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Phytoplasma-induced changes in the acetylome and succinylome of *Paulownia tomentosa* provide evidence for involvement of acetylated proteins in witches’ broom disease

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Abbreviations

PTM: post-translational modification. GO: gene ontology. KEGG: kyoto Encyclopedia of Genes and genomes. PaWB: paulownia witches’ broom. RuBisCO: ribulose bisphosphate carboxylase oxygenase. POR: protochlorophyllide reductase. HS: healthy seedlings. PIS: phytoplasma-infected seedlings. HS-60: 60 mg·L$^{-1}$ methyl methanesulfonate-treated healthy P. tomentosa seedlings. PIS-60: 60 mg·L$^{-1}$ methyl methanesulfonate-treated phytoplasma-infected seedlings. PVDF: polyvinylidene fluoride fluoropolymer. FDR: false discovery rate. LB: luria-bertani. Pn: net photosynthesis rate. CE: carboxylation efficiency. DEPs: differentially expressed proteins. H: histidine. R: arginine. K: lysine. E: glutamic acid. D: aspartic acid. Y: tyrosine. S: serine. T: threonine. N: asparagine. L: leucine. P: proline. ROS: reactive oxygen species. PPI: protein-protein interaction

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Summary

Lysine acetylation and succinylation are post-translational modifications of proteins that have been shown to play roles in plants response to pathogen infection. Phytoplasma infection can directly alter multiple metabolic processes in the deciduous plant Paulownia and lead to Paulownia witches’ broom (PaWB) disease, the major cause of Paulownia mortality worldwide. However, the extent and function of lysine acetylation and succinylation during phytoplasma infection have yet to be explored. Here, we investigated the changes in the proteome, acetylome, and succinylome of phytoplasma-infected Paulownia tomentosa seedlings using quantitative mass spectrometry. In total, we identified 8963 proteins, 2893 acetylated proteins (5558 acetylation sites), and 1271 succinylated proteins (1970 succinylation sites), with 425 (533 sites) simultaneously acetylated and succinylated. Comparative analysis revealed that 276 proteins, 546 acetylated proteins (741 acetylation sites) and 5 succinylated proteins (5 succinylation sites) were regulated in response to phytoplasma infection, suggesting that acetylation may be more important than succinylation in PaWB. Enzymatic assays showed that acetylation of specific sites in protochlorophyllide reductase and RuBisCO, key enzymes in chlorophyll and starch biosynthesis, respectively, modifies their activity in phytoplasma-infected seedlings. On the basis of these results, we propose a model to elucidate the molecular mechanism of responses to PaWB and offer a resource for functional studies on the effects of acetylation on protein function.
Introduction

Paulownia is a deciduous tree species native to China, where it has been cultivated for more than 2000 years. The tree has large and dense leaves and beautiful flowers, and is prized for its ornamental value and its ability to improve air quality. Paulownia has straight-grained timber and high ignition point, making it ideal for manufacturing furniture, artificial board, and musical instrument (1). However, Paulownia witches’ broom (PaWB), caused by phytoplasma invasion, is the most widespread infectious disease of Paulownia. Paulownia infected with phytoplasma shows stunted growth and various malformations, including dwarfism, bushy shoots (hence, “witches’ broom”), short internodes, and yellow leaves (2). Thus, PaWB seriously affects the yield of the Paulownia and leads to large economic losses.

Phytoplasmas are specialized obligate bacteria of plants in the class *Mollicutes*, which lack cell walls and exhibit highly diverse morphology. Generally, they primarily inhabit in nutrient-rich phloem cells in plants and transmit by leafhoppers, psyllids, and plant hoppers (2). Till now, more than 1000 plant species worldwide are known to be infected with phytoplasma, which cause great economic losses to agriculture, forestry, and horticultural crop production. Due to the complexity of phytoplasma, only six phytoplasma genomes have been completely assembled, and nine phytoplasma draft genomes have been obtained (3,4). Since the genomic information is not available for the phytoplasma that infects Paulownia, little is known about this host-pathogen interaction. With the rapid development of omics-based approaches, an increasing body of evidence showed that the metabolic pathways of Paulownia can be regulated at transcriptional, translational, and metabolic levels (5-9), enabling it to survive during phytoplasma invasion. Nevertheless, researches on the role of post-translational modification (PTM) of proteins in phytoplasma-infected Paulownia are limit. PTM can reveal how
genes are regulated, and how the stability and activity of proteins changes in plants under stresses. A recent study reported that lysine acetylation proteins in fungus infected maize suppressed the defense response of the host (10), suggesting that PTM of proteins in plants are an important aspect of the plants response to biotic stress.

PTMs are dynamic and reversible protein processing events that affect the proteins stability, cell signaling transduction, gene expression, and enzymatic activity (11). To date, over 450 different PTMs have been identified, including acetylation, succinylation, ubiquitination, SUMOylation, and crotonylation (12). Among these PTMs, lysine acetylation, a reversible, covalent, and highly dynamic PTM, is one of the most extensively studied PTM. Early investigations on lysine acetylation are mainly focused on histone, transcription factor and other regulators. Hence, the best known function of lysine acetylation is regulate the expression of genes. However, recent breakthroughs in identification of acetylated proteins have uncovered that the substrates of lysine acetylation are not limited to histone, especially intensive studies of acetylation modification were performed by Wang (13) and Zhao et al. (14), which revealed multiple lysine acetylation sites on non-histone and metabolic enzymes. This finding not only verified the significant roles of non-histone acetylation in protein function, but greatly expanded our understanding on the functions of this PTM. Till now, non-histone acetylation has been detected in several organisms, including microorganisms (15, 16), plants (10,17-20) and humans (14, 21), and is known to regulate many cellular processes, containing cell morphology (22), protein stability (23), energy metabolism (24), enzymatic activity (25-27), and cell apoptosis (28). Thus, lysine acetylation is a major PTM that is widespread and has diverse biological functions.

Succinylation is another conserved modification of lysine residues, in which succinyl-CoA is transferred to a specific lysine residue (29). Usually, succinylation of proteins result in changes of
charge and/or protein structural, which in turn leads to substantial changes in the chemical properties of the target proteins. Consequently, this type of PTM would likely alter protein function. To date, systemic identification of lysine succinylation has been conducted in plants, mammals and bacteria (12, 30, 31), while the study of lysine succinylation in woody plants has lagged behind, to our knowledge. Moreover, lysine succinylation can confer distinctive advantages in response to changes in the growth environment (31). Thus, research on lysine succinylation may provide clues about a new regulatory mechanism during PaWB occurring.

Phytoplasma infection leads to a series of physiological and biochemical changes in Paulownia, such as imbalanced hormone levels and a decreased photosynthetic rate (7, 32, 33). To well understand the impacts of phytoplasma on these results production, it is important to explore the details of Paulownia metabolic process under phytoplasma stress. In previous studies, lower chlorophyll content and accumulated starch content were considered as the main factors related to the decreased photosynthetic rate (34). RuBisCO is a key enzyme in starch biosynthesis and lysine acetylation and succinylation have been found widely existed in RuBisCO (30). In addition, it has been demonstrated that lysine acetylation can change the activity of RuBisCO (35). Thus, we speculated that lysine acetylation may be a main reason for this phenomenon emergence in phytoplasma-infected seedlings. However, no lysine acetylation data are available after phytoplasma invasion, which is a huge barrier for further understanding the functions of acetylated proteins in PaWB. In this study, therefore, we conducted lysine acetylation and succinylation proteome profiling of Paulownia tomentosa seedlings infected with phytoplasma to reveal the central roles of these PTMs in the occurrence of PaWB.

Experimental Procedures

Plant materials
The tissue-cultured plant materials used in this study were obtained from the Institute of Paulownia (Henan Agricultural University, Henan, China). Four samples include healthy *P. tomentosa* seedlings (HS), phytoplasma-infected seedlings (PIS), 60 mg·L\(^{-1}\) methyl methanesulfonate-treated healthy *P. tomentosa* seedlings (HS-60) and 60 mg·L\(^{-1}\) methyl methanesulfonate-treated phytoplasma-infected seedlings (PIS-60) were collected and prepared as described previously (36) with three biological replicates. Tissues from the four samples were immediately frozen and stored at \(-80^\circ\text{C}\) until analysis.

**Protein extraction and western blot**

Samples were ground into a fine powder in liquid nitrogen and then transferred to 5mL centrifuge tubes containing four volumes of lysis buffer (8 M urea, 1% Triton-100, 10 mM dithiothreitol, and 1% Protease Inhibitor Cocktail). Protein extracts were sonicated three times on ice using a high intensity ultrasonic processor (Scientz) and the remaining debris were centrifuged at 20,000 g at 4 °C for 10 min to obtain the soluble protein fraction. Soluble proteins were precipitated with 20% cold trifluoroacetic acid for 2 h at \(-20^\circ\text{C}\). After centrifugation at 12,000 g at 4 °C for 10 min, the supernatant was discarded. The remaining protein precipitates was washed three times with cold acetone and then redissolved in 8 M urea. The protein concentration was determined with BCA kit according to the manufacturer’s instructions.

Proteins from each sample were separated on 12% SDS-PAGE gels and transferred to a polyvinylidene fluoride fluoropolymer (PVDF) membrane (0.45 m, Millipore, Darmstadt, Germany) using Trans-Blot Turbo transfer system (Bio-Rad, California, CA, USA), and then blocked with TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, pH 8.0) containing 5% BSA overnight at 4 °C. The primary antibodies (1:1000 dilution, PTM Biolabs, Hangzhou, China) used were commercially available antibodies against acetylated-lysine residues or succinylated-lysine residues.
Trypsin digestion, TMT labeling and HPLC fractionation

For digestion, the protein solution was reduced with 5 mM dithiothreitol for 30 min at 56 °C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The trypsin digestion was performed as described by Zhou et al (30). Digested peptides from 3 mg protein of each sample were labeled by TMT regent and then mixed for HPLC fractionation and enrichment. TMT labeling was processed according to the manufacturers’ protocol for six-plex of TMT kit. The labeling information for each sample was list in supplemental Table S1.

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 min into 60 fractions. Then, the peptides were combined into 6 fractions and dried by vacuum centrifuging.

Affinity enrichment of lysine acetylated and succinylated peptides

To enrich lysine-acetylated and lysine-succinylated peptides, tryptic peptides were dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0) and then incubated with pre-washed antibody beads (100 μl anti-succinyllysine: PTM-402 and 200 μl anti-acetyllysine: PTM-104, PTM Biolabs, Hangzhou, China) at 4°C overnight with gentle shaking. The detail methods were according to Fang et al (37) and Zhou et al (30). For the LC-MS/MS analysis, the resulting peptides were desalted with C18 ZipTips (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions.

LC-MS/MS analysis and database search

The tryptic peptides were dissolved in 0.1% formic acid (solvent A), and directly loaded onto a reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific), and separated on a
reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Fisher Scientific) using an EASY-nLC 1000 UPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The solvent B (0.1% formic acid in 98% acetonitrile) was comprised of an increase gradient following: 6% to 23% over 26 min, 23% to 35% in 8 min and climbing to 80% in 3 min, then holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system. All peptides were analyzed using a Orbitrap Fusion™ (Thermo Fisher Scientific) coupled with an ultra performance liquid chromatography. The mass window for precursor ion selection was 2.0 m/z. An Orbitrap with a resolution setting at 60,000 was used to detect intact peptides and NCE set as 35 was used for MS/MS analysis. Ion fragments were detected in the Orbitrap at a resolution of 15,000. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold intensity greater than 5E3 in the MS survey scan with 15.0 s dynamic exclusion. The electrospray voltage was 2.0 kV. Automatic gain control was used to prevent overfilling of the Orbitrap; 5E4 ions were accumulated to generate MS/MS spectra. The m/z scan range was 350 to 1550. The fixed first mass was set as 100 m/z.

The resulting MS/MS data were processed using the Maxquant search engine (v.1.5.2.8) and tandem mass spectra were searched against the database of *P. tomentosa* transcriptome (a total of 31105 sequences combined from the healthy seedlings, phytoplasma-infected seedlings, 60 mg·L⁻¹ methyl methanesulfonate-treated healthy *P. tomentosa* seedlings and 60 mg·L⁻¹ methyl methanesulfonate-treated phytoplasma-infected seedlings) and the reverse decoy database. Specific trypsin/p was specified as the cleavage enzyme with no more than four missing cleavages permitted. The mass tolerance for precursor ions was set to 20 ppm in First search and 5 ppm in Main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carboxymethyl on cysteine was specified as
a fixed modification and acetylation or succinylation of lysine and oxidation of methionine were specified as variable modifications. False discovery rate (FDR) was adjusted to < 1% and the minimum score for modified peptides was set to 40. Minimum peptide length and site localization probability were set to 6 and ≥ 0.75, respectively. The other parameters in the MaxQuant analysis were set to default values.

**Bioinformatics analysis**

Annotation of proteins and modified proteins based on the Gene ontology (GO) annotation were derived according to the methods described by Zhou et al (30). Identified proteins domain functional descriptions were annotated by InterPro domain database (http://www.ebi.ac.uk/interpro/) (38). KEGG pathway was annotated using the KEGG Automatic Annotation Server, and the annotation results were mapped by KEGG Mapper. Subcellular localization predication was performed according to Horton et al (39). Amino acid sequence motifs were detected using motif-X (http://motif-x.med.harvard.edu/). All the database protein sequences obtained from the *P. tomentosa* transcriptome dataset (31105 entries) were used as a background database parameter and other parameters were set to default values. Conservation of modified proteins across different species was determined using BLASTP (40). Secondary structures of protein surrounding the modified lysine residue were determined by using NetSurfP. Conserved domain analysis was conducted in NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The interaction network analysis was performed using STRING. First, all protein name identifiers of differential expression of succinylation, acetylation and proteome were blast to *Vitis vinifera* species. Then searched against the STRING database version 10.5 for protein-protein interactions (41). Only interactions between the proteins belonging to the searched dataset were selected (confidence score ≥ 0.9).
Analysis of differentially expressed modified and no-modified proteins

For TMT quantification, the ratios of the TMT reporter ion intensities in MS/MS spectra (m/z) from raw data sets were used to calculate fold changes between samples. For each sample, the quantification was mean-normalized at peptide level to center the distribution of quantitative values. Protein quantitation was then calculated as the median ratio of corresponding unique or razor peptides for a given protein. Two-sample, two-sided t-tests were used to compare expression of proteins. In general, a significance level of $P \leq 0.05$ was used for statistical testing. The differentially expressed modified and no-modified proteins were defined as fold-change $\geq 1.50$ or $\leq 0.67$, and $P < 0.05$.

Site-directed mutagenesis

Site-directed mutagenesis of K339 and K78 in protochlorophyllide reductase (POR), K55 and K764 in two RuBisCO large subunit-binding protein subunits alpha were conducted using the method of whole gene synthesis. Mutants were selected by DNA sequencing.

Expression and purification of recombinant POR and RuBisCO

To generate homogenously acetylated proteins, a three-plasmid based system as Chen et al. (18) described was used. Constructs containing wild-type and mutated genes encoding POR and RuBisCO were transformed into *Escherichia coli* BL21 (DE3) cells. The cells were cultured in Luria-Bertani (LB) medium containing ampicillin, spectinomycin and kanamycin for expression. Plasmid construction, western blotting, and recombinant enzymes expression and purification were performed according to the previous study (18).

Enzyme assay

RuBisCO and POR activity were measured as previous studies described (35, 42)

Starch and pigment content analysis
Leaf samples from HS and PIS were immediately frozen and dried. Dried samples (50 mg) were ground into a fine power and boiled 2 min in 80% (v/v) ethanol for extraction. The extracts from the two samples were vortex oscillated for 20 min and centrifuged 5 min, respectively, and then re-extracted twice in the same way. Starch content was measured as Stitt et al. (43) previously described. Chlorophyll a and chlorophyll b levels were determined as described by Tan et al. (44).

**Measurement of photosynthetic activity**

The net photosynthesis rate (Pn) and carboxylation efficiency (CE) of HS and PIS samples was assessed under natural conditions as described previously (34).

**Experimental Design and Statistical Rationale**

The proteome, lysine acetylome and lysine succinylome analysis was performed by analyzing four samples with three biological preparation. Acetylated and succinylated peptides from four samples were fractionated and enriched using high efficiency immune-affinity enrichment strategies and analyzed with high-accuracy nanoflow HPLC-MS/MS. FDR thresholds for proteins, peptides, and modification sites were specified at maximum 0.01. GO enrichment analysis was conducted using Hypergeometric test (P<0.05) as previous study described (30). Two-sample, two-sided t-tests were used to compare expression of proteins. In general, a significance level of P ≤ 0.05 was used for statistical testing. Motifs analysis of acetylated and succinylated were analyzed by motif-x (http://motif-x.med.harvard.edu/) with a significance level of 0.000001. Conservation analysis were performed by BLASTP (40). Secondary structures were predicted using NetSurfP.

In the functional study, sequence- and lys-acetylation antibodies were prepared to confirm acetylated K339 and K78 in protochlorophyllide reductase (POR), K55 and K764 in two RuBisCO large subunit-binding protein subunits alpha. To determine the effect of lysine acetylation on these two
enzymes, we investigated the effect on these two enzyme activity. Each biological sample was prepared and analyzed with three replicates: WT, site directed mutagenesis of K78 and K339 of POR, and K55 and K764 in two RuBisCO large subunit-binding protein subunits alpha. The mutagenesis included a nonacetylated state K78R, K339R, K55R and K764R, and a mimic-acetylated state K78Q, K339Q, K55Q and K764Q. To generate homogenously acetylated proteins, a three-plasmid based system as Chen et al. (18) described was used. WT, site directed mutagenesis of K78 and K339 of POR, and K55 and K764 in two RuBisCO large subunit-binding protein subunits alpha recombinant proteins were purified and used for enzyme activity assays. The means and standard deviation of the three independent experiments were provided and the statistical tests were used to analyze the data.

Results

Phytoplasma infection triggered starch accumulation and decreased photosynthetic activity and pigment contents

Seedlings with phytoplasma infection exhibited a series of visible symptoms including yellowing leaves and witches’ broom as previous study described (36). Compared with uninfected seedlings, the PIS had a higher starch content (Fig. 1a), lower content of pigments (Fig. 1b). Photosynthetic activity was also lower in PIS than in HS (Fig. 1c).

Basic information on quantitative proteome

To explore the response of Paulownia to phytoplasma infection from a proteomic perspective, we conducted a comprehensive proteome analysis that included TMT labeling and quantitative proteomics to detect proteins showing differences in abundance among four Paulownia samples. Biological replicates were validated by Pearson’s correlation coefficients, which demonstrated the high repeatability of our data (Fig. 2a). After a strict quality control, we gained many peptides. The
distribution of mass errors was close to zero with most distributed within 0.02Da (Fig. 2b), indicating the data obtained by MS were satisfactory for the following analyses. Further analysis found that most peptides were in the size range from 7 to 20 amino acids (Fig. 2c), consistent with the characteristics of tryptic peptides, indicating that the results were reliable. Finally, by a series of bioinformatics analyses, 8963 proteins were identified, of which 7826 were quantified.

To elucidate the function and distribution of the quantified proteins in Paulownia response to phytoplasma infection, GO classification, protein domain, protein annotation, and subcellular localization analyses were performed. The results showed that most proteins located in the chloroplast, and had AAA+ ATPase and ABC transport-like domains. The result of the GO analysis showed that proteins mainly involved in DNA binding, structural constituent of ribosome, membrane, ribosome and transport processes. Detail information was shown in Supplemental Table S2.

**Phytoplasma infection alters protein expression levels in Paulownia**

Phytoplasma infection changed the whole proteome profile of Paulownia. In this study, differentially expressed proteins (DEPs) (fold-change ≥1.50 or ≤0.67, P <0.05) in different comparisons were listed in Table 1. According to the comparison scheme as Fan et al. (36) described, 276 proteins were related to the occurrence of PaWB. Among them, 141 proteins were up-regulated in PIS compared in HS, and 135 DEPs were down-regulated. Moreover, Eight proteins showed >10-fold change (six up-regulated and two down-regulated in PIS compared with HS) (Supplemental Table S3).

To gain a comprehensive view of the proteins related to PaWB, Go functional classification were performed as shown in Supplemental Figure. S1a-c. More detail, the majority of DEPs (88%) in the biological processes category were involved in metabolic process, single-organism process, and cellular process. The majority of proteins in molecular function category were related to cell, membrane, and
organelle. Proteins in cellular component category were related to catalytic activity and binding. These results suggested that phytoplasma infection may affect many biological processes in Paulownia. A subcellular localization analysis revealed the following distribution of these proteins among the cellular compartments: chloroplast (37%), cytoplasm (25%), nucleus (13%), extracellular areas (8%), plasma membrane (6%), endoplasmic reticulum (3%), and mitochondria (2%) (Supplemental Figure S1d). GO enrichment analysis demonstrated that DEPs were abundant in the extracellular region, oxidoreductase activity, oxidation-reduction process and chlorophyll biosynthetic process (Fig. 3a). In addition, KEGG pathway enrichment analyses showed that 87 proteins were involved in 37 pathways, of which 15 were significantly enriched (P <0.05), including biosynthesis of secondary metabolites, porphyrin and chlorophyll metabolism, metabolic pathways, and phenylpropanoid biosynthesis (Fig. 3b).

A protein domain analysis indicated that these PaWB-related proteins contained the following domains: ‘NAD(P)-binding domain’, ‘alcohol dehydrogenase, N-terminal’, ‘glycoside hydrolase family 16, catalytic’, ‘glycoside hydrolase, family 19, catalytic’, ‘START-like domain’, ‘ATP-binding cassette transporter, and ‘DSBA-like thioredoxin domain’ (Fig. 3c).

Comparative analysis of the PaWB related proteins and transcripts

Phytoplasma infection of Paulownia can affect many biological processes, which can be regulated at both the mRNA and protein levels. To delineate and compare changes in transcriptional level with post-transcriptional level, we compared the 276 PaWB related proteins with protein-coding genes from a transcriptome analysis that performed on the same seedlings. In HS vs. PIS comparison, 273 proteins or transcripts were identified both in proteomics and transcriptomics datasets (correlation, r=0.256), regardless of whether the protein-coding genes were differentially expressed. In these 273 proteins, 56 showed a same expression trend with their corresponding transcripts (Supplemental Table S4-1),
functional annotation suggested they were assigned to 40 Go groups, including oxidoreductase activity, iron ion binding, cell wall organization or biogenesis, and response to biotic stimulus. In the PIS-60 vs. PIS comparison, 271 protein were identified both in proteomics and transcriptomics datasets (r=0.1). 67 proteins showed a same expression trend with their corresponding transcripts, they main involved in defense response and response to biotic stimulus (Supplemental Table S4-2). While in the PIS-60 vs. HS comparison, 272 proteins were identified both in the proteomics and transcriptomics datasets (r =0.014), and only 2 proteins showed a same trend with their corresponding transcripts. All the above results suggested that these is no straightforward correlation of the expression level between proteins and their corresponding coding genes.

**Detection of total acetylated and succinylated proteins in Paulownia**

To gain a global view of the level of protein acetylation and succinylation in Paulownia, western-blot analyses with specific antibodies for acetylated and succinylated proteins were performed, respectively. As shown in Supplemental Figure. S2, multiple lys-acetylated and lys-succinylated proteins of different sizes were present in the four samples, indicating that lysine acetylation and succinylation are widespread in Paulownia.

**Identification of acetylated and succinylated proteins by mass spectrometry**

Lysine acetylation or succinylation can alter the structure or function of proteins involved in diverse biological processes. Recent researches have validated that lys-acetylation and lys-succinylation events occur widely in plants (10, 27). In this study, enrichment of acetylated or succinylated polypeptides by HPLC-MS/MS was performed to identify the acetylated and succinylated proteins in Paulownia. In total, we detected 5558 unique modified lysine-acetylated sites in four samples, belonging to 2893 acetylated substrates (Supplemental Table S5-1). Supplