 kDa polypeptide, chloroplastic
Q07473	1.225[c]	–	174	*Arabidopsis thaliana*	Chlorophyll a-b binding protein CP29.1, chloroplastic
Q9S7H1	–	0.619[c]	323	*Arabidopsis thaliana*	Photosystem I reaction center subunit II-1, chloroplastic
Q43088	–	0.639[c]	62.8	*Pisum sativum*	Ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit *N*-methyltransferase, chloroplastic
Q02060	–	0.716[d]	297	*Spinacia oleracea*	Photosystem II 22 kDa protein, chloroplastic
P14279	–	0.734[d]	461	*Solanum lycopersicum*	Chlorophyll a-b binding protein 5, chloroplastic, CAB5
Q9M0V6	–	0.62[d]	460	*Arabidopsis thaliana*	Ferredoxin–NADP reductase, root isozyme 1, chloroplastic, RFNR1

Superscription, [a, b, c and d] refers to CnDB-0_VS_CnDB-2, CnDB-0_VS_CnDB-5, CnXS-0_VS_CnXS-2 and CnXS-0_VS_CnXS-5 respectively.

[3], alfalfa [61] and barely leaf proteomic studies [62] in response to cold stress. ATP synthase delta/gamma chains (P32980 and Q2LGZ2) and photosynthetic NDH subunit of lumenal location 4 (PNSL4), chloroplastic (Q9SCY3) proteins were suppressed mainly in BD under cold stress and this result is an agreement with that of maize leaf proteomic analysis [2].

Ferredoxin–NADP reductase, root isozyme 1, chloroplastic (Q9M0V6), ferredoxin, root R-B1 (P14936) and ferredoxin-1, chloroplastic (P27787) were reduced severely in XS, while, ferredoxin (P83527) was enhanced in proteomic data under cold stress (Fig. 9; Table 3). Ferredoxin-NADP reductase increased abundance in previously reported proteomic study in winter barely under cold stress [62]. Ribulose bisphosphate carboxylase small chain (A6N117) was only found suppressed in BD. The same protein differentially expressed in winter barely proteomic analysis under cold stress [62].

During photosynthesis the light energy trapping and charge-separation based utilization of this energy is mainly temperature independent [63]. The more inhibition of photosynthesis proteins including, chlorophyll *a*, b binding proteins, (PNSL4), ATP synthases, ribulose bisphosphate carboxylase small chain and oxygen-evolving enhancer protein 3 etc., could lessen the over energized state of thylakoid membrane lead to photodamage and resulting generation of more ROS in leaves of BD [2,26] (Tables 1 and 2; Table S1–4). Thus, these data suggest that BD Hainan Tall coconut is more cold-tolerant variety compared to XS aromatic coconut variety.

5. Conclusion

To our best knowledge, this is the first iTRAQ-based proteomic technique applied in coconut crop to analyze the differentially expressed proteins under cold stress. Base on proteomics data, major proteins involved in metabolic pathways through accumulating differentially expressed proteins in both coconut varieties under cold stress. Cold tolerance of Hainan tall might be related to accumulating the stress-responsive proteins including biotic, abiotic and oxidative stress. However, Hainan tall variety could cope with cold stress by enhancing the scavenging capacity of ROS during oxidative stress. Additionally, posttranslational modification, protein turnover, chaperones (PTMPTC) proteins may also play role in coconut crop adaptation to low temperature stress. This study would assist to decipher the further functions of differentially expressed proteins under cold stress in coconut. In the future, the coupling of multi-omics approaches including transcriptomics and metabolomics could elucidate the molecular mechanisms and biological interaction underlying the cold-tolerant or cold-sensitive varieties of coconut.

Contributions

JZ, WA, YW and JL performed the coconut cold treatment and subsequent sampling. YY and MS participated in the design of the study and performed the data analysis. YY, and MS wrote the paper. YY, HF and FW participated in coordination of the research and discussion. All authors reviewed the manuscript and have given final approval of the version to be published.

