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ARTICLE

Global Lysine Acetylome Analysis of Flower Bud Development in *Catalpa bungei*

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ABSTRACT

Lysine acetylation, a reversible and dynamic post-translational modification, plays pivotal roles in regulating many biological processes in plants. However, the information of lysine acetylation during flower development of woody angiosperm remains unclear. Herein, we identified a total of 667 lysine acetylation sites in 467 proteins in flower buds of *Catalpa Bungei*. The acetylated proteins were mainly involved in the pathways of carbon metabolism, glycometabolism and oxidative phosphorylation. Using functional enrichment analysis, 61 and 13 acetylated proteins were involved in the glycometabolism and oxidative phosphorylation pathways, respectively. This suggests that lysine acetylated proteins play critical roles in the energy metabolism pathways. Accordingly, the protein–protein interactions were also highly enriched in the carbon metabolic pathway. Integrative analysis of lysine acetylome and transcriptome showed that transcript level changes of seven key lysine-acetylated proteins/genes involved in energy metabolism were significant different during floral transition. Importantly, compared with the normal flowering variety, the expression levels of phosphoribulokinase, phosphoenolpyruvate carboxykinase, ATP synthase and pyruvate dehydrogenase complex genes were significantly up-regulated at the stages of vegetative and transition buds during early flowering of *C. bungei*. These data represent the first lysine acetylome in the *C. bungei* flowers, and can serve as an essential basis for exploring the function of lysine acetylated proteins in the flower bud development of plants.

KEYWORDS

Lysine acetylome; *Catalpa bungei*; flower development; energy metabolism

Abbreviations

PTMs	Post-translational modifications
LC-MS/MS	liquid chromatography-tandem mass spectrometry
ppm	parts per million
GPI	glucose-6-phosphate isomerase
FBP	fructose-1,6-bisphosphatase I
pfp	pyrophosphate-fructose-6-phosphate

ALDO	fructose-bisphosphate aldolase
talA/B	transaldolase
PGLS	6-phosphogluconolactonase
PGD	6-phosphogluconate dehydrogenase
RPE	ribulose-phosphate 3-epimerase
rpiA	ribose 5-phosphate isomerase A
ACO	aconitate hydratase
IDH	isocitrate dehydrogenase
DLD	dihydrolipoamide dehydrogenase
DLST	dihydrolipoamide succinyltransferase
LSC	succinyl-CoA synthetase
SDH	succinate dehydrogenase
DTT	dithiothreitol
FA	formic acid
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
FPKM	Fragments Per Kilobase of exon model per Million mapped fragments.

1 Introduction

Post-translational modifications (PTMs) play important roles in modulating diverse protein properties including localization, activity, protein-protein interaction, and protein-nucleic acid interaction [1–3]. To date, more than 630 PTMs have been identified in the Uniprot database. Among them, lysine acetylation, which extensively occurs on the ε-amino groups of lysine residues in both histones and non-histone proteins, is a reversible, dynamic and ubiquitous PTMs [4–6]. Since the first identification in histone proteins [7], lysine acetylation has been extensively investigated in gene expression regulation and metabolic stability [8–10]. In recent years, proteome-wide analysis of lysine acetylation has been present in humans [4], mouse [11], eukaryotes [12,13] and plants [14–19]. These analyses of lysine acetylome revealed that acetylated proteins regulate various cellular processes, such as enzymatic activities, stress responses and metabolic pathways.

The first lysine acetylome on plants was performed in *Arabidopsis* by Wu et al. [14] and Finkemeier et al. [19]. More recently, lysine acetylome of strawberry leaves indicated important regulatory roles of the acetylated proteins in photosynthesis [18]. Acetylome analysis of grape mesocarp and exocarp revealed that the acetylated proteins are involved in plant defense [15]. Proteomic analysis of lysine-acetylated proteins in rice seeds demonstrated that the proteins are mainly involved in regulating central metabolisms, especially for the citrate cycle [20]. The lysine acetylome study of rice seedlings showed that the acetylated proteins are mainly associated with the regulation of the glyoxylate and dicarboxylate metabolisms, carbon metabolism, and photosynthesis pathways [21]. However, lysine acetylome in flower buds of woody angiosperms remains unclear.

Catalpa bungei, which belongs to the family Bignoniaceae, is not only cultivated broadly for garden landscaping, but also provides ideal materials of flower development for woody angiosperms [22]. Here we used the analyses of anti-acetyllysine-based enrichment and high-sensitive mass spectrometry to investigate the lysine acetylation proteome in the *C. bungei* flower buds. These identified acetylated proteins are mainly involved in energy metabolism, including the pentose phosphate pathway, glycometabolism, citrate cycle and oxidative phosphorylation pathways. Importantly, transcript level changes of seven key lysine-acetylated proteins/genes showed significant differences at the floral

transition stage. With this global proteomic analysis, we revealed important energy metabolism-related functions of lysine acetylated proteins in flower buds.

2 Materials and Methods

2.1 Plant Materials and Growth Conditions

Flower buds of 5, 10, 15 and 20 mm length were collected (Figs. 1A and 1B), respectively, from the double-flower *C. bungei* trees growing in a natural secondary forest in Xiangyang city, Hubei Province, China. These samples were frozen immediately in liquid nitrogen, and stored at −80°C until used.

2.2 Protein Extraction and Western Blotting

The buds were ground in liquid nitrogen. Then, the obtained powder was suspended in extraction buffer containing 8 M urea, 10 mM dithiothreitol (DTT), 50 mM nicotinamide, 3 μM trichostatin A and 0.1% Protease Inhibitor Cocktail Set VI (Calbiochem, Darmstadt, Germany). This was followed by sonication three times on ice using a high intensity ultrasonic processor (scientz-IID, SCIENTZ GmbH, Ningbo, China). After centrifugation at 20,000 g at 4°C for 10 min, the remaining debris was removed. The supernatant containing protein was precipitated with cold 15% trichloroacetic acid at −20°C for 2 h. The precipitated protein was centrifuged again for 10 min at 4°C at 20,000 g, and the pellet was washed three times with cold acetone. The pellet, which was concentrated in a SpeedVac concentrator (Thermo Fisher Scientific, Inc., Waltham, MA, USA), was re-dissolved in buffer (8 M urea, 100 mM $(NH4)_2CO_3$, pH 8.0) and sonicated for 5 min. The protein concentration was measured using a 2-D Quant kit (GE Healthcare) following the manufacturer's instructions.

