TMT-Based Proteomic Profiling Reveals the Molecular Mechanism in the Cold Tolerance of *Rhododendron Aureum* Georgi.

Xiaofu Zhou
Jilin Normal University
Kun Cao
Jilin Normal University
Ziyao Zhang
Jilin Normal University
Yun Tan
Jilin Normal University
Hang Fan
Jilin Normal University
Hongwei Xu (xuhongwei@jlnu.edu.cn)
Jilin Normal University

Research Article

Keywords: Cold stress, Rhododendron aureum Georgi, proteomic, differential accumulation proteins

Posted Date: August 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-668077/v1

Abstract

Background: Cold stress is one of the important factors that restrict plant growth and regional distribution. Under cold acclimation conditions, plants growing in temperate zones reprogram the gene expression in the cells, making the plants better cope with the coming cold stress. However, under natural environmental conditions, the climate is complex and changeable. The sudden large temperature drop will bring serious disasters to plants. *Rhododendron aureum* Georgi, as an evergreen plant growing in high altitude areas of Changbai Mountain, the harsh ecological environment may endow it with different cold tolerance characteristics.

Results: In this study, the proteomic difference between samples under control and cold stress were compared pairwise. A total of 360 DAPs were identified, of which 175 were down-regulated and 185 were up-regulated when comparing these two sets of data. The high cold tolerance of *Rhododendron* plants can be attributed to: accumulation of chaperone proteins; the up-regulation of components related to translation; enhancement of catabolism and reduction in anabolism, provide energy for plants and fight against cold stress; enhanced cellular antioxidant capacity; modification of components in cell wall, membrane, and cytoskeleton.

Conclusion: These results provide an in-depth understanding of the cold tolerance mechanism of *Rhododendron*. The identified genes and metabolic pathways provide a certain reference for the genetic improvement of plant cold tolerance.

Background

Rhododendron has been reported to be the largest genus in the Ericaceae. The flowers of *Rhododendron* are colorful, including white, yellow and red. As a garden ornamental plant, it is popular in many countries. *Rhododendrons* are distributed from the northern temperate zone to the northeastern part of Australia[1]. The wide geographical distribution confers *Rhododendrons* different ecological characteristics, either evergreen or deciduous. For example, *R. hyperythrum* is native to Taiwan and grows at an altitude of 900–1200 m. It grows well in the warm environment of Louisiana[2]. *R. russutum*, which grows in Yunnan Province in southwestern China, is a typical alpine plant that grows between elevations of 3400–4300 m and does not grow well under warm climate[3]. Therefore, they can be used as potential genetic resources to develop new plant varieties to adapt to different environmental conditions.

Adversity environment forces plants to adopt different strategies to fight against environmental stimuli, and ultimately forces plants to continuously adapt to changes in the environment to survive in adversity. One of the environmental challenges faced by plants is low temperature. The low temperature above 0 °C and below 15 °C is called cold stress. The damage of cold stress to plants is first manifested in the plasma membrane, leading to cell leakage and destruction. Similar to other stresses, some symptoms of cold stress are also manifested in plant wilting. The leaf mesophyll cells, vascular tubes, roots and epidermis form crystalline deposits, which damage the vacuole membrane and accelerate cell senescence. In addition, cold stress leads to disturbances in plant metabolism and physiological functions, including reduction of net

photosynthetic rate, obstacles to conversion of starch to sugar, degradation of chlorophyll, reduction of respiratory activity and fatty acid synthesis, resulting in plant starvation and even death[4].

Proteomics is a powerful tool for large-scale analysis of protein abundance, protein interaction, and post-translational modification. It performs a global analysis of proteins in different tissues or organs and cells at different developmental stages. The plant genome is relatively static structure, while the proteome is extremely dynamic. Not only are there great differences between cells, different developmental and environmental stimuli will also cause great fluctuations in the proteomic. Protein participates in various and important life activities, including enzyme catalysis, molecular chaperones, energy transfer, transcription factors, and stress responses that reflect the level of transcription and translation efficiency. Stress-responsive proteins are key contributors to stress adaptation by changing the characteristics and composition of the plasma membrane, intracellular compartments and cytoskeleton[4].

Changbai Mountain, located between the boundary of China and Korea, has a long frosty season, and the top of the mountain is covered with snow all year round. To cope with the harsh climatic conditions, most of the plants growing in Changbai Mountain are resistant to low temperature and ultraviolet radiation. As an evergreen shrub growing in the Changbai Mountain area, *Rhododendron aureum* Georgi (*R. aureum*) can withstand low temperatures around − 40°C[5]. Therefore, *R. aureum* not only has ornamental value because of its beauty, but also has become a potential genetic resource for mining key cold-tolerant metabolic pathways and genes because of its ability to withstand extreme cold. To have a global understanding of the cold tolerance mechanism of *R. aureum*, leaves collected from Rhododendron plants grown under normal and cold stress conditions were used as the experimental material, and obtained 360 differential accumulation proteins (DAPs) through TMT experiment. The analysis of DAPs and its metabolic pathways will deepen our understanding of the molecular mechanisms of plants dealing with cold stress.

Materials And Methods

2.1 Plant materials and treatment conditions

According to the morphological appearance and the guidance of the staff of the Academy of Forestry, the stems from the *R. aureum* taken from Changbai Mountain were used for vegetative propagation (hardwood cuttings) and grown in 25-liter plastic pots. Six seedlings with similar morphology were used for this experiment. Before the cold treatment, all individuals were grown for 8 months at 25 °C /25 °C day /night temperature, 14 hours/10 hours (day/night) cycles. Then we randomly selected 3 plants from them and transferred them to the growth chamber at 4 °C, and underwent low temperature treatment under the same photoperiod conditions. Forty-eight hours later, the leaves of the two groups of *Rhododendron* materials at the same position were harvested and immediately frozen in liquid nitrogen to prepare for protein extraction.

2.2 protein extraction

Sample was first grinded with liquid nitrogen, then the powder was transferred to a 5-mL centrifuge tube and sonicated three times on ice using a high intensity ultrasonic processor (Scientz) in lysis buffer (including 1% TritonX-100, 10 mM dithiothreitol, and 1% protease inhibitor cocktail□1% phosphatase inhibitor for

phosphorylation.). An equal volume of Tris-saturated phenol (pH 8.0) was added. Then, the mixture was further vortexed for 5 min. After centrifugation (4 °C, 10 min, 5 000g), the upper phenol phase was transferred to a new centrifuge tube. Proteins were precipitated by adding at least four volumes of ammonium sulfate-saturated methanol and incubated at -20 °C for at least 12 h. After centrifugation at 4 °C for 10 min, the supernatant was discarded. The remaining precipitate was washed with ice-cold methanol, followed by ice-cold acetone for three times. The protein was redissolved in 8 M urea and the protein concentration was determined with BCA kit according to the manufacturer's instructions[6].

2.3 Trypsin Digestion, TMT labeling, and HPLC fraction

Dithiothreitol was added to each protein sample to reach a final concentration of 5 mM, and the reduction reaction was performed under conditions of 56 °C for 30 minutes. Then adding iodoacetamide to make the final concentration of 11 mM, and incubate for 15 minutes at room temperature in darkness. The concentration of urea in protein sample was diluted to less than 2 M by adding 100 mM TEAB for digestion with trypsin. Protein sample was digested with trypsin at a ratio of 1:50 overnight at 37 °C and then adding new trypsin at a ratio of 1:100 to continue enzymatic hydrolysis for another 4 hours. The peptide mixture was desalted by Strata X C18 SPE column (Phenomenex) and labelled with TMT reagent. The tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using Thermo Betasil C18 column (5 μm particles, 10 mm ID, 250 mm length). Briefly, peptides were first separated with a gradient of 8 % to 32 % acetonitrile (pH 9.0) over 60 minutes into 60 fractions. Then, the peptides were combined into 6 fractions and dried by vacuum centrifuging[7].

2.4 LC-MS/MS Analysis

The tryptic peptides were dissolved in buffer A (0.1 % formic acid and 2 % acetonitrile), directly loaded onto a reversed-phase analytical column and separated with a linear gradient of buffer B (0.1 % formic acid and 90 % acetonitrile) at a flow rate of 500 nL/min on an EASY-nLC 1000 UPLC system: held in 6–23 % buffer B for 26 minutes, 23–35 % buffer B for 8 minutes, 35–80 % buffer B for 3 minutes and 80 % buffer B for 3 minutes.

The peptides are separated by the ultra-high performance liquid system and injected into the NSI ion source for ionization and then analyzed by Q Exactive mass spectrometry. The ion source voltage was set to 2.0 kV, and the peptide precursor ions and their secondary fragments were detected and analyzed by high-resolution Orbitrap. The scanning range of the primary mass spectrum is set to 350-1800 m/z, and the scanning resolution is set to 70,000; the scanning range of the secondary mass spectrum is set to a fixed starting point of 100 m/z, and the secondary scanning resolution is set to 17,500. The data acquisition mode uses the data-dependent scanning (DDA) program, that is, the first 20 peptide precursor ions with the highest signal intensity are selected to enter the HCD collision cell sequentially using 28 % and 31 % of the fragmentation energy for fragmentation[8].

2.5 Database search

The raw data were searched against *Rhododendron_lapponicum*_313330 protein database (45945 sequences) using Maxquant (v1.5.2.8). The parameter setting method of protein identification is in accordance with Zhou's method[5].

Results

Identification of DAPs

To get an overview of the protein changes of *Rhododendron* under low temperature, we conducted a proteomic analysis of *Rhododendron* leaves before and after cold stress. DAPs are the key players that we are most concerned about that can be used to clarify the cold-resistant performance of *Rhododendron* plants. When the fold change ratio of the protein species before and after the cold treatment is greater than 1.2 or less than 0.833 and the p value is less than 0.05, we define such proteins as up-regulated proteins or down-regulated proteins. Using this cut-off point standard, a total of 360 DAPs were identified, of which 175 were down-regulated and 185 were up-regulated under cold stress versus room temperature growth.