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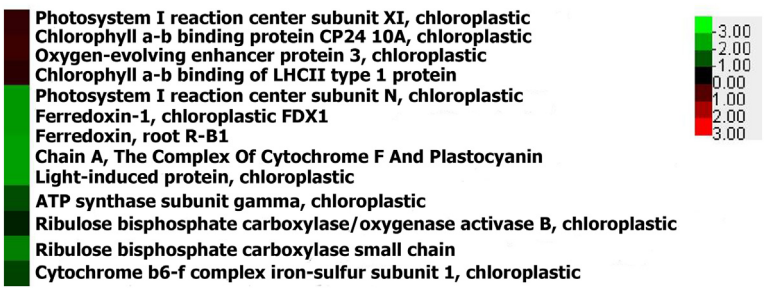

Fig. 9. Decreased abundance of photosynthesis related proteins observed between two coconut varieties in response to cold stress. Red and green colors are indicating the up and down-regulated proteins.

Table 3
Proteins involved in respiration under cold stress in two coconut varieties.

Protein accession	Regulation		Score	Species	Description
	Up	Down			
Q42954	1.999[a]	–	770	*Nicotiana tabacum*	Pyruvate kinase, cytosolic isozyme
B9MYJ3	–	0.714[a]	416	*Populus trichocarpa*	Fructose-2,6-bisphosphatase (FBP)
Q8GTQ9	2.028[a] 2.437[b]	–	478	*Solanum lycopersicum*	Succinyl-CoA ligase [ADP-forming] subunit alpha-1, mitochondrial
H6TNP0	1.276[b]	–	664	*Elaeis guineensis*	Glucose-6-phosphate isomerase
Q9SXU6	1.728[b]	–	1006	*Cicer arietinum*	Pyruvate kinase
Q20H34	1.604[b]	–	557	*Arrabidaea patellifera*	Phosphoenolpyruvate carboxylase
B9SXB6	1.776[b]	–	1660	*Ricinus communis*	Aconitate hydratase
P51134	–	0.618[b]	415	*Nicotiana tabacum*	Cytochrome b-c1 complex subunit Rieske-4, mitochondrial
Q7XMA0	–	0.537[b]	726	*Oryza sativa*	Isocitrate dehydrogenase [NADP]
P29610	–	0.289[b]	504	*Solanum tuberosum*	Cytochrome c1–2, heme protein, mitochondrial, CYCL
P46269	–	0.644[b]	–	*Solanum tuberosum*	Cytochrome b-c1 complex subunit 8
Q9SXX7	–	0.5[b]	–	*Oryza sativa*	Cytochrome c oxidase subunit 5C
Q9LHI0	1.625[c] 1.501[d]	0.764[b]	217	*Arabidopsis thaliana*	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6
Q9FWR5	1.232[b]	–	–	*Arabidopsis thaliana*	Gamma carbonic anhydrase 1, mitochondrial
Q43117	1.551[c]	–	944	*Ricinus communis*	Pyruvate kinase isozyme A, chloroplastic
Q2KNB9	1.318[c]	–	703	*Oryza sativa*	Hexokinase-2, HXK2
P12862	1.253[c]	–	961	*Triticum aestivum*	ATP synthase subunit alpha, mitochondrial
P49608	1.225[c]	–	1673	*Cucurbita maxima*	Aconitate hydratase, cytoplasmic
O80433	1.713[c]	–	704	*Daucus carota*	Citrate synthase, mitochondrial
Q9FLQ4	1.617[c] 1.668[d]	–	430	*Arabidopsis thaliana*	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex 1, mitochondrial
O22769	–	0.455[c]	452	*Arabidopsis thaliana*	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial
B9T6R6	1.626[d]	–	927	*Ricinus communis*	Pyruvate kinase, putative
B3TLL4	1.211[d]	–	445	*Elaeis guineensis*	Triose phosphate isomerase cytosolic isoform
Q41141	1.681[d]	–	753	*Ricinus communis*	Pyrophosphate–fructose 6-phosphate 1-phosphotransferase subunit beta
P19023	2.048[d]	–	803	*Zea mays*	ATP synthase subunit beta, mitochondrial
O49313	–	0.566[d]	141	*Arabidopsis thaliana*	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13-B

Superscription, [a, b, c and d] refers to CnDB-0_VS_CnDB-2, CnDB-0_VS_CnDB-5, CnXS-0_VS_CnXS-2 and CnXS-0_VS_CnXS-5 respectively.

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Declaration of Competing Interest

Authors declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.103766.

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