For western blot, protein was diluted with SDS loading buffer, and 20 μg protein of each sample was separated by 12% SDS-PAGE (Fig. 1C), and transferred to a PVDF (Millipore) membrane. The blot was probed using a pan anti-acetyllysine antibody (PTM Biolabs, Hangzhou, China) in a 1:1000 dilution. Secondary anti-horseradish peroxidase antibody (HuaAn Biotechnology, Hangzhou, China) was used in a 1:10,000 dilution. Plot density of western blot was analyzed using software of Quantity One (Bio-Rad, USA).

2.3 Trypsin Digestion

Protein sample (∼10 mg) of 5 mm flower bud was reduced using 10 mM DTT at 37°C for 1 h, and then alkylated with 20 mM iodoacetamide in darkness at room temperature for 45 min. To ensure that the urea concentration was less than 2 M, the treated protein was subsequently diluted by adding 100 mM $(NH_4)_2CO_3$. The re-suspended protein was finally digested with trypsin/P (Promega) (Product code V5111, Promega Corp., Madison, WI, USA) as described previously [23]. Briefly, trypsin was added at a 1:50 mass ratio of trypsin-to-protein for the first digestion at 37°C overnight. Then, a 1:100 trypsin-to-protein mass ratio was used, and 4 h of digestion was conducted at 37°C. After digestion, the peptides were lyophilized using the SpeedVac.

2.4 Acetylated Peptides Enrichment

Lyophilized peptides were firstly re-dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0), and then incubated overnight at 4°C using pre-washed pan-anti-acetyllysine agarose beads (Catalog No. PTM-104, PTM Biolabs, Hangzhou, China). After incubation with gentle shaking, the agarose beads were washed by centrifugation four times with NETN buffer and twice with ddH_2O. Then, 0.1% trifluoroacetic acid was used to elute the peptides. The peptides were concentrated again using the SpeedVac concentrator. The collected peptides were finally cleaned by C18 ZipTips (Millipore Corp, billerica, MA, USA).

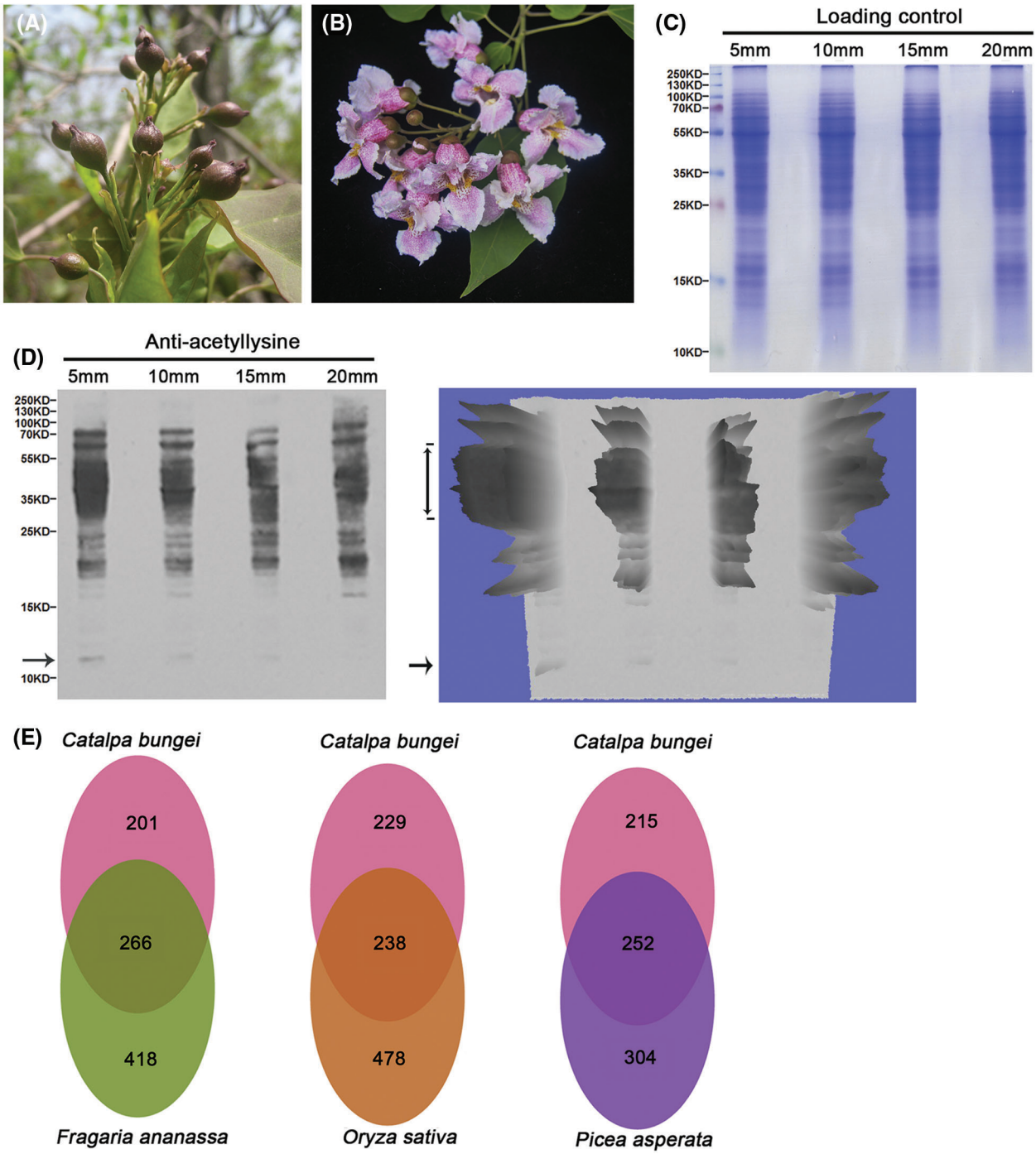

Figure 1: Morphological observation of *C. bungei* flowers and global identification of lysine acetylation sites. (A) an inflorescence of *C. bungei*. (B) full bloom. (C) SDS-PAGE of the proteins. (D) blotting signals and 3-D view of western blotting of the acetylated proteins. The signals of 11 kD and 30–55 kD bands were enhanced for anti-acetyllysine antibody detection at 5 mm flower bud (black arrow). (E) homologous analysis of acetylated proteins in *C. bungei*, *F. ananassa*, *O. sativa* and *P. asperata*

2.5 High-Resolution LC-MS/MS Analysis

Enriched peptides were re-dissolved in mobile phase A [0.1% formic acid (FA) in 2% acetonitrile (ACN)] and directly loaded onto a reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific). The peptides were separated via a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific). A linear gradient of 3% to 22% mobile phase B (0.1% FA in 98% ACN) was applied for 26 min, and followed by 22% to 35% for 8 min and a climb to 80% in 3 min, and then a hold at 80% for the eventual 3 min, all at a fixed flow rate of 300 nl/min in an EASY-nLC 1000 UPLC system (Thermo Fisher Scientific Inc., USA). The obtained peptides were then subjected to a nanospray ion source, followed by MS/MS in Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific, Inc, USA.) coupled online to the UPLC. Intact peptides and ion fragments were detected in the Orbitrap at a resolution of 70,000 and 17,500 (m/z 200), respectively. Additionally, peptides were selected for MS/MS using normalized collisional energy setting as 28. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was used for the top 20 precursor ions above a threshold ion count of 5E3 in the MS survey scan with 30.0 s dynamic exclusion. The electrospray voltage was set at 2.0 kV. Automatic gain control was used to prevent overfilling of the Orbitrap, and 5E4 ions were accumulated to generate the MS/MS spectra. For MS scans, the range of m/z scans was from 350 to 1800.

2.6 Database Search

The resulting MS/MS data were processed by using MaxQuant software with an integrated Andromeda search engine (version 1.5.1.8). Tandem mass spectra were searched against the *Catalpa bungei* transcriptome database concatenated with a reverse decoy database [24]. Trypsin/P was specified as cleavage enzyme and the search allowed up to four missing cleavages, five charges and five modifications per peptide. The 10 ppm and 0.02 Da of mass error were set for precursor ions and fragment ions, respectively. Carbamidomethylation on cysteine was defined as a fixed modification; the oxidation on methionine, acetylation on lysine, and acetylation at the protein N-terminus were defined as variable modifications. The false discovery rate (FDR) threshold for protein, peptide and modification site were set as 1%. The minimum peptide length was specified at 7 amino acids. Default values were set in other parameters of the MaxQuant: the Min and Max peptide length for unspecific search were 7 and 25, respectively. The top MS/MS peaks per 100 Da was 12, MS/MS deisotoping was set as true. The site localization probability was set at more than 0.75.

2.7 Bioinformatics Analyses

Homologs of acetylated proteins were analyzed in *Catalpa bungei*, *Fragaria ananassa* [18], *Oryza sativa* [21] and *Picea asperata* [23]. Gene Ontology (GO) annotation proteome information was derived from the UniProt-GOA database (www.ebi.ac.uk/GOA/). Lysine acetylated protein IDs were converted to UniProt ID and mapped to GO IDs. Then, the remaining lysine acetylation substrates, which were not annotated by UniProt-GOA database, were further annotated GO function by the InterProScan soft based on protein sequence alignment. The identified acetylated proteins were classified by GO terms based on three categories: biological process, cellular component, and molecular function. For each category, a two-tailed Fisher's exact test was used to test the enrichment of the identified protein against all database proteins. The GO with a corrected P-value < 0.05 was considered significant. Kyoto Encyclopedia of Genes and Genomes (KEGG) [25], domain description, and subcellular localization prediction were performed through Web-based interfaces and services, along with the bioinformatics tools, including KAAS, KEGG mapper, InterProScan, and WoLF PSORT (PSORT/PSORT II). Secondary structures were predicted using NetSurfP. The annotation categories showing P-values < 0.05 were considered to be significantly enriched [26]. The filtered P-value matrix was transformed by the function $x = -\log10$ (P-value). The results were visualized using a "ggplot2" R project (http://cran.r-project.org/web/packages/ggplot2/).

All acetylated protein name identifiers were searched against the STRING database version 9.1 for protein-protein interactions and blast to *Solanum lycopersicum* species. Interaction networks from STRING database were visualized in Cytoscape software [27,28]. The lysine acetylated proteins of *C. bungei* flower buds and transcriptome of buds from early flowering (EF) and normal flowering (NF) were analysed. Then, the relative expression levels for differentially expressed proteins were calculated based on the FPKM [29].

3 Results

3.1 Identification of Lysine Acetylated Proteins in C. bungei Flower

To acquire an initial overview of the extent of lysine acetylation during flower bud development, we detected the lysine acetylated proteins using western blotting analysis. Multiple major protein bands with a molecular weight higher than histones (10–30 kD) were successfully detected (Fig. 1D). Compared with the flower buds of 10, 15 and 20 mm in length, the signals of 11 kD and 30–55 kD bands were enhanced for anti-acetyllysine antibody detection at 5 mm flower buds (Fig. 1D and Tab. S1). Therefore, the flower buds of 5 mm were chosen to profile the acetylated proteins.

To determine the lysine acetylome, we used anti-acetyllysine-based enrichment and high-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify acetylated proteins and their modification sites. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org/) via the PRIDE partner repository with the dataset identifier PXD005121. For the enriched acetylated peptides, the distributions of mass errors were investigated and all of them were less than 5 parts per million (ppm), showing the high accuracy of the MS data which fits the requirement (Fig. S2A). The lengths of the identified peptides were distributed between 7 and 30 amino acids, which were consistent with the property of tryptic peptides (Fig. S2B). In total, 667 lysine acetylation sites in 467 proteins were identified (Tab. S2).

Comparative proteomic analysis of *Catalpa bungei*, *Fragaria ananassa*, *Oryza sativa* and *Picea asperata* was analysed to investigate the homology and specificity of acetylated proteins (Fig. 1E). We identified 266, 238, and 252 overlapped proteins in *C. bungei vs. F. ananassa*, *C. bungei vs. O. sativa*, and *C. bungei vs. P. asperata*, respectively.

3.2 Distribution Analysis of Lysine Acetylation Sites

To elucidate the distribution of acetylation sites in the identified proteins, we calculated the degree of lysine acetylation. A survey on the 667 acetylation sites in 467 proteins demonstrated that the average number of acetylation was 1.4 sites per protein with the level ranging from 1 to 8 (Fig. 2A). Among these acetylated proteins, 75% contained only one acetylation site; the remaining 25% were modified on multiple lysine residues.

To explore the relationship between lysine acetylation and protein structure, we performed local secondary structural analysis of all identified proteins. The results demonstrated that 56% of the acetylation sites were detected in regions with ordered secondary structures of proteins (Fig. 2B). Among them, 45% were distributed in alpha-helix and 11% were located in beta-strand; the remaining 44% of the acetylation sites were located at regions with the disordered structures.

3.3 Functional Characterization of Lysine Acetylated Proteins

To acquire a better understanding of the acetylated proteins identified in the flower buds, GO classification analysis was performed. The biological process analysis indicated that the acetylated proteins were involved in metabolic, cellular, and single-organism processes, accounting for 37%, 29% and 23% of all the identified acetylated proteins, respectively (Fig. 3A). For the molecular functions ontology, acetylated proteins were mainly associated with catalytic activity, binding, and

structural-molecule activity (Fig. 3B). Consistently, in the cellular components classification, the acetylated proteins mainly focused on cell (39%), macromolecular complex (25%), organelle membrane (24%), and membrane (11%) (Fig. 3C). The GO classification analysis revealed that the proteins mainly involved in various metabolic processes and catalytic activities could be acetylated at lysine sites.

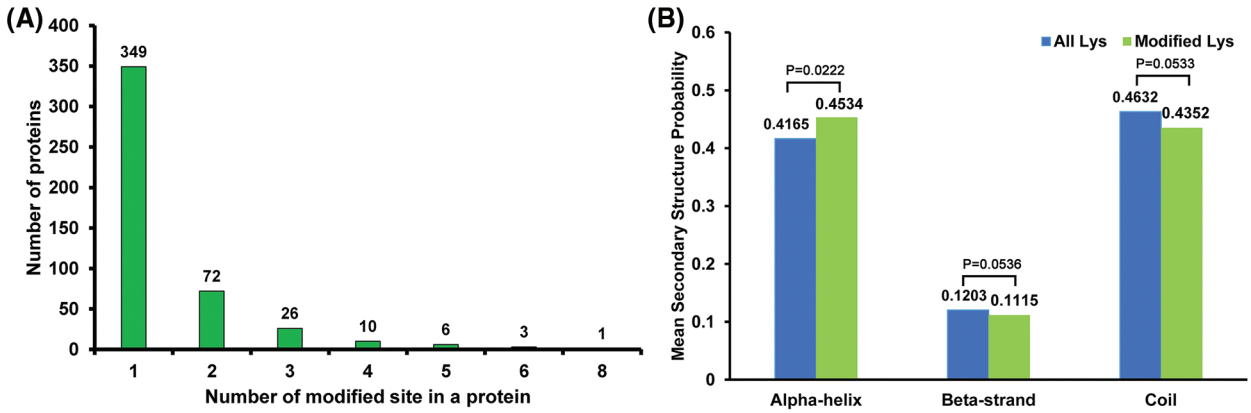

Figure 2: Properties of acetylated sites. (A) acetylated site distribution in the acetylated proteins. (B) probabilities of localization on different protein secondary structures (alpha helix, beta strand and coil)

Subcellular localization of the acetylated proteins was performed, and the results showed that a larger proportion of identified acetylated proteins were distributed in the cytosol (38%), indicating that acetylated proteins play important roles in this compartment in flower buds (Fig. 3D). Meanwhile, a number of acetylated proteins were also localized in the chloroplast (30%), nucleus (14%) and mitochondria (7%). Furthermore, some proteins were distributed in other components such as the plasma membrane (4%), cytoskeleton (2%) and endoplasmic reticulum (2%).

To reveal the preferential functional terms of lysine acetylation, the enrichment analysis of all the acetylated proteins was performed. The top significantly enriched GO terms and KEGG pathways were shown in Fig. 4. In the ontology of biological process, many metabolic and biosynthetic processes had a higher tendency to be acetylated (Fig. S3). Consistently, proteins, which were involved in structural molecule activity, structural constituent of ribosome, oxidoreductase activity and cofactor binding, were more likely to be acetylated based on the molecular function analysis. The cellular components of the proteins were mainly classified into cytoplasm, cell, cell part, and intracellular part. Meanwhile, we found that carbon metabolism, carbon fixation in photosynthetic organisms, glycolysis/gluconeogenesis, citrate cycle, pentose phosphate pathway, pyruvate metabolism, fructose and mannose metabolism, and oxidative phosphorylation pathways related terms were significantly enriched on the basis of KEGG pathway enrichment analysis (Fig. 4).

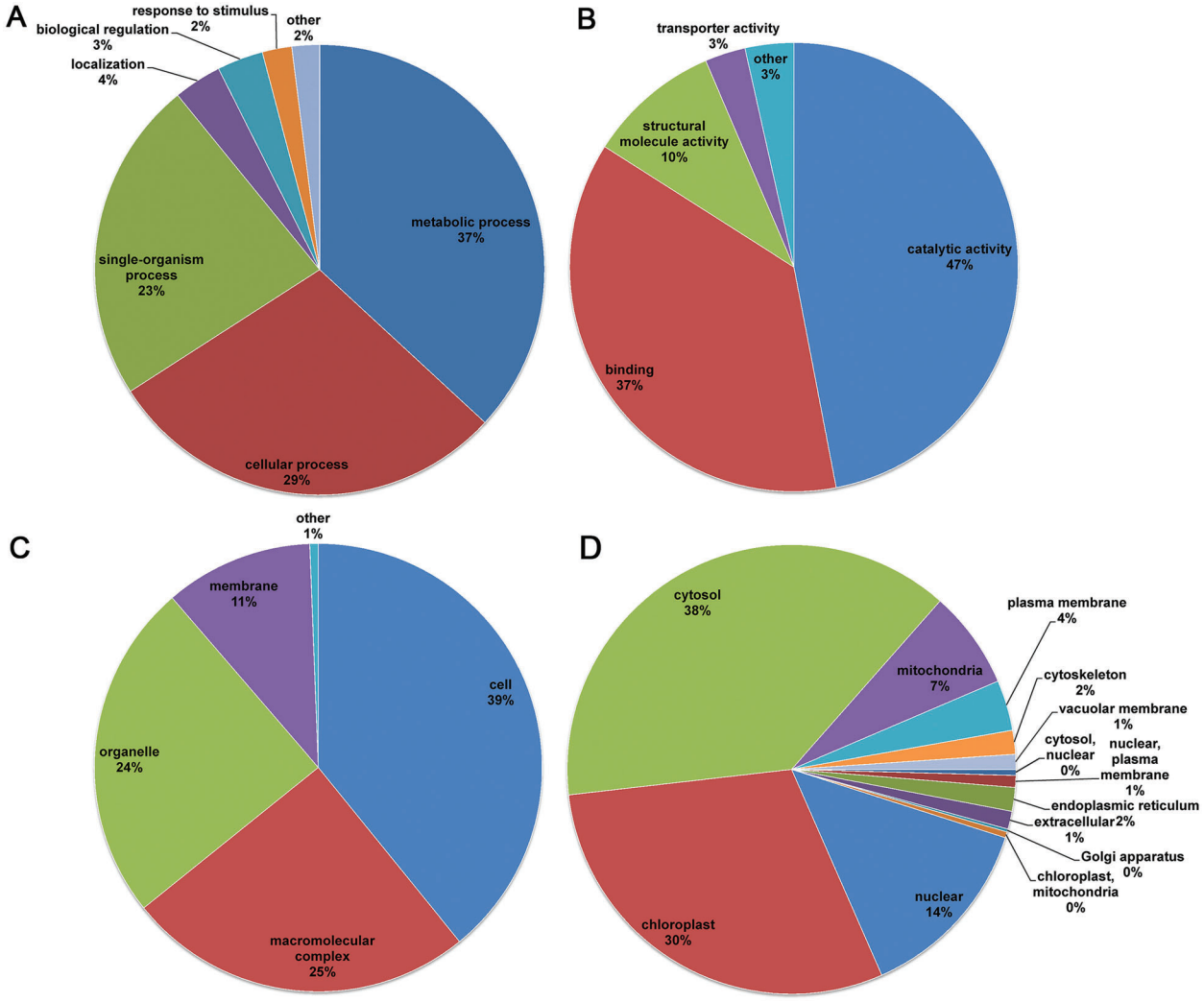

Figure 3: Distribution of the acetylated proteins in the *C. bungei* flower. (A) Biological process classifications. (B) Molecular function classification. (C) Cellular component classification. (D) Subcellular location predictions

3.4 Clustering Analysis of Functional Enrichment

To investigate the preferential target substrates of lysine acetylation, we conducted functional enrichment on the basis of clustering analysis of subcellular locations. In KEGG pathway enrichment, the acetylated proteins were mainly focused on the pentose phosphate pathway, photosynthesis, and the fructose and mannose metabolisms in the chloroplast. The proteins were mainly classified into aminoacyl-tRNA biosynthesis in the cytosol. The proteins were significantly enriched in the citrate cycle, oxidative phosphorylation, and carbon metablism in the mitochondria (Fig. S4). Meanwhile, on the basis of biological process category of GO enrichment analysis, the proteins were mainly classified into photosynthesis in the chloroplast. The main proteins were involved in gene expression, macromolecule biosynthetic process, and nucleic acid metabolic process in the cytosol. The proteins were mainly enriched in generation of precursor metabolites and energy, electron transport chain, and oxidation-reduction processes in the mitochondria (Fig. S5). The KEGG pathway enrichment of acetylated proteins corresponded well to the biological process of GO enrichment analysis.

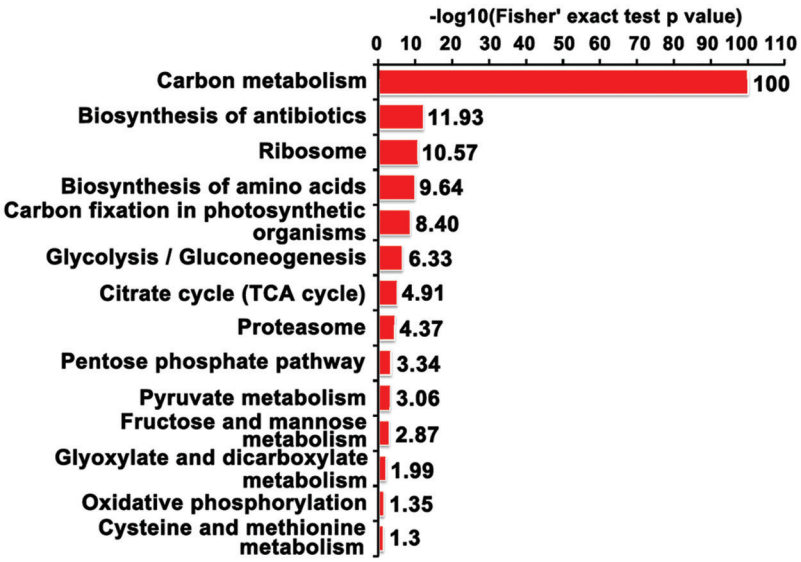

Figure 4: KEGG pathways of acetylated proteins

Accordingly, the cellular components of GO enrichment analysis were involved in photosystem I, photosystem, and photosynthetic membrane in the chloroplast. The main cellular components were classified into ribosome in the cytosol. The respiratory chain, oxidoreductase complex, and respiratory chain complex III were also significantly enriched in the cellular component category in the mitochondria (Fig. S6). Furthermore, on the basis of the molecular function category of GO enrichment analysis, iron ion binding, organic acid binding, and pyridoxal phosphate binding were mainly enriched in the chloroplast. The RNA bingding and aminoacyl-tRNA ligase activity were significantly enriched in the cytosol. The transmembrane transporter activity, oxidoreductase activity, antioxidant activity and ion channel activity were mainly enriched in the mitochondria (Fig. S7). Consistently, the major domains of the pyridoxal phosphate-dependent transferase, chlorophyll a/b binding protein, translation protein, ribosomal protein, aminoacyl-tRNA synthetase and ATPase were significantly enriched based on the classification of protein domain (Fig. S8).

3.5 Acetylated Proteins Involved in the Glycometabolism and Oxidative Phosphorylation Pathways

The pathway enrichment analysis of acetylated proteins revealed that lysine acetylation may play important roles in the energy-metabolism pathway. Consistent with this hypothesis, we found that 61 and 13 acetylated proteins were associated with the glycometabolism and oxidative phosphorylation pathways (Tab. S2), respectively. As shown in Fig. 5a wide array of enzymes in glycometabolism were detected as being acetylated.

In higher plants, the oxidation of carbohydrates involves enzymatic conversion to glucose-6-P, which is further metabolized via the oxidative pentose phosphate or glycolysis pathway [30,31]. In the pentose phosphate pathway, various enzymes were shown to be acetylated. They included the glucose-6-phosphate isomerase (GPI), fructose-1,6-bisphosphatase I (FBP), pyrophosphate-fructose-6-phosphate (pfp), fructose-bisphosphate aldolase (ALDO), transaldolase (talA/B), 6-phosphogluconolactonase (PGLS), 6-phosphogluconate dehydrogenase (PGD), ribulose-phosphate 3-epimerase (RPE), and ribose 5-phosphate isomerase A (rpiA). In addition to the enzymes in the pentose phosphate pathway, many enzymes involved in glycolysis were identified to be acetylated.

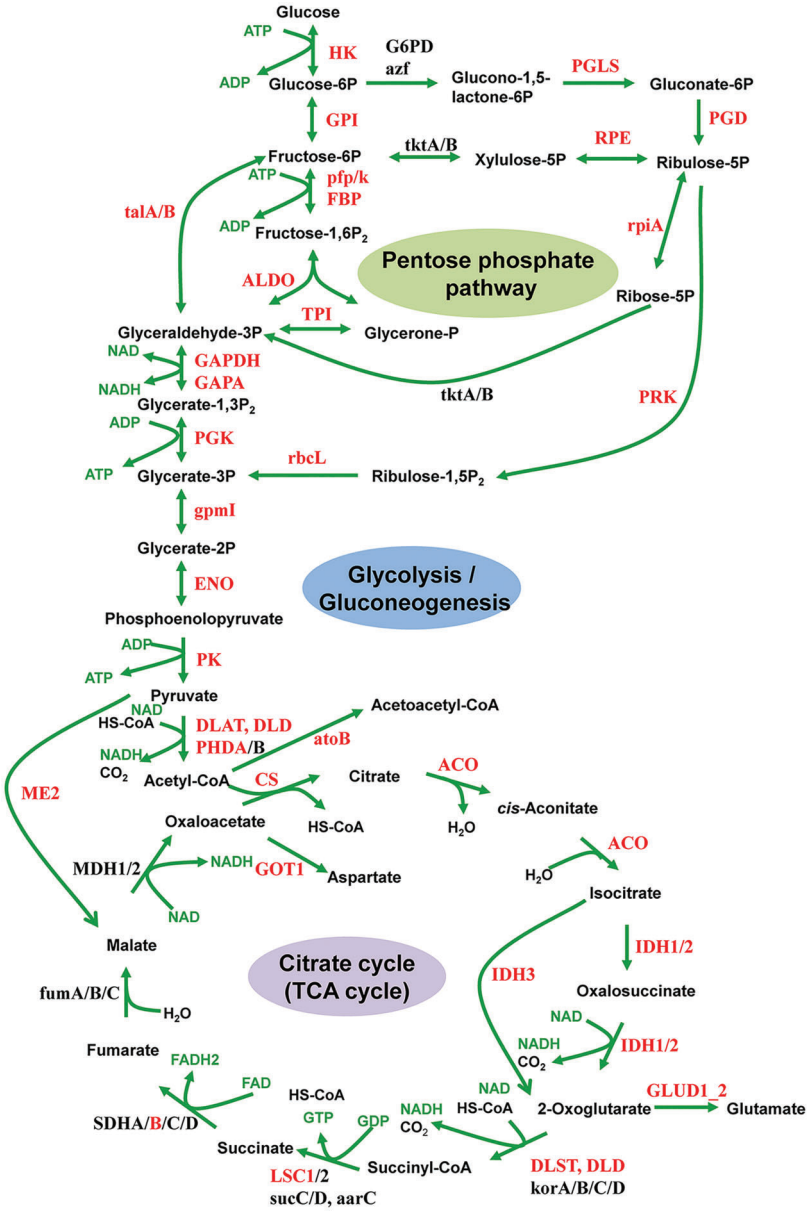

Figure 5: Representative significantly enriched glycometabolism pathways of the acetylated proteins. The characters in red show acetylated enzymes

The citrate cycle, a series of chemical reactions to generate energy, provides precursors of certain amino acids and the reducing agent NADH that are needed in many important biochemical reactions [32]. In this study, the proteins involved in the citrate cycle, such as the aconitate hydratase (ACO), isocitrate dehydrogenase (IDH), dihydrolipoamide dehydrogenase (DLD), dihydrolipoamide succinyltransferase (DLST), succinyl-CoA synthetase (LSC), and succinate dehydrogenase (SDH), were identified to be acetylated in the flower buds.

The oxidative phosphorylation system is the process in which ATP is synthesized as a result of transfer of electrons from NADH and $FADH_2$ to oxygen [33]. Our data demonstrated that 33 of the acetylated proteins were localized in the mitochondria in the flower buds. Among them, 13 were involved in the

oxidative phosphorylation pathway, including the NADH dehydrogenase, succinate dehydrogenase, ubiquinol-cytochrome c reductase complex protein, cytochrome b-c1 complex, and ATPase (Tab. S3 and Fig. S9).

3.6 Interaction Network Analysis of Acetylated Proteins

To better understand the cross-linked pathways of these acetylated proteins, the protein-protein interaction networks were established. In the whole network, a total of 255 acetylated proteins were predicted to be involved in 18 clusters of protein-protein interaction networks, presenting functional diversity of acetylated proteins in the flower development (Tab. S4). We extracted the top three highly interconnected clusters (Fig. 6). The top clusters I, II and III comprised 43, 18 and 13 proteins were associated with ribosome, carbon metabolism and proteasome. Meanwhile, these carbon-metabolism associated proteins involved in the processes of pentose phosphate, glycolysis/gluconeogenesis, fructose and mannose metabolism, and carbon fixation (Table 1).

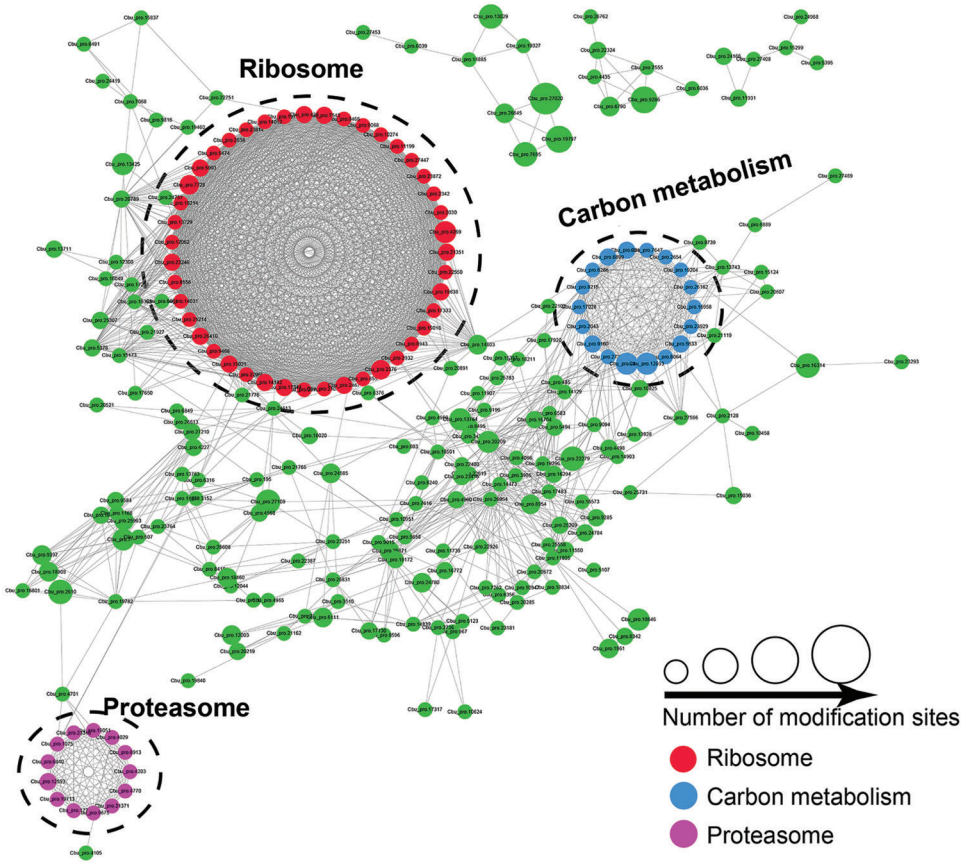

Figure 6: Protein-protein interaction network of lysine acetylated proteins

Table 1: Lysined acetylome involved in carbon metabolism

Protein accession	Protein name	Metabolic pathway
Cbu_pro.23929	Transaldolase	Pentose phosphate pathway
Cbu_pro.8064	Fructose-bisphosphate aldolase	Pentose phosphate pathway
Cbu_pro.24469	Fructose-bisphosphate aldolase	Pentose phosphate pathway
Cbu_pro.9160	Fructose-bisphosphate aldolase	Pentose phosphate pathway
Cbu_pro.17028	Pyrophosphate-fructose-6-phosphate	Pentose phosphate pathway
Cbu_pro.6266	Glucose-6-phosphate isomerase	Pentose phosphate pathway
Cbu_pro.9215	Pyruvate kinase	Pentose phosphate pathway
Cbu_pro.27340	Pyruvate kinase	Pentose phosphate pathway
Cbu_pro.5633	Glyceraldehyde 3-phosphate dehydrogenase	Glycolysis/Gluconeogenesis
Cbu_pro.7647	Glyceraldehyde 3-phosphate dehydrogenase	Glycolysis/Gluconeogenesis
Cbu_pro.12693	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis/Gluconeogenesis
Cbu_pro.9212	Enolase	Glycolysis/Gluconeogenesis
Cbu_pro.2043	Enolase	Glycolysis/Gluconeogenesis
Cbu_pro.19204	Phosphoglycerate kinase	Glycolysis/Gluconeogenesis
Cbu_pro.26182	Triosephosphate isomerase	Fructose and mannose metabolism
Cbu_pro.2654	Triosephosphate isomerase	Fructose and mannose metabolism
Cbu_pro.6899	Glyceraldehyde-3-phosphate dehydrogenase	Carbon fixation
Cbu_pro.16958	Glyceraldehyde-3-phosphate dehydrogenase	Carbon fixation

3.7 Transcript Level Changes in Important Lysine-Acetylated Proteins of the Energy Metabolism

We further analyzed transcript level changes of important lysine-acetylated proteins in *C. bungei* buds from early flowering (EF) and normal flowering (NF) varieties at vegetative buds (Vb), transition buds (Tb) and reproductive buds (Rb). We identified 66, 73 and 70 overlapped proteins/genes in EF-Vb *vs.* NF-Vb, EF-Tb *vs.* NF-Tb, and EF-Rb *vs.* NF-Rb (Tab. S5 and Fig. 7). The expression level changes of some lysine-acetylated proteins, such as the phosphoribulokinase, phosphoenolpyruvate carboxykinase, ATP synthase, ribulose bisphosphate carboxylase large chain, pyruvate dehydrogenase complex, H(+)-transporting ATP synthase and fructokinase-1, showed different expression trends in the EF and NF buds (Fig. 7). The expression levels of the phosphoribulokinase, phosphoenolpyruvate carboxykinase, ATP synthase and pyruvate dehydrogenase complex were up-regulated at EF-Vb and EF-Tb of early flowering *C. bungei* (Fig. 7D and Table 2).

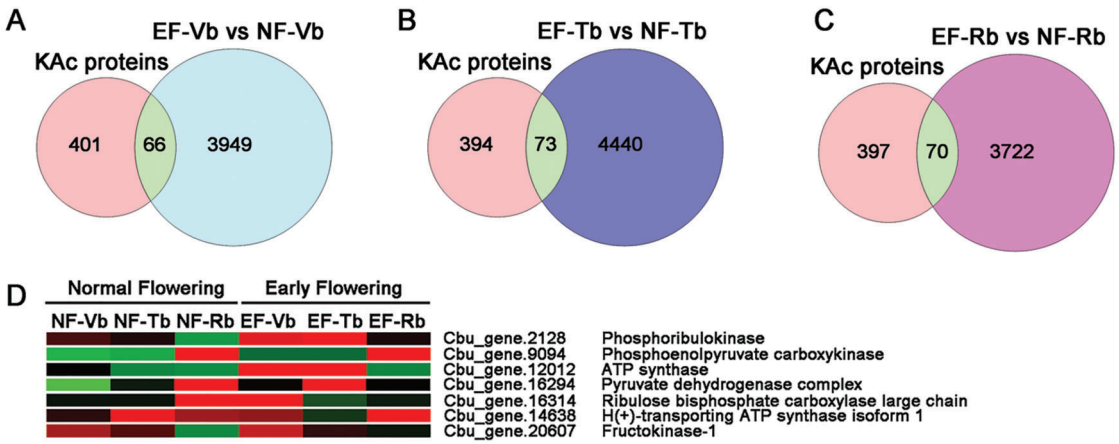

Figure 7: The overlapped proteins and expression levels of some lysine-acetylated proteins in *C. bungei* buds from early flowering (EF) and normal flowering (NF). (A) The overlapped proteins in EF-Vb *vs.* NF-Vb. (B) The overlapped proteins in EF-Tb *vs.* NF-Tb. (C) The overlapped proteins in EF-Rb *vs.* NF-Rb. (D) The expression levels of some lysine-acetylated proteins. KAc: lysine-acetylated proteins. Vb: vegetative buds. Tb: transition buds. Rb: reproductive buds

Table 2: Transcript level changes in important lysine-acetylated proteins involved in energy metabolism

Gene_ID	Annotation	Fold Change (EF-Vb/NF-Vb)	Fold Change (EF-Tb/NF-Tb)	Fold Change (EF-Rb/NF-Rb)
Cbu_gene.2128	Phosphoribulokinase	1.44	3.00	2.72
Cbu_gene.9094	Phosphoenolpyruvate carboxykinase	1.88	1.65	1.00
Cbu_gene.12012	ATP synthase	133.00	2.55	1.06
Cbu_gene.16294	Pyruvate dehydrogenase complex	13.00	12.13	12.33
Cbu_gene.16314	Ribulose bisphosphate carboxylase large chain	1.16	0.73	0.84
Cbu_gene.14638	H(+)-transporting ATP synthase	1.05	0.69	1.13
Cbu_gene.20607	Fructokinase-1	1.11	0.83	1.99

4 Discussion

4.1 Global Proteomic Analysis of Lysine-Acetylated Proteins in C. bungei Flower Buds

Lysine acetylation is emerging as a dynamic, reversible and highly conserved post-translation modification in both eukaryotes and prokaryotes with multiple biological functions [34]. However, the lysine acetylome has been reported only in leaves [17,18], seeds [20,35] and seedlings [16] of a limited number of plants. In this study, we determined the lysine acetylome of *C. bungei* flower buds. Compared to lysine acetylome in leaves of *F. ananassa*, seeds of *O. sativa* and somatic embryos of *P. asperata*, the acetylome of the *C. bungei* flower has a large quantity of unique acetylated proteins, revealing an indispensable role of lysine acetylation in flower development (Fig. 1C). Meanwhile, the acetylated proteins were mainly associated with carbon metabolism, glycometabolism and oxidative phosphorylation pathways. The protein–protein interactions were also highly enriched in the process of carbon metabolism to confirm this hypothesis. Meanwhile, transcript level changes of some lysine-acetylated proteins

indicated that these proteins were associated with floral transition. These results reveal that the lysine acetylated proteins play important roles in many regulatory pathways during flower development.

4.2 Lysine Acetylated Proteins Involved in Glycometabolism

In plants, glycometabolism is one of the most important metabolic processes [36,37]. Lysine acetylation of glycometabolism related proteins plays important roles in plant life activities such as plant development and growth [18,20]. In our work, 212 acetylated proteins were localized in the cytosol and the mitochondria, and many of them were involved in glycometabolism including glycolysis/gluconeogenesis, citrate cycle, pentose phosphate pathway, pyruvate metabolism, and fructose and mannose metabolisms. Similarly, acetylated proteins of three glycometabolism related pathways, which is citrate cycle, glycolysis/gluconeogenesis, and pyruvate metabolism, were significantly enriched in strawberry leaves [18]. It was also reported that the enzymes of the citrate cycle were identified to contain lysine acetylation sites in pea seedlings [16]. Meanwhile, Zhang et al. [17] found that the acetylated proteins were involved in the pentose phosphate pathway and glycolysis/gluconeogenesis in common wheat leaves .

4.3 Lysine Acetylated Proteins Involved in Oxidative Phosphorylation

As the primary source of metabolic energy, oxidative phosphorylation is a highly efficient way to form ATP using energy derived from the transfer of electron donors to electron acceptors, and takes place inside the mitochondria in higher plants [38]. Notably, 13 acetylated proteins were significantly enriched in the oxidative phosphorylation pathway in our study. Similarly, several researches have shown that the acetylated proteins of the oxidative phosphorylation pathway are significantly enriched in pea seedlings [16], rice embryos [20], and HL60 cells [39]. These studies may facilitate the revealing of the mechanism of the lysine acetylation regulated oxidative phosphorylation pathway in both plants and human beings.

4.4 Transcript Level Changes of the Acetylated Proteins Involved in Energy Metabolism

The protein-protein interaction networks of the acetylated proteins that were mainly enriched in the pathways of the ribosome and carbon metabolism, might be associated with protein synthesis and energy metabolism. Previously, the ribosome is associated with the efficiency of protein synthesis at the codon level in plants [40]. At the floral anthesis stage of *Eriobotrya japonica*, the ribosome pathway is significantly enriched [41]. Similarly, carbon metabolism is significantly enriched at the flowering stage in *Lonicera japonica* [42].

The expression level of some lysine-acetylated proteins, which are involved in energy metabolism such as phosphoribulokinase, phosphoenolpyruvate carboxykinase, ATP synthase, ribulose bisphosphate carboxylase large chain, pyruvate dehydrogenase complex, H(+)-transporting ATP synthase and fructokinase-1, were significantly differentially expressed in the flower buds. Previously, as an important photosynthetic enzyme, phosphoribulokinase is involved in the carbon and energy metabolism by reversible oxidation/reduction [43]. Phosphoenolpyruvate carboxykinase catalyzes the conversion of some amino acids and lipids to sugars by gluconeogenesis, and its activity was significantly higher in the floral buds than that in the leaves in plants [44]. Meanwhile, ATP synthase and ribulose bisphosphate carboxylase large chain and pyruvate dehydrogenase complex are the key and important factors in energy conversion, and thus, they are important for plant growth [45–48]. Furthermore, fructokinase plays key roles in the carbohydrate metabolism by catalyzing fructose phosphorylation, and fructokinase-1 participates in regulating flower development [49,50]. Taken together, the expression level analysis suggests that the lysine acetylation on energy metabolism-related proteins plays important roles in flower bud development.

5 Conclusion

In the study, we provided the most extensive lysine acetylome in *C. bungei* flower buds to date. These results reveal that, in flower buds, lysine acetylation might play critical roles in energy metabolism including glycometabolism and oxidative phosphorylation pathway. These data not only expand our knowledge of the physiological role of lysine acetylation, but also provide bioinformatic resources for the functional analysis of lysine acetylation in regulating flower development on *C. bungei* and in all plants.

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