Classification of DAPs

Gene Ontology (GO) is an important bioinformatics analysis method used to characterize various attributes of genes and gene products. GO annotations are divided into 3 categories: Biological Process, Cellular Component and Molecular Function, explaining the biological role of proteins from different perspectives. We have made statistics on the distribution of DAPs in GO secondary annotations. A total of 179 DAPs are enriched in biological processes, where metabolic processes account for the largest proportion, followed by single-organism process categories and cellular process categories. Among the cell components, the membrane ranks first in terms of the number of DAPs, the other components identified correspond to cells, macromolecular protein complexes, organelles, and extracellular regions. Catalytic activity is the most representative category of molecular functions, accounting for 50 % of this category. The results of GO data suggest that cold exposure mainly affects the metabolic activities, intracellular material transport and signal transduction in *Rhododendron* plants. 360 DAPs are assigned to 21 categories according to the Clusters of Orthologous Groups (COG) database. The main functional categories include General function prediction only (17.24%), posttranslational modification, protein turnover, chaperones (10.78 %), Energy production and conversion (9.91 %), Translation, ribosomal structure and biogenesis (8.62 %), Carbohydrate transport and metabolism (8.19 %), Inorganic ion transport and metabolism (6.9 %), Intracellular trafficking, secretion, and vesicular transport (6.03 %), Signal transduction mechanisms (6.03 %), and amino acid transport and metabolism (5.17 %) (Fig. 1).

Correlation analysis of DAPs and DEGs

Proteomics provides some clues on translation and post-translational levels in response to cold stress. However, the integration of transcriptome and proteome data can show a global picture of gene expression regulation in *Rhododendron* plants. In this study, we performed a correlation analysis on the results of the transcriptome and proteome. To identify those DEGs and DAPs that respond to cold stress, we used the following criteria as a threshold for screening ($|\log_2 FC|>1$, p value <0.001 for DEGs; Proteins with FC value

>1.2 or <0.83 and P value <0.05 are regarded as significant DAPs). A total of 6378 genes had corresponding proteins in the transcriptome, we have got a very poor positive correlation between the changes in the transcript level and the protein level for DAPs (Pearson correlation coefficient r = 0.042). More DEGs (n=587) and DAPs (n=306) are not correlated. 54 genes are shared between the transcriptome and proteome, among which 34 genes have similar expression trend, and the remaining 20 genes have opposite expression trends, which implies that complicated regulation process exist from transcription to translation when *Rhododendron* plants suffer from cold stress(Fig. 2). For those genes whose transcription is down-regulated but the translation level is up-regulated, it means that these genes may enter the translation machinery more efficiently to synthesize protein products during periods of cold stress. To obtain the metabolic pathways most closely related to cold stress, we performed KEGG enrichment analysis on the data in the correlation analysis, and the results are shown in Fig. 3 We observed that these DAPs were enriched in 9 pathways, including circadian rhythm; oxidative phosphorylation; cyanoamino acid metabolism; ascorbate and aldarate metabolism; SNARE interactions in vesicular transport; nitrogen metabolism; amino sugar and nucleotide sugar metabolism; flavonoid biosynthesis; phenylpropanoid biosynthesis. These results indicate that the proteins involved in various metabolic pathways, photoperiod and vesicle transport respond to cold stress.

Discussion

DAPs involved in Posttranslational modification, protein turnover, chaperones

Low temperature stress increases the risk of protein unfolding, misfolding, degradation and oxidation. The accumulation of these abnormal proteins in the cell can have harmful consequences for the cell. Therefore, increasing the abundance of proteins with protective functions is necessary to protect proteins from damage[9]. Correspondingly, in our proteomics experimental results, we observed that the abundance of some proteins such as molecular chaperones and heat shock proteins increased under low temperature stress. The obvious feature of chloroplast photosystem II is that it is particularly susceptible to photo-oxidative damage during cold stress. DnaJ acts as molecular chaperone that plays essential role in contributing to maintenance of photosystem II. In our current research, the up-regulated levels of DnaJ may provide protection for the photosystem II under cold stress environments. DnaJ has been reported to have an interaction with HSP70. Consistent with the up-regulation of DnaJ, the abundance of HSP70 also appeared to accumulate after *Rhododendron* was exposed to cold stress[10]. Therefore, here DnaJ and HSP70 may form a complex to work together. This interaction will prompt HSP70 to hydrolyze ATP to recruit other client proteins to perform functional diversification. Obviously, this combined machine will be more efficient and have broader functions. In addition to HSP70, other chaperone-related proteins such as Chaperonin 60 and 10 kDa chaperonin 1 (CPN10-1) have also been shown to accumulate in response to cold stress[11]. These two proteins together with Cpn20 that form a complex are required for the fold of the RuBisCo small subunit after they translocate from cytoplasm to chloroplast. The cpn60α1 knockdown mutant exhibits yellow leaf phenotypes, dwarfing, and chloroplast collapse that disrupt both photosynthesis and photorespiration[12]. In addition to being recognized as aiding the folding of proteins in the chloroplast, other roles of Chaperonin 60 and its co-molecular chaperones in cold stress still need to be further explored. Protein degradation mediated by protease and ubiquitin modification system plays an important role in the

process of plants coping with cold stress. In our study, the abundance of 26S proteasome non-ATPase regulatory subunit 13 (RPN9B), serine protease EDA2, and Aspartic proteinase A1 identified as being involved in protein degradation were all down-regulated under cold stress. It has been revealed that Aspartic proteinase A1 is involved in biotic and abiotic stress response, reproductive development and chloroplast metabolism, while, to our best knowledge the report on the functional analysis of the other two proteins in plant abiotic stress has not been discovered. As we know, similar to drought stress, cold stress can also bring about the imbalance of plant water status. As an important hub gene for ubiquitin mediated proteolysis pathway, SUMO-activating enzyme subunit 2 (SEA2) plays an important role in avoiding excessive water loss during drought stress[13]. In our study, cold stress also induced the accumulation of SEA2, and the up-regulation of this protein may play a positive role in the adaptation of *Rhododendron* to severe cold (Table 1).

DAPs involved in translation, ribosomal structure and biogenesis

In eukaryotic cells, the nucleus and ribosomes are considered to be the central hub for integrating stress responses. The protein synthesis process includes four stages: initiation, extension, termination, and ribosomal cycle, among which translation initiation is the main regulatory step of protein synthesis[14]. So far, there is not much direct evidence that ribosomal proteins (RP) are involved in cold stress. Tronchoni et al. found that the improvement of translation efficiency is an important means for S. kudriavzevii strain to adapt to low temperature environment[15]. Rogalski et al. reported Rpl33 knockout plants exhibited a compromised recovery when transferred from chilling stress condition to standard growth conditions, indicating Rpl33 is required for efficient translation under cold stress[16]. However, Cheng et al.'s research in soybeans found that overexpression of sense and antisense ribosomal protein L34-like gene in transgenic plants showed characteristics of cold sensitivity and cold resistance, respectively, indicating that SOL34 plays a negative regulatory role in the metabolic process that adapts to low temperature during seed imbibition[17]. However, most evidence pointed to the up-regulation of RP accumulation under cold condition. In our study, we also noticed that 6 plastic ribosomal proteins (RPS) and 7 RP were up-regulated after cold stress (Fig. 4) (Table 1). Therefore, the experimental results of the up-regulation of RP abundance are consistent with previous studies on model organisms such as Arabidopsis. Regarding the explanation of this phenomenon, here we adopt the viewpoint of Molina et al.: Under cold acclimation, the *Rhododendron* needs to change the composition of ribosomes to meet the proper translation rate of protein[18].

DAPs involved in intracellular trafficking

For eukaryotes, vesicle transport not only plays a role in plant growth and development by maintaining the specificity and integrity of the compartment, but also plays a role in the response to abiotic and biotic stress, although the latter studies are relatively small, but it has been supported by increasing evidence. The main internal membrane transport pathways in plant cells include secretory and endocytic pathways[19]. The transport of protein cargo from one organelle to another is mediated by vesicle transport, it has been demonstrated that cold stress affects the intracellular protein transport pathway to varying degrees. The SYP51/SYP52 of the SYP5 family is located on PVC or tonoplast, and forms a complex with other SNARE proteins, including VAMP722, SYP22, and VTI11, which is critical for protein transport and PSV

formation[20]. Our proteomics results showed that the abundance of SYP51, SYP52, and SYP22 were down-regulated, which implies that the trafficking destined to the vacuole may be affected. Exposure to high salt or high osmotic pressure conditions causes excessive accumulation of intracellular ROS, which is carried by vesicles into vacuoles. Suppressing the expression of AtVAMP7C gene makes these vesicles unable to fuse with the tonoplast and stay in the cytoplasm, which can maintain the function of the vacuole[21]. In our research, cold acclimation may lead to an increase in ROS accumulated in cells and a decrease in the abundance of SNARE protein, which reminds us whether *Rhododendron* plants alleviate oxidative stress in the same way to preserve the function of tonoplast. FAB1A/B function as PtdIns 3,5-kinase to catalyze PtdIns 3-P to produce PtdIns (3,5) P2, which is necessary for maintaining endomembrane homeostasis[22]. Increased FAB1A/B abundance was detected in the cold acclimation of *Rhododendron*. The above results let us speculate that *Rhododendron* actively preserve the homeostasis of the endometrial system during cold acclimation. Annexins are an evolutionarily conserved multi-gene family that relies on Ca^{2+} to bind the negatively charged phospholipids on the membrane. In addition to Ca^{2+} binding properties, other protein domains also endow it with various other functions including membrane traffic, cytoskeletal responses, ion transport, and stress responses[23]. Regarding the performance of Annexins in stress response, the existing conclusions are not completely consistent, and many of their functions are inferred from their expression in stress. In Arabidopsis, the two genes encoding Annexins (AtANN1 and AtANN4) are regulated by abiotic stress[24]. Single mutant *annAt1* and *annAt4* plants both show drought and salt tolerance, while their overexpression plants show Stress-sensitive characteristics[24]. In wheat, *TaANN3* was induced by cold stress for 1h. In a time-course experiment, *TaANN3* decline to a level lower than the initial expression 24h after treatment with cold stress[25]. However, Li et al. discovered that *OsANN3* was induced by drought and ABA in rice, and overexpression plants showed better drought resistance[26]. In our research, the abundance of Annexin3 has been down-regulated (Table 1). Therefore, the exact role of Annexins in plant cold stress response should be further investigated according to plant species and cold stress treatment conditions.

DAPs involved in carbohydrate transport and metabolism

In higher plants, NADPH is mainly synthesized through two pathways. One is produced by photosynthetic machinery through the photosynthetic electron transport chain. The other is produced by the pentose phosphate pathway. Glucose 6-phosphate dehydrogenase is the key enzyme that oxidizes glucose 6-phosphate to NADPH. But under light, this pathway in photosynthetic tissue is inhibited by the reduction of F-type thiooxidized protein[27]. In our study, we noticed that the abundance of glucose 6 phosphate dehydrogenase increases during cold stress. In addition, the abundance of F-type thioredoxin also increased. The glucose 6 phosphate dehydrogenase of Chlorella was transferred to Saccharomyces cerevisiae. Although the increase in the abundance of glucose 6 phosphate dehydrogenase did not increase the enzyme activity, it could effectively alleviate the oxidative stress caused by freezing damage[28]. The second enzyme of the pentose phosphate pathway, 6-phosphogluconolactonase, also increased its protein abundance when subjected to cold stress, which indicated that the pentose phosphate pathway of *Rhododendron* plants was activated after being induced by cold stress. The pentose phosphate pathway not only provides the reducing equivalent of NADPH and the necessary carbon skeleton for plants, but is also related to the assimilation of nitrogen[29]. The pentose phosphate pathway provides NADPH for NIR. In

our study, the expression of NIR was also up-regulated after being induced by cold stress. Reduced carbon flows into the pentose phosphate pathway and leaves assimilate nitrite ions to produce organic molecules, which help plants maintain vitality. Glucose-6-phosphate, as the interface carbon source of glycolysis and pentose phosphate pathway, can be catalyzed by glucose 6-phosphate dehydrogenase to generate 6-phosphogluconic acid to enter the pentose phosphate pathway, or it can be converted to Fructose 1,6-bisphosphate under the action of phosphohexose isomerase and phosphofructokinases to enters the glycolytic pathway[30]. The accumulation of glucose 6-phosphate dehydrogenase and the decrease in abundance of phosphofructokinase in our study suggest that the carbon flow shifts from glycolysis in the catabolic pathway to the anabolic pentose phosphate pathway. Adversity environments often compromise photosynthesis of plants, and the sugar concentration will be reduced to the level of “sugar deficit”, which triggers a sugar starvation response in plants. If the duration of the stress is prolonged, the plant will be depleted of sugar. Therefore, in most cases, people generally accept the conclusion that cold stress triggers starch degradation[31]. In our research, we found that the abundance of Alpha-amylase related to starch hydrolysis increased under cold stress (Table 1). This implies that the cold stress environment may make *Rhododendron* plants in a state of sugar deficit, requiring the conversion of starch to sugar to respond adaptively to stress.

DAPs involved in amino acid transport and metabolism

In addition to being a building block for protein synthesis, amino acids are also involved in plant physiological processes[32]. Some differentially expressed proteins related to amino acid metabolism were identified after *Rhododendron* was exposed to cold stress. Branched-chain amino acid aminotransferase 2 and Methionine gamma-lyase showed a tendency of up-regulation after cold induction. This result indicates that the anabolism of branched amino acids is positively regulated in response to cold signals compared to controls. The precursor molecule of isoleucine synthesis is α-ketobutyric acid which can be generated either from the decomposition of methionine catalyzed by methionine gamma-lyase or the deamination of threonine. The last four steps of branched amino acid (isoleucine and valine) synthesis share the same enzymes, and Branched-chain amino acid aminotransferase 2 is responsible for catalyzing the final step of this pathway[33]. Therefore, the up-regulation of these two enzymes implies an increase in the accumulation of branched amino acids following cold treatment. It is reported that the branch amino acid content in potato leaves increases more than the proline content after drought stress. Isoleucine and valine can also be served as osmotic regulators to deal with osmotic stress, although they are not as familiar as proline[34]. In addition, it has been proposed that the accumulation of free branched amino acids can be used as a substrate for stress-induced proteins or as a signal molecule to regulate gene expression. Therefore, we suggested that the two enzymes related to branch amino acid synthesis induced by low temperature stress play a positive regulatory role in the process of *Rhododendron* cold resistance. The reactive oxygen species (ROS) brought about by oxidative stress is the main factors affecting plant fitness and reproduction when plants encounter cold stress. Plants evolved a powerful defense system including antioxidant enzymes and antioxidant compounds to reduce or eliminate ROS. Tocopherols and flavonoids are such antioxidants. Tocopherol is a class of lipophilic compounds mainly found in plastids, and its main function is to protect the chloroplast membrane from oxidative stress. Overexpression of 4-Hydroxyphenylpyruvate dioxygenase (HPPD) in Arabidopsis resulted in an up-regulation of the tocopherol

content in the leaves by 37%[35]. In our study, the abundance of HPPD also showed a significant up-regulation after the cold stress treatment. As an important category of flavonoids, anthocyanins not only provide colorful colors to plants, but are also important antioxidants. The supply of phenylalanine is one of the factors affecting the biosynthesis of anthocyanins. Arogenate dehydratases are the key enzymes that catalyze the formation of phenylalanine from arogenate[36]. Genetic analysis showed that arogenate dehydratases2 (ADT2) contributed the most to the promotion of anthocyanin synthesis among the six isoenzymes[36]. Moreover, we noticed that the abundance of 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHD-SDH), a key enzyme in the synthetic pathway of aromatic amino acids, also increased significantly after the *Rhododendron* was exposed to cold stress. The up-regulated expression of these three enzymes means that *Rhododendron* shifts the metabolic flux from the synthesis of aromatic amino acids to the production of antioxidant substances under cold stress. We cannot determine the role of up-regulated expression of HISN2 in cold stress. Energetically, it has been proved that the synthesis of histidine requires a high metabolic cost, which is obviously unsuitable for *Rhododendron* under cold stress. More importantly, the overexpression of HISN2 has no effect on the level of histidine[37, 38]. Therefore, we speculate that the elevated accumulation of HISN2 is not to increase the abundance of histidine, but to synthesize metabolic intermediates and divert the metabolic flow to the corresponding pathway. It is known that asparagine plays an important role in nitrogen storage and long-distance transportation of nitrogen. The level of asparagine synthase is closely related to the amount of free asparagine[39]. In our study, we observed that asparagine synthase accumulates to high levels during cold stress. The up-regulation of asparagine synthase may be related to the turnover of protein during cold stress, which produces more ammonia that is toxic to living cells (Table 1). In addition to the detoxification of ammonia and the carrier of nitrogen, the relationship between increased abundance of asparagine synthase and cold stress still needs further exploration.

DAPs involved in energy production and conversion

It is reported that the components of complex I, II and III in the respiratory chain are significantly down-regulated under cold stress[40]. In our study, we also observed decrease in the abundance of Cytochrome c1, which led us to speculate that this may be related to the disruption of the metabolism or the reduction of the reducing equivalents during cold stress. In addition, we found that Iron-sulfur cluster assembly protein 1 (ISU1) is also sensitive to cold stress, and its abundance is down-regulated compared to the control. However, the abundance of Cytochrome c has been up-regulated, which may be related to the functional diversification of this protein. In addition to the classic role as electron transport carrier, Cytochrome c also participates in the synthesis of ascorbic acid and the stabilization of the electron transport chain. Moreover, it is also the target of cyanide attacking the electron transport chain, and electrons cannot be transferred to molecular oxygen via complex III. Under normal conditions, cyanide is combined into non-toxic glycosides and stored in vacuoles, while glycosidases are localized in the cytoplasm. The vacuole membrane under environmental stress is damaged; glycosidase directly hydrolyzes the glycosidic bond and releases hydrogen cyanide, causing damage to plants. β-cyanoalanine synthase is directly involved in the detoxification of cyanide, concentrating cyanide and cysteine to synthesize cyanoalanine[41]. In our study, we observed increased abundance of β-cyanoalanine synthase under cold stress. The fuel required for oxidative phosphorylation comes from the reducing equivalent produced by metabolic pathways such as the tricarboxylic acid (TCA) cycle and β-oxidation pathway[42]. Glyoxylate cycle involves the concentration

of acetyl-CoA and oxaloacetate catalyzed by citrate synthase to produce citric acid and coenzyme A. The up-regulation of citrate synthase 2, a key enzyme in glyoxylate cycle, indicates that more acetyl groups may enter TCA cycle from peroxisome. The substrate of the glyoxylate cycle comes from the beta oxidation of fatty acids in the peroxisome. Evidence suggests that acetyl-CoA oxidase 3 is responsible for catalyzing the first step of β-oxidation of medium-length fatty acids in peroxisome[43]. In our study, the enhanced accumulation of acetyl-CoA oxidase 3 implies that the mobilized lipids may be oxidized to meet the substrate requirements of the glyoxylate cycle. Furthermore, the abundance of acylcarnitine carriers (BOU) related to acetyl or acyl transport also increased after being induced by cold stress. This indicates that in addition to the glyoxylate pathway, alternative pathway, the BOU pathway, which also participates in providing metabolic substrates to the TCA cycle. 2-oxoglutarate dehydrogenase complex is responsible for catalyzing the second oxidative decarboxylation reaction in the TCA cycle. In our study, the level of dihydrolipoyl succinyl transferase, E2 subunit of 2-oxoglutarate dehydrogenase (OGDHC) complex1, was down-regulated, suggesting that this enzyme is sensitive to cold stress (Table 1). The combined effect of the instability of OGDHC and the increased influx of acetyl groups into the TCA cycle may cause the accumulation of 2-oxoglutarate. As an important organic molecule in the cell, 2-oxoglutarate plays regulatory role at least in three aspects: (i) carbon skeleton for nitrogen assimilation; (ii) modulation in amino acid metabolic network; (iii) regulation in carbon-nitrogen interaction. The inhibition of 2-OGDHC in potato, via chemical inhibitors, culminated with reduction of the intermediate of the TCA cycle except for succinic acid and down-regulation of amino acids related to nitrogen assimilation. Our results may differ from the above conclusions: (i) The acetyl group derived from β oxidation feeds the TCA cycle, allowing metabolism to flow from citrate synthase to isocitrate dehydrogenase; (ii) although not significant, the down-regulated glutamate decarboxylase (required for the GABA pathway) may not be able to supplement succinate. The lack of concerted up-regulation of glutamine synthase and glutamate synthase makes us uncertain that the carbon skeleton of 2-oxoglutarate will switch to nitrogen assimilation. Therefore, we cannot determine which metabolic pathway the accumulated 2-oxoglutarate will lead to with our existing data.

DAPs involved in antioxidation, inorganic ion transport and metabolism

Superoxide dismutase (SOD) converts O^{2-} into H_2O_2 and O_2 through disproportionation. It is the first line of defense for antioxidant enzymes against reactive oxygen species. According to the metal cofactors in the active center, SOD in plants is divided into three categories: Copper/zinc SOD (CuZnSOD), manganese SOD (MnSOD), and iron SOD (FeSOD). CuZnSODs are localized in cytoplasm, chloroplast and peroxisome. MnSODs are found in the chloroplast. FeSODs are mainly located in chloroplasts, and parts of them are presented in peroxisomes and apoplasts. Proteomic results revealed that CuZnSODs showed increased abundance after cold stress treatment in wheat leaves, while opposite conclusion was obtained in rice under such treatment. In our study, among the three types of SOD, only the abundance of CuZnSODs changed significantly and showed up-regulation under cold stress. CuZnSOD is a homodimer enzyme containing copper and zinc. Copper is a key cofactor necessary for enzymes to perform catalytic functions[44]. Copper chaperones are involved in the trafficing of coppers and release them to copper-containing proteins. Those proteins that insert coppers into CuZnSODs are called Cu chaperone of SOD (CCS)[45]. In our study, we

observed a concerted up-regulation of CCS after cold stress treatment. In addition to copper ions, another metal ion, iron, is also interesting because it participates in the electron transport chain. Ferritin is an iron storage protein synthesized in the cytoplasm and transported to mitochondria or chloroplasts. Mutants devoid of ferritin did not show obvious growth and development defects under normal conditions, but is sensitive to oxidative stress caused by methylviologen. Transgenic mutants overexpressing alfalfa ferredoxin showed resistance to photoinhibition induced by low temperature stress[46]. In our study, we observed increased abundance of Ferritin-1 and Ferritin-3 after plants were exposed to cold stress (Table 1). This indicates that ferritin is required to relieve the oxidative stress caused by cold stress to maintain the redox homeostasis in *Rhododendron* plants. As the main soluble antioxidant substance in plants, Ascorbic acid (ASA) plays a vital role in detoxifying ROS produced by photosynthesis, respiration and abiotic stress. In the process of ASA biosynthesis, D-galacturonate reductase catalyzes the reduction of D-galacturonic acid to L-galactonic acid. L-galactonic acid is then converted to L-galactono-1,4-lactone, and is further oxidized to produce ASA. It has been shown that heterologous overexpression of strawberry GalUR, the content of ASA increases to the original 2 to 3 times in Arabidopsis[47]. In tomato overexpression lines, although the content of ASA only increased moderately, the transgenic lines showed the characteristics of photoprotection against photooxidation[47]. Therefore, we believe that D-galacturonate reductase plays a positive role in enhancing the cold tolerance of *Rhododendrons*.

DAPs involved in cytoskeleton

It is well known that when plants encounter cold stress, they are often accompanied by osmotic stress. Actin-depolymerizing factor 2 was identified as an up-regulation of abundance after cold exposure, indicating that this protein may be required in response to osmotic stress. In fact, the correlation between the regulation of potassium channels in guard cells and osmotic stress has long been reported. During the period of cold stress, the actin filaments depolymerization caused by osmotic stress further strengthens the influx of potassium ions in the guard cells[48]. Therefore, the up-regulation of Actin-depolymerizing factor 2 abundance here can be regarded as sensors of osmotic stress protect cells from excessive water loss. Another protein (Tubulin β-2) related to the cytoskeleton also accumulated after being induced by cold stress. This accumulation may come from the biosynthesis of the protein or the disassembly of microtubule fibrils. The assumption of microtubule depolymerization is consistent with the consensus that cold stress induces a decrease in membrane fluidity, accompanied by calcium ion influx and microtubule depolymerization[49]. In addition to microtubules and microfilaments, phospholipase D is also an important part of the cold signaling[50]. In our study, the abundance of PLD-α1 was down-regulated when *Rhododendron* was exposed to cold stress (Table 1). As we know that PLD-α1 can aggravate the damage of freezing stress to plants, the reduction of PLD-α1 abundance can be seen as a manifestation of *Rhododendron* plants actively responding to cold stress and reducing freezing damage.

DAPs involved in cell wall, aquaporins, and H^+-ATPase

The cell wall protects plants from environmental stress, provides structural support and acts as a barrier to diffusion. UDP-xylose is a direct donor for the synthesis of cell wall polysaccharides xylose and xylan[51]. Our results indicate that the enzymes UDP-glucuronic acid decarboxylase (UXS), UXS2 and UXS6, involved

in the synthesis of UDP-xylose, were down-regulated after the *Rhododendron* plants were subjected to cold stress. However, in view of the irregular xylose structure in the *uxs3 xus5 uxs6* triple mutants and no obvious phenotype in all 6 single *uxs*, we cannot determine the effect of down-regulation of UXS2 and UXS6 on the xylose content in the cell wall[52]. Ferulic acid extensively dimerizes, facilitating formation of cross-links between cell wall polysaccharides, and contributes to the recalcitrance of cell wall[53]. In this study, the abundance of Aldehyde dehydrogenase family 2 member C4 (ALDH2C4), the enzyme responsible for oxidizing coniferaldehyde to ferulic acid, was down-regulated. Another protein involved in the regulation of cell wall polymer is Secretory Carrier-Associated Membrane Proteins (SCAMP), which may influence the composition of the cell wall by finely regulating the level of cell wall precursors and the secretion of proteins participated in cell wall synthesis and transport. It has been reported that PttSCAMP3 knockdown mutants increase the accumulation of carbohydrates and phenolics on the secondary cell wall[54]. Therefore, the down-accumulation of SCAMP3 in this study may play an important role in the cold tolerance of *Rhododendron* plants by changing the composition of the cell wall. We assume that the altered cell wall composition may be an adjustment to cold stress, and perception of cold stress triggers the cold response of plants. The cell membrane plays a very important role in the interaction between the cell and the environment. For example, it can act as thermo sensors, permeable barrier, and the boundary of a cell. Many functions of the cell membrane are completed with the participation of membrane-bound proteins[55]. Aquaporin and plasma membrane H^+-ATPase, as important proteins on the plasma membrane, play an important role in regulating the entry and exit of various materials. In addition to transporting water, aquaporins can also transport neutral solutes and ammonia[56]. The response of aquaporin to abiotic stress has been widely reported in the literature. For example, transgenic plants overexpressing wheat *TaAQP7* (*PIP2*) show cold tolerance[57]. However, opposite pattern was revealed in our research. All the DEPs, PIP1-2, PIP1-3, PIP2-4, PIP2-1, and PIP2-8 related to the water channel were down-regulated after being subjected to cold stress. We speculate that this is related to the growth environment of the *Rhododendron*. Cold memory allows plants to reduce water into the apoplast to avoid the formation of ice crystals in the apoplast when freezing stress comes, causing further dehydration of the plant and physical damage to the plasma membrane. The plasma membrane H^+-ATPase is responsible for the active transport of cations. This process is accompanied by the hydrolysis of ATP and the efflux of protons. In addition, H^+-ATPase is also involved in other physiological processes such as salt resistance and pH adjustment. The results of the time course experiment showed that the amount of H^+-ATPase increased by the induction of cold stress[58]. However, in our study, the abundance of plasma membrane ATPase 4 and 10 both decreased after being subjected to cold stress (Table 1). For the opposite result that appeared in the experiment, we cannot give further explanation for the time being.

Conclusion

In this study, we made a comparative analysis of *Rhododendron* plants under normal temperature and cold stress according to their proteomic response. Our results indicate that *Rhododendron* plants have divergent cold stress tolerance mechanisms, which can be summarized as follows: (a) Accumulation of chaperone proteins like DnaJ, HSP70, and Chaperonin 60 in order to stabilize the protein and facilitate protein folding, and protect the protein from cold stress-induced denaturation; (b) The up-regulation of components related

to translation, such as ribosomal proteins, Translation initiation factor, and translation initiation factor, may be crucial to the cold tolerance of rhododendrons; (c) Enhancement of carbohydrate and lipid metabolism and reduction in anabolism, provide energy for plants and fight against cold stress; (d) Change the metabolic pathway of aromatic amino acids towards the synthesis of antioxidants, such as tocopherol, and synthesize other antioxidants-related substances such as ascorbic acid and SOD to detoxify the attack of ROS; (e) Modification of components in cell wall, membrane, and cytoskeleton, contributing to the transmission of stress signals and adjusts the water state in the cell. According to the proteomics data, the response of Rhododendron to cold stress can be summarized in Fig. 5 and Fig. 6. These results provide an in-depth understanding of the cold tolerance mechanism of *Rhododendron*.

Abbreviations

TMTs: Tandem mass tags; FC: Fold change; DEGs: Differentially expressed genes; GalUR: D-galacturonate reductase; GABA: g-aminobutyric acid; TGN: trans-Golgi network; PVC: prevacuolar compartment

Declarations

Ethics approval and consent to participate

We obtained permission for our field study from Changbai Mountain Planning and Natural Resources Bureau.

Consent for publication

Not Applicable

Availability of data and material

The data sets supporting the results of this article are included in this article.

Competing interests

The authors declare that they have no conflict of interest.

Funding

This work was supported by the National Natural Science Foundation of China (Grant No. 31070224)

Authors' contributions

XFZ and HWH provided the materials and designed the experiment. KC wrote the manuscript. ZYZ, YT, and HF reviewed the full text.

Acknowledgements

Not Applicable

References

1. Hall R: **The plate tectonics of Cenozoic SE Asia and the distribution of land and sea**. *biogeography & geological evolution of se asia* 1998, 99-131.
2. Huang CC, Hung KH, Hwang CC, Huang JC, Chiang TY: **Genetic population structure of the alpine species Rhododendron pseudochrysanthum sensu lato (Ericaceae) inferred from chloroplast and nuclear DNA**. *Bmc Evolutionary Biology* 2011, **11**(1):108-123.
3. Hallenberg N, Nilsson RH, Robledo G: **Species complexes in Hericium (Russulales, Agaricomycota) and a new species - Hericium rajchenbergii - from southern South America**. *Mycological Progress* 2013, **12**(2):413-420.
4. Jan N, Qazi HA, Raja V, John R: **Proteomics: A tool to decipher cold tolerance**. *Theoretical and Experimental Plant Physiology* 2019, 31: 183-213.
5. Zhou X, Chen S, Wu H, Yang Y, Xu H: **Biochemical and proteomics analyses of antioxidant enzymes reveal the potential stress tolerance inRhododendron chrysanthumPall**. *Biology Direct* 2017, **12**(1):10-18.
6. Isaacson T, Damasceno CM, Saravanan RS, He Y, Catalá C, Saladié M, Rose JK: **Sample extraction techniques for enhanced proteomic analysis of plant tissues**. *Nature protocols* 2006, **1**(2):769-774.
7. Zhang N, Jiang N, Zhang K, Zheng L, Zhang D, Sang X, Feng Y, Chen R, Yang N, Wang X *et al*: **Landscapes of Protein Posttranslational Modifications of African Trypanosoma Parasites**. *iScience* 2020, **23**(5):101074-101104.
8. Zhao Z, Liu J, Jia R, Bao S, Haixia, Chen X: **Physiological and TMT-based proteomic analysis of oat early seedlings in response to alkali stress**. *J Proteomics* 2019, **193**:10-26.
9. Nakajima Y, Suzuki S: **Environmental stresses induce misfolded protein aggregation in plant cells in a microtubule-dependent manner**. *Int J Mol Sci* 2013, **14**(4):7771-7783.
10. Kong F, Deng Y, Zhou B, Wang G, Wang Y, Meng Q: **A chloroplast-targeted DnaJ protein contributes to maintenance of photosystem II under chilling stress**. *J Exp Bot* 2014, **65**(1):143-158.
11. Holland, Neta, d B, Anna, Doron, Pick, Uri, Edelman, Marvin: **Stress-responsive accumulation of plastid chaperonin 60 during seedling development**. *Plant Journal* 1998, **13**(3):311-316.
12. Atsushi I, Hideaki T, Masato N, Tadashi A: **Deletion of a chaperonin 60 beta gene leads to cell death in the Arabidopsis lesion initiation 1 mutant**. *Plant and Cell Physiology* 2003(3):255-261.
13. Liu S, Hu ZM, Zhang Q, Yang X, Critchley AT, Duan D: **PI signal transduction and ubiquitination respond to dehydration stress in the red seaweed Gloiopeltis furcata under successive tidal cycles**. *BMC plant biology* 2019, **19**(1):516-529.
14. Pelletier J, Sonenberg N: **The Organizing Principles of Eukaryotic Ribosome Recruitment**. *Annual review of biochemistry* 2019, **88**:307-335.
15. Tronchoni J, Medina V, Guillamón JM, Querol A, Pérez-Torrado R: **Transcriptomics of cryophilic Saccharomyces kudriavzevii reveals the key role of gene translation efficiency in cold stress adaptations**. *BMC genomics* 2014, **15**(1):432-441.

16. Rogalski M, Schöttler MA, Thiele W, Schulze WX, Bock R: **Rpl33, a nonessential plastid-encoded ribosomal protein in tobacco, is required under cold stress conditions**. *Plant Cell* 2008, **20**(8):2221-2237.
17. Cheng LB, Li SY, Yang GX, Jing XM, He GY, Mones NG: **Overexpression of Soybean (Glycine max (L.) Meer.) L34 Gene Leads to Reduced Survival to Cold Stress in Transgenic Arabidopsis**. *Plant Molecular Biology Reporter* 2010, **28**(1):41-48.
18. Garcia-Molina A, Kleine T, Schneider K, Mühlhaus T, Lehmann M, Leister D: **Translational Components Contribute to Acclimation Responses to High Light, Heat, and Cold in Arabidopsis**. *iScience* 2020, **23**(7):101331-101371.
19. Wang X, Xu M, Gao C, Zeng Y, Cui Y, Shen W, Jiang L: **The roles of endomembrane trafficking in plant abiotic stress responses**. *Journal of integrative plant biology* 2020, **62**(1):55-69.
20. De Benedictis M, Bleve G, Faraco M, Stigliano E, Grieco F, Piro G, Dalessandro G, Di Sansebastiano GP: **AtSYP51/52 functions diverge in the post-Golgi traffic and differently affect vacuolar sorting**. *Molecular plant* 2013, **6**(3):916-930.
21. Leshem Y, Melamed-Book N, Cagnac O, Ronen G, Nishri Y, Solomon M, Cohen G, Levine A: **Suppression of Arabidopsis vesicle-SNARE expression inhibited fusion of H2O2-containing vesicles with tonoplast and increased salt tolerance**. *Proc Natl Acad Sci U S A* 2006, **103**(47):18008-18013.
22. Hirano T, Matsuzawa T, Takegawa K, Sato MH: **Loss-of-function and gain-of-function mutations in FAB1A/B impair endomembrane homeostasis, conferring pleiotropic developmental abnormalities in Arabidopsis**. *Plant Signaling & Behavior* 2011, **155**(4):797-807.
23. Saad RB, Ben Romdhane W, Ben Hsouna A, Mihoubi W, Harbaoui M, Brini F: **Insights into plant annexins function in abiotic and biotic stress tolerance**. *Plant Signal Behav* 2020, **15**(1):1699264-1699267.
24. Huh SM, Noh EK, Kim HG, Jeon BW, Bae K, Hu HC, Kwak JM, Park OK: **Arabidopsis annexins AnnAt1 and AnnAt4 interact with each other and regulate drought and salt stress responses**. *Plant Cell Physiol* 2010, **51**(9):1499-1514.
25. Xu L, Tang Y, Gao S, Su S, Hong L, Wang W, Fang Z, Li X, Ma J, Quan W *et al*: **Comprehensive analyses of the annexin gene family in wheat**. *BMC genomics* 2016, **17**:415-432.
26. Li X, Zhang Q, Yang X, Han J, Zhu Z: **OsANN3, a calcium-dependent lipid binding annexin is a positive regulator of ABA-dependent stress tolerance in rice**. *Plant Science* 2019, 212-220.
27. Tian Y, Peng K, Bao Y, Zhang D, Meng J, Wang D, Wang X, Cang J: **Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase genes of winter wheat enhance the cold tolerance of transgenic Arabidopsis**. *Plant Physiol Biochem* 2021, **161**:86-97.
28. Honjoh, Ken-ichi, Miyamoto, Takahisa, Ohashi, Naoto, Masayoshi, Machida, Takeshi, Mimura: **Chloroplastic Glucose 6-Phosphate Dehydrogenase from Chlorella vulgaris Alleviates Freezing and Menadione-Induced Oxidative Stresses in Saccharomyces cerevisiae**. *Journal of the Faculty of Agriculture, Kyushu University* 2010, **55**(1):29-38.
29. Esposito S: **Nitrogen Assimilation, Abiotic Stress and Glucose 6-Phosphate Dehydrogenase: The Full Circle of Reductants**. *Plants (Basel, Switzerland)* 2016, **5**(2) :24-37..
30. Chen X, Schreiber K, Appel J, Makowka A, Fähnrich B, Roettger M, Hajirezaei MR, Sönnichsen FD, Schönheit P, Martin WF *et al*: **The Entner-Doudoroff pathway is an overlooked glycolytic route in**

cyanobacteria and plants. *Proc Natl Acad Sci U S A* 2016, **113**(19):5441-5446.

31. Dong S, Beckles DM: **Dynamic changes in the starch-sugar interconversion within plant source and sink tissues promote a better abiotic stress response**. *J Plant Physiol* 2019, **234-235**:80-93.
32. Hildebrandt TM: **Synthesis versus degradation: directions of amino acid metabolism during Arabidopsis abiotic stress response**. *Plant molecular biology* 2018, **98**(1-2):121-135.
33. Peng C, Uygun S, Shiu SH, Last RL: **The Impact of the Branched-Chain Ketoacid Dehydrogenase Complex on Amino Acid Homeostasis in Arabidopsis**. *Plant Physiol* 2015, **169**(3):1807-1820.
34. SHEN, FOSTER, JG, ORCUTT, DM: **COMPOSITION AND DISTRIBUTION OF FREE AMINO-ACIDS IN FLATPEA (LATHYRUS-SYLVESTRIS L) AS INFLUENCED BY WATER DEFICIT AND PLANT-AGE**. *J Exp Bot* 1989, **40**(1):71-79.
35. Moran GR: **4-Hydroxyphenylpyruvate dioxygenase**. *Archives of Biochemistry & Biophysics* 2005, **433**(1):117-128.
36. Chen Q, Man C, Li D, Tan H, Xie Y, Huang J: **Arogenate Dehydratase Isoforms Differentially Regulate Anthocyanin Biosynthesis in Arabidopsis thaliana**. *Molecular plant* 2016, **9**(12):1609-1619.
37. Stepansky A, Leustek T: **Histidine biosynthesis in plants**. *Amino acids* 2006, **30**(2):127-142.
38. Rees JD, Ingle RA, Smith JA: **Relative contributions of nine genes in the pathway of histidine biosynthesis to the control of free histidine concentrations in Arabidopsis thaliana**. *Plant biotechnology journal* 2009, **7**(6):499-511.
39. Lea PJ, Sodek L, Parry M, Shewry PR, Halford NG: **Asparagine in plants**. *Annals of Applied Biology* 2010, **150**(1):1-26.
40. Svensson AS, Johansson FI, Møller IM, Rasmusson AG: **Cold stress decreases the capacity for respiratory NADH oxidation in potato leaves**. *FEBS letters* 2002, **517**(1-3):79-82.
41. Niang AS: **Effect of abiotic stresses and cyanide treatment on the cyanide assimilatory pathway in Arabidopsis thaliana**. *Dissertations & Theses Gradworks* 2008.
42. Braun HP: **The Oxidative Phosphorylation system of the mitochondria in plants**. *Mitochondrion* 2020, **53**:66-75.
43. Pracharoenwattana I, Smith SM: **When is a peroxisome not a peroxisome?** *Trends in Plant Science* 2008, **13**(10):522-525.
44. Sarvajeet, Singh, Gill, Naser, A., Anjum, Ritu, Sandeep, Yadav: **Superoxide dismutase—mentor of abiotic stress tolerance in crop plants**. *Environmental Science&Pollution Research* 2015, **22**(14):10375-10394.
45. Chu CC: **A copper chaperone for superoxide dismutase that confers three types of copper/zinc superoxide dismutase activity in Arabidopsis**. *Plant Physiol* 2005, **139**(1): 425-436.
46. Hegedus A, Janda T, Horváth GV, Dudits D: **Accumulation of overproduced ferritin in the chloroplast provides protection against photoinhibition induced by low temperature in tobacco plants**. *J Plant Physiol* 2008, **165**(15):1647-1651.
47. Amaya I, Osorio S, Martinez-Ferri E, Lima-Silva V, Doblas VG, Fernández-Muñoz R, Fernie AR, Botella MA, Valpuesta V: **Increased antioxidant capacity in tomato by ectopic expression of the strawberry D-galacturonate reductase gene**. *Biotechnology journal* 2015, **10**(3):490-500.

48. MacRobbie EAC, Kurup S: **Signalling mechanisms in the regulation of vacuolar ion release in guard cells**. *The New phytologist* 2007, **175**(4):630-640.
49. Pokorná J, Schwarzerová K, Zelenková S, Petrá?Ek J, Janotová I, ?Apková V, Opatrny Z: **Sites of actin filament initiation and reorganization in cold-treated tobacco cells**. *Plant Cell & Environment* 2010, **27**(5):641-653.
50. Wang X, Liang G, Wang G, Li M: **PLD: Phospholipase Ds in Plant Signaling**: Phospholipases in Plant Signaling; 2014, **20**:121-134.
51. Lerouxel O, Cavalier DM, Liepman AH, Keegstra K: **Biosynthesis of plant cell wall polysaccharides - a complex process**. *Curr Opin Plant Biol* 2006, **9**(6):621-630.
52. Kuang B, Zhao X, Zhou C, Zeng W, Ren J, Ebert B, Beahan CT, Deng X, Zeng Q, Zhou G *et al*: **Role of UDP-Glucuronic Acid Decarboxylase in Xylan Biosynthesis in Arabidopsis**. *Molecular plant* 2016, **9**(8):1119-1131.
53. Missihoun TD, Kotchoni SO, Bartels D, Bonham-Smith P: **Active Sites of Reduced Epidermal Fluorescence1 (REF1) Isoforms Contain Amino Acid Substitutions That Are Different between Monocots and Dicots**. *PloS one* 2016, **11**(10):e0165867.
54. Obudulu O, Mähler N, Skotare T, Bygdell J, Abreu IN, Ahnlund M, Latha Gandla M, Petterle A, Moritz T, Hvidsten TR *et al*: **A multi-omics approach reveals function of Secretory Carrier-Associated Membrane Proteins in wood formation of Populus trees**. *BMC genomics* 2018, **19**(1):11-28.
55. Luschnig C, Vert G: **The dynamics of plant plasma membrane proteins: PINs and beyond**. *Development (Cambridge, England)* 2014, **141**(15):2924-2938.
56. Maurel C, Verdoucq L, Luu DT, Santoni V: **Plant aquaporins: membrane channels with multiple integrated functions**. *Annual review of plant biology* 2008, **59**:595-624.
57. Huang C, Zhou S, Hu W, Deng X, Wei S, Yang G, He G: **The wheat aquaporin gene TaAQP7 confers tolerance to cold stress in transgenic tobacco**. *Zeitschrift fur Naturforschung C, Journal of biosciences* 2014, **69**(3-4):142-148.
58. Sadura I, Libik-Konieczny M, Jurczyk B, Gruszka D, Janeczko A: **Plasma membrane ATPase and the aquaporin HvPIP1 in barley brassinosteroid mutants acclimated to high and low temperature**. *J Plant Physiol* 2020, **244**:153090-153097.

Tables

Table 1 DAPs potentially associated with cold stress in *R. aureum*

Category	Protein accession	Protein description	Ratio	MW [kDa]
Posttranslational modification, protein turnover, chaperones	Gene.73635_Unigene12134_All	HSP90	2.17	94.031
	Gene.59323_Unigene1842_All	Thioredoxin F	1.26	12.58
	Gene.77347_Unigene15610_All	Thioredoxin-like 2	1.22	26.168
	Gene.83438_Unigene21137_All	DNAJ1	1.31	46.475
	Gene.39619_CL4992.Contig2_All	RPN9A	1.22	46.446
	Gene.84965_Unigene22649_All	CPN60A2	1.2	63.202
	Gene.53323_CL8052.Contig3_All	HSP70	1.35	19.895
	Gene.68609_Unigene8266_All	CPN10-1	1.21	14.646
	Gene.23804_CL2221.Contig4_All	SAE2	1.2	71.528
	Gene.58610_Unigene848_All	HSP70-6	1.23	75.401
	Gene.48883_CL7175.Contig2_All	HSP70-17	1.55	98.672
	Gene.38950_CL4821.Contig1_All	PDIL1-3	1.42	51.137
	Gene.25023_CL2387.Contig2_All	APA1	0.793	55.332
	Gene.39618_CL4992.Contig1_All	RPN9B	0.827	44.155
	Gene.57951_Unigene397_All	EDA2	0.678	56.53
Intracellular trafficking, secretion, and vesicular transport	Gene.13630_CL1062.Contig1_All	SYP51	0.807	25.799
	Gene.73390_Unigene11969_All	SYP52	0.806	25.758
	Gene.47948_CL6912.Contig1_All	SYP22	0.789	29.459
	Gene.76804_Unigene15287_All	FAB1B	1.37	112.15
	Gene.6538_CL447.Contig5_All	Annexin D3	0.778	35.95
	Gene.6539_CL447.Contig6_All	Annexin D3	0.788	36.157
	Gene.36610_CL4341.Contig2_All	SCAMP3	0.738	33.765
Carbohydrate transport and metabolism	Gene.26870_CL2627.Contig3_All	Glucose-6-phosphate 1-dehydrogenase	1.309	67.996
	Gene.23476_CL2196.Contig3_All	6-phosphogluconolactonase	1.231	29.403

	Gene.16014_CL1325.Contig2_All	NIR1	1.244	41.543
	Gene.39120_CL4862.Contig2_All	PFK2	0.762	52.744
	Gene.21264_CL1913.Contig3_All	AMY1.1	1.27	38.562
Amino acid transport and metabolism	Gene.47259_CL6832.Contig5_All	BCAT2	1.239	45.137
	Gene.39758_CL5024.Contig5_All	MGL	1.406	52.576
	Gene.39939_CL5069.Contig1_All	HPPD	1.213	48.549
	Gene.47103_CL6796.Contig2_All	ADT2	1.243	37.392
	Gene.19856_CL1763.Contig2_All	DHD-SDH	1.206	43.819
	Gene.87181_Unigene30322_All	DHD-SDH	1.257	12.874
	Gene.34866_CL3972.Contig4_All	HISN2	1.218	32.111
	Gene.22551_CL2090.Contig1_All	Asparagine synthetase	1.706	66.171
Energy production and conversion	Gene.78096_Unigene16411_All	Cytochrome c1-1	0.778	33.701
	Gene.75410_Unigene13887_All	ISU1	0.81	18.21
	Gene.76554_Unigene15118_All	Cytochrome c	1.256	15.161
	Gene.35451_CL4075.Contig6_All	PCAS-2	1.208	39.904
	Gene.49116_CL7232.Contig2_All	ACLA-2	1.212	49.101
	Gene.71167_Unigene10022_All	ACX3	1.224	75.857
	Gene.77475_Unigene15712_All	BOU	1.282	30.679
	Gene.76231_Unigene14931_All	Dihydrolipoyl succinyl transferase	0.815	51.291
Antioxidation	Gene.75130_Unigene13691_All	SODCC	1.31	15.293
	Gene.43665_CL5950.Contig1_All	Superoxide dismutase	1.209	15.159
	Gene.21962_CL2014.Contig3_All	CCS	1.349	35.64
	Gene.61436_Unigene3284_All	Ferritin-3	1.283	27.194
	Gene.81273_Unigene19206_All	Ferritin-3	1.264	28.02
	Gene.37414_CL4489.Contig4_All	Ferritin-1	1.205	15.978
	Gene.73607_Unigene12118_All	GALUR	1.462	38.574
Cytoskeleton	Gene.35965_CL4190.Contig3_All	ADF2	1.253	16.16

	Gene.53474_CL8078.Contig14_All	TUBB2	1.404	50.13
	Gene.65690_Unigene6208_All	Actin-depolymerizing factor	1.428	16.623
	Gene.35789_CL4153.Contig3_All	Phospholipase D alpha 1	0.589	94.795
Cell wall, aquaporins, and H+-ATPase	Gene.13316_CL1042.Contig5_All	UXS6	0.804	42.837
	Gene.33802_CL3784.Contig4_All	UXS2	0.756	49.089
	Gene.43588_CL5928.Contig2_All	ALDH2C4	0.753	54.119
	Gene.36610_CL4341.Contig2_All	SCAMP3	0.738	33.765
	Gene.39268_CL4899.Contig4_All	PIP1-3	0.749	30.604
	Gene.48649_CL7101.Contig1_All	PIP2-1	0.646	30.175
	Gene.39270_CL4899.Contig6_All	PIP1-3	0.648	30.693
	Gene.40027_CL5088.Contig2_All	PIP1-2	0.668	30.666
	Gene.44173_CL6057.Contig2_All	PIP2-4	0.609	34.387
	Gene.6848_CL464.Contig2_All	PIP2-8	0.774	30.241
	Gene.28694_CL2922.Contig4_All	AHA10	0.752	104.4
	Gene.39207_CL4882.Contig1_All	PMA4	0.755	104.88

Figures

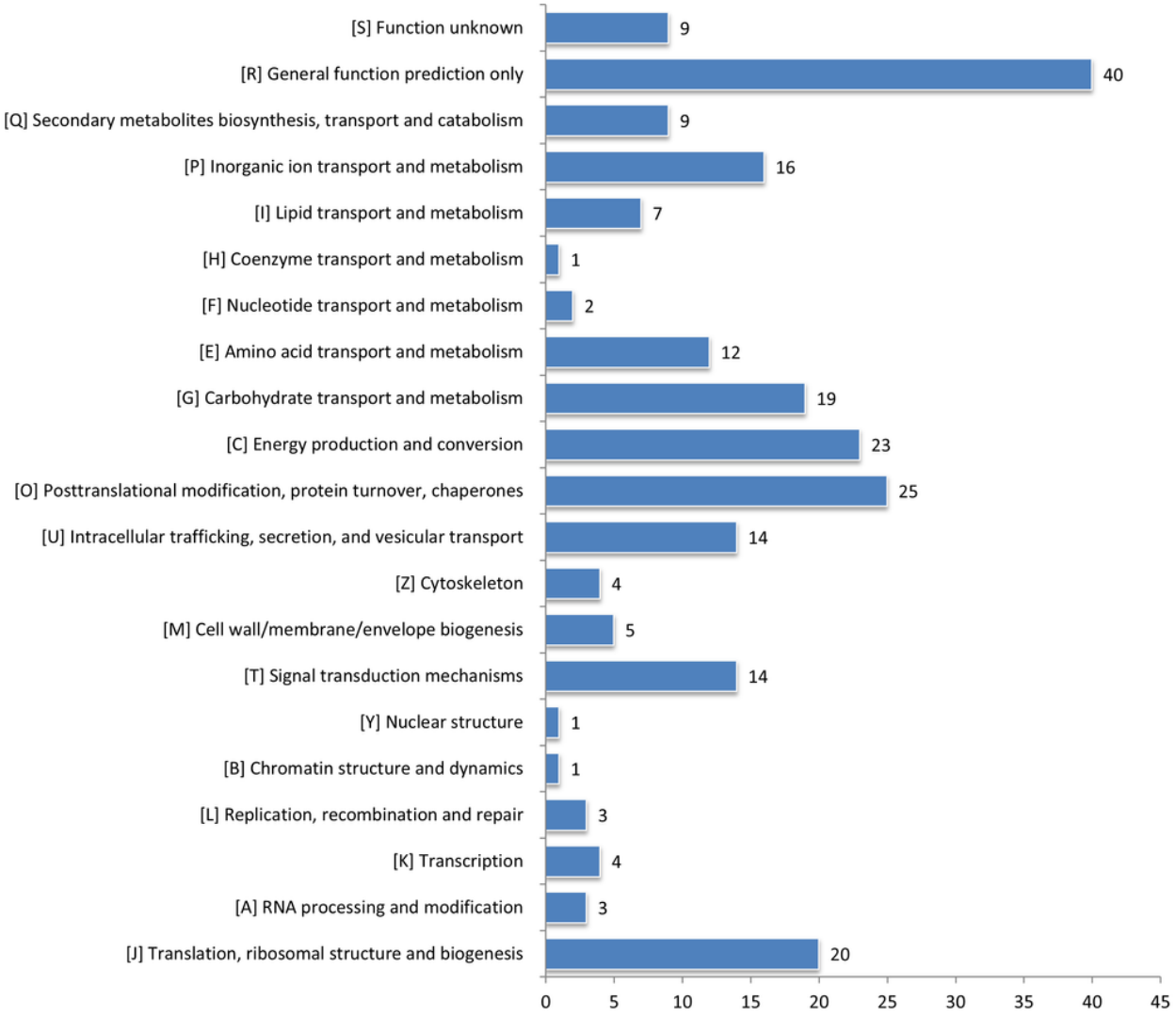

Figure 1

Clusters of Orthologous Groups of proteins (COG) classification of DAPs in leaves of R. aureum under normal and cold condition.

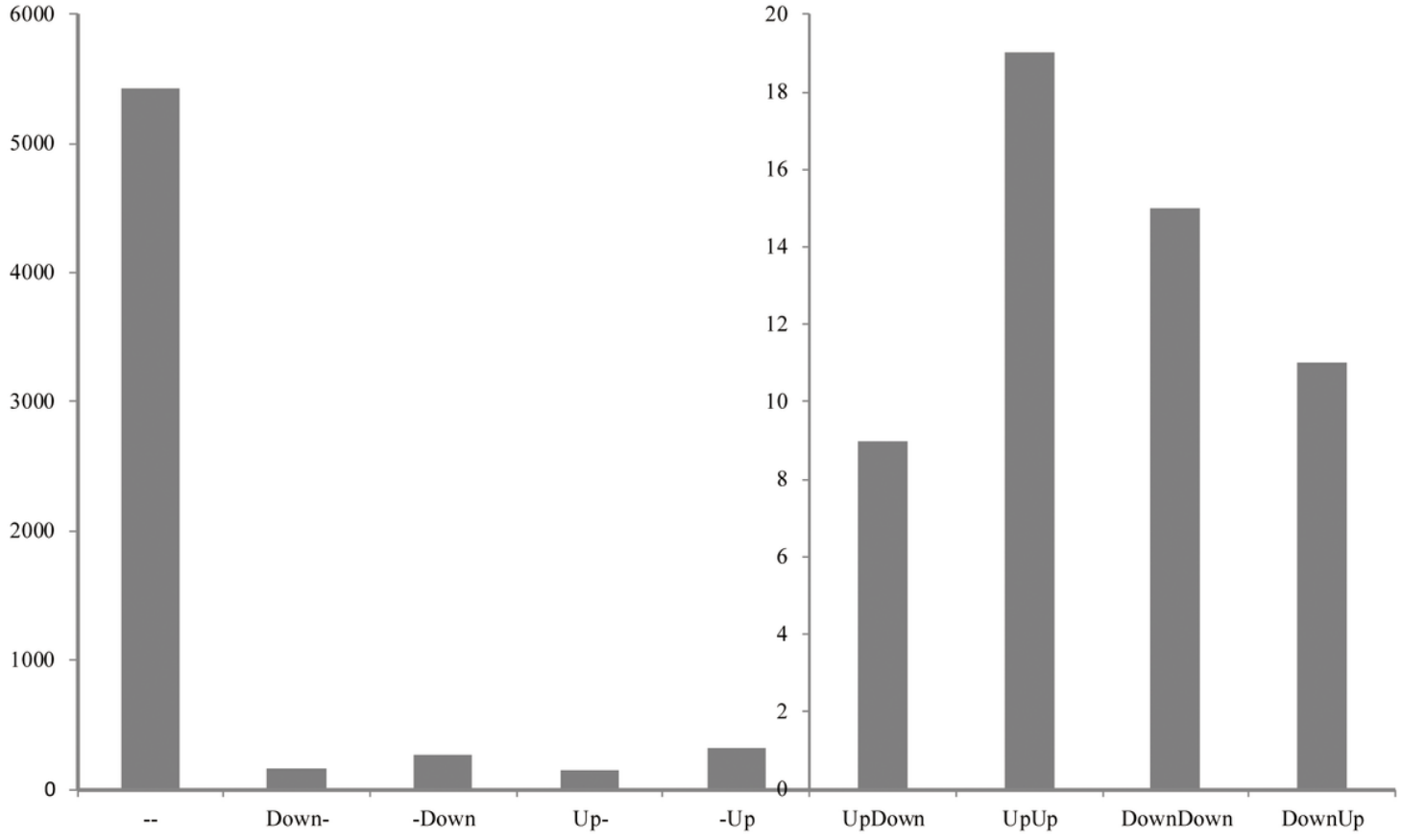

Figure 2

Correlation analysis between transcriptome and proteomics of R. aureum under normal and cold condition. “- -“ indicated non-DEGs and non-DAPs. “Down –“ indicated down-accumulated DAPs and non-DEGs. “Up –“ indicated up-accumulated DAPs and non-DEGs. “- Down” indicated non-DAPs and down-regulated DEGs. “- Up” indicated non-DAPs and up-regulated DEGs. “Up Down“ indicated up-accumulated DAPs and down-regulated DEGs. “Up Up “ indicated up- accumulated DAPs and up- regulated DEGs. “Down Down“ indicated down-accumulated DAPs and down-regulated DEGs. “Down Up“indicated down-accumulated DAPs and up-regulated DEGs.

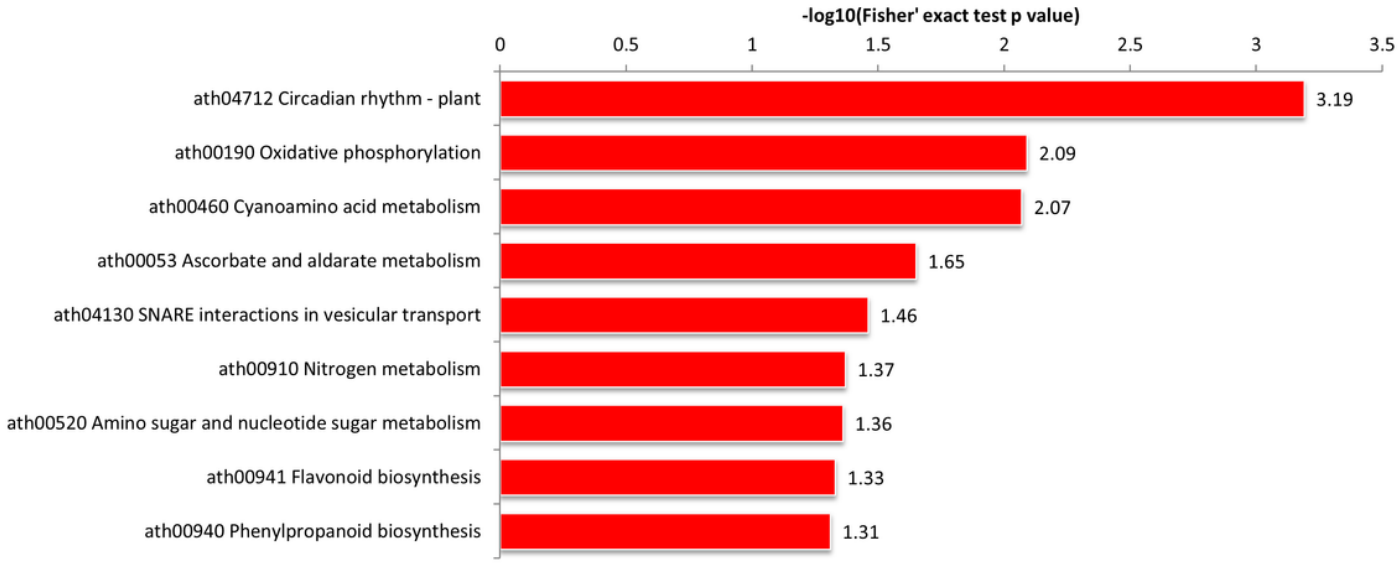

Figure 3

KEGG enrichment analysis on the data in the correlation analysis of R. aureum under normal and cold condition.

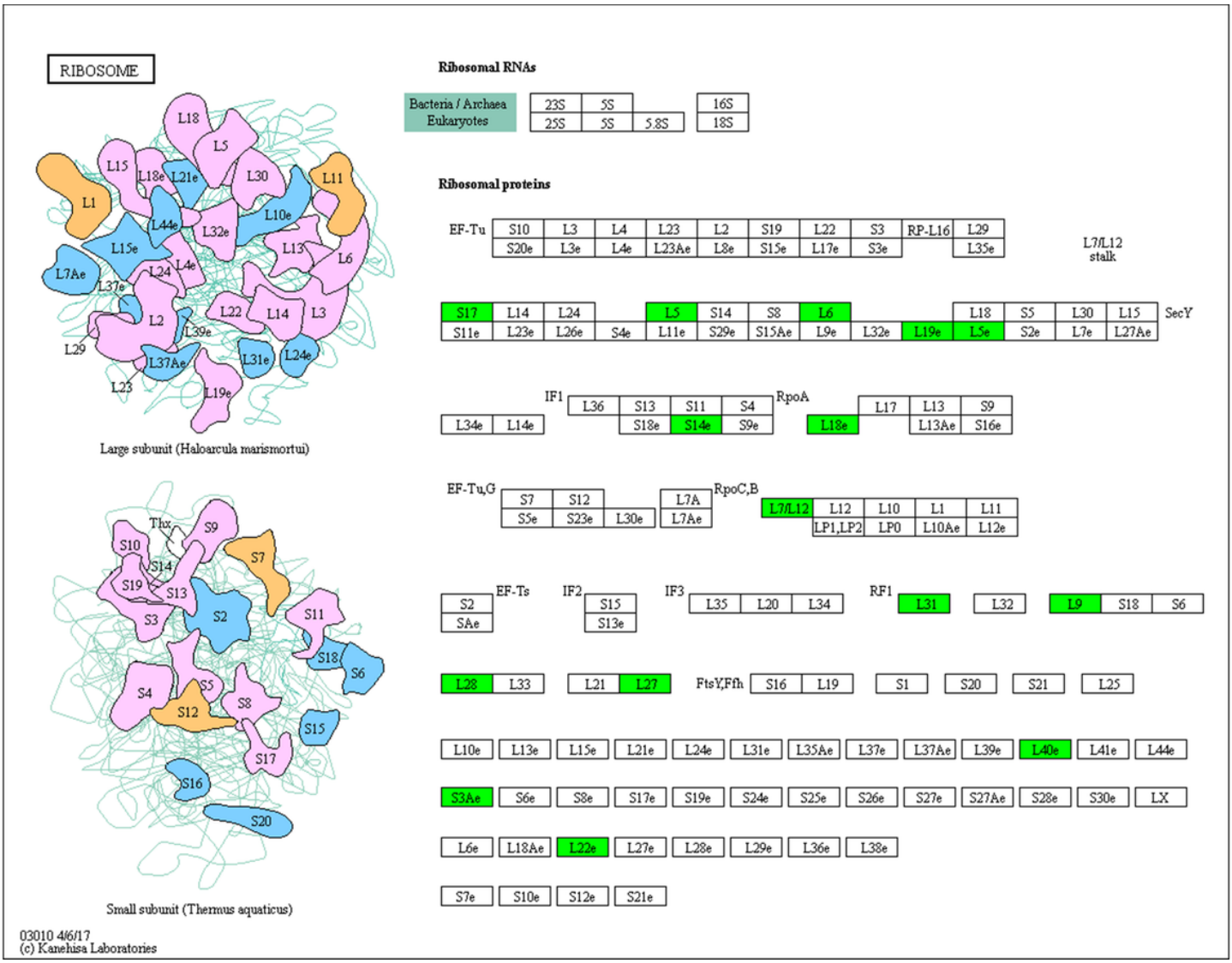

Figure 4

DAPs involved in translation, ribosomal structure and biogenesis. The green boxes represented up-regulated proteins under cold stress.

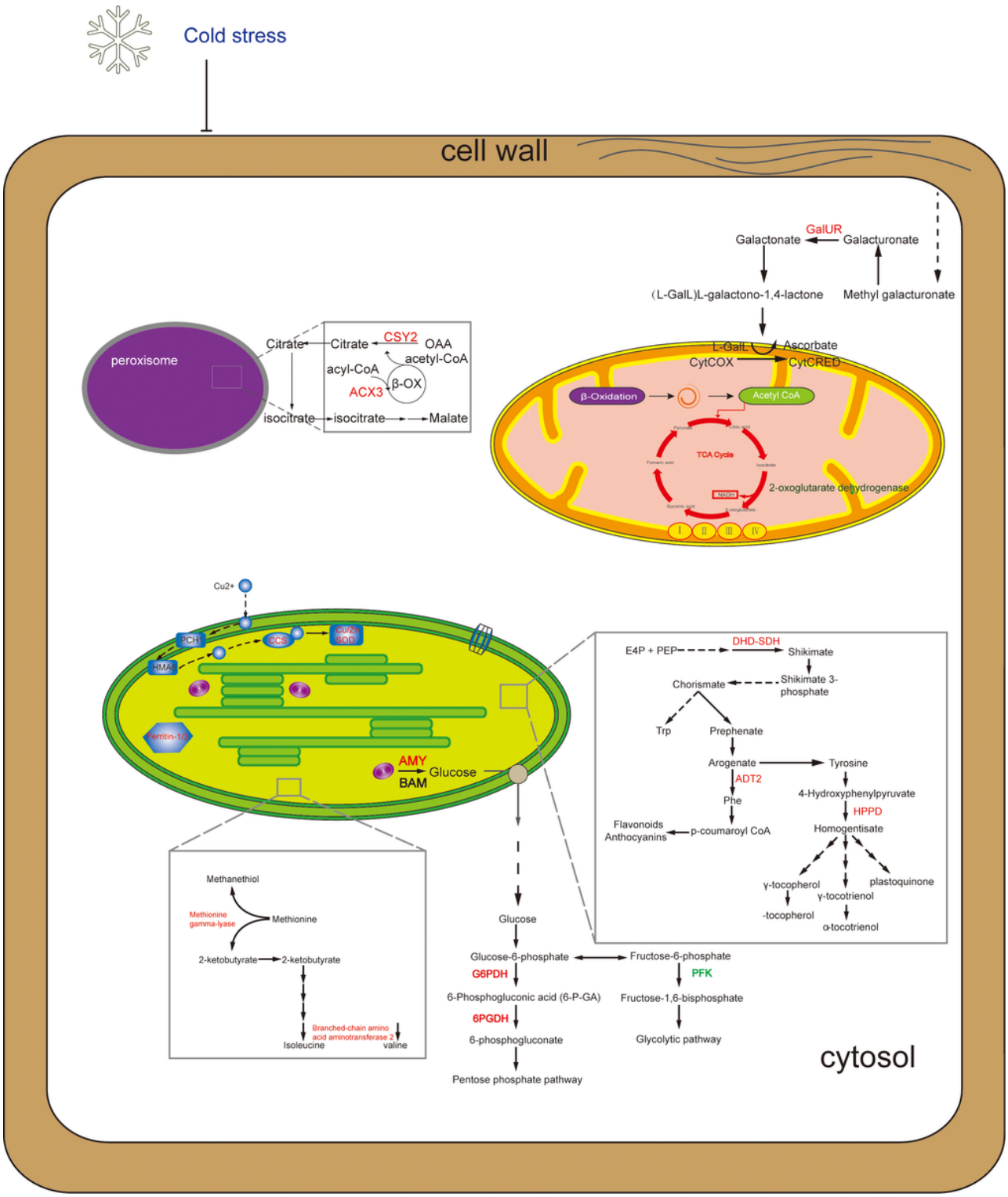

Figure 5

Schematic diagram of proteins involved in cold stress in R. aureum. Abbreviations: ACX3, Acyl-coenzyme A oxidase 3; GalUR, D-galacturonate reductase; CSY2, Citrate synthase 2; CCS, Copper chaperone for superoxide dismutase; Cu/Zn SOD, Superoxide dismutase; AMY, Alpha-amylase; DHD-SDH, 3-dehydroquinate dehydratase/shikimate dehydrogenase; ADT2, Arogenate dehydratase/prephenate dehydratase 2; HPPD, 4-Hydroxyphenylpyruvate dioxygenase; G6PDH, Glucose-6-phosphate 1-dehydrogenase; 6PGDH, 6-phosphogluconolactonase 2; PFK, ATP-dependent 6-phosphofructokinase

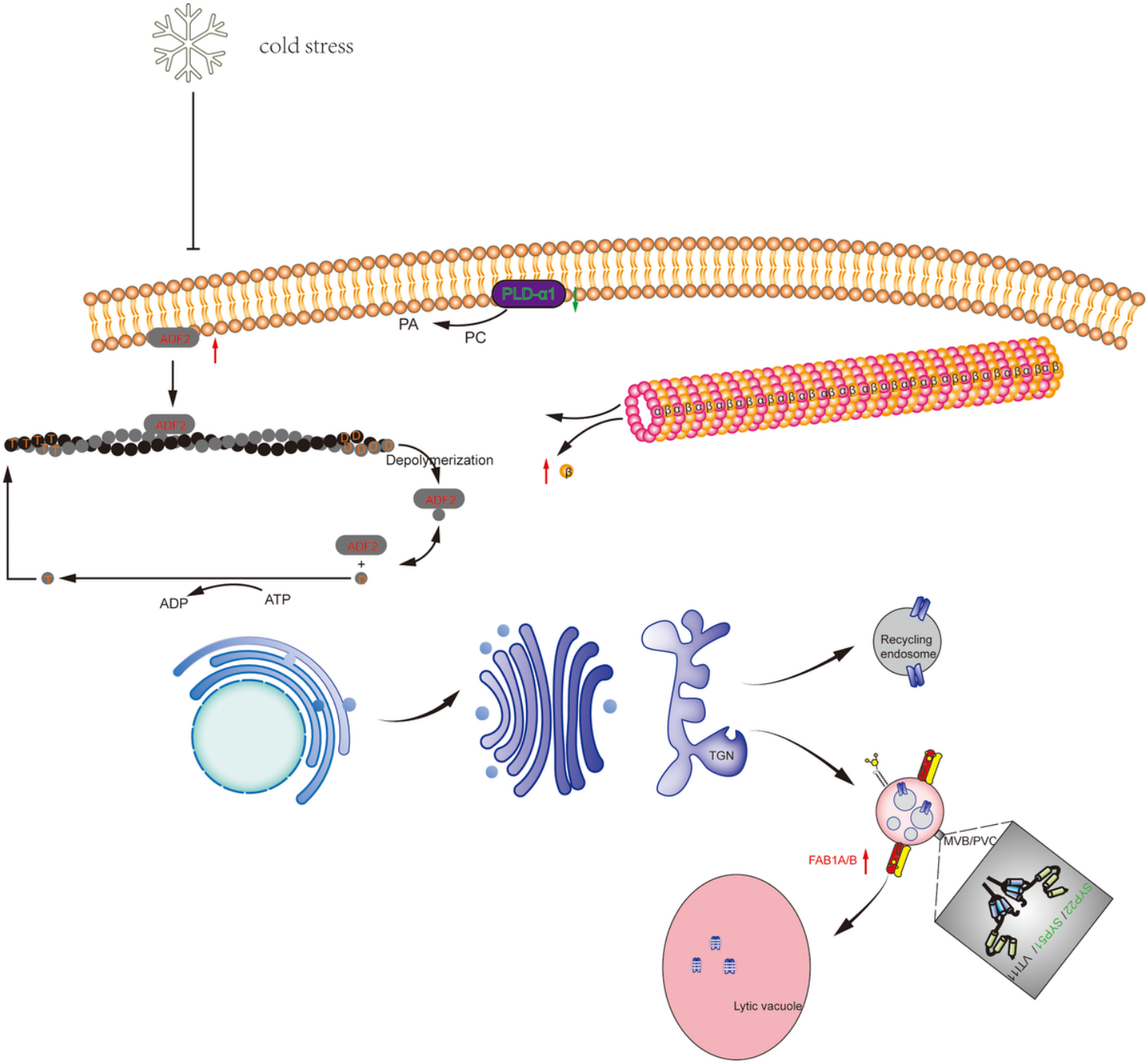

Figure 6

Overview of proteins involved in cold stress in R. aureum. Abbreviations: ADF2, Actin-depolymerizing factor 2; FAB1B, 1-phosphatidylinositol-3-phosphate 5-kinase; SYP51, Syntaxin-51; SYP22, Syntaxin-22; PLDα1, Phospholipase D alpha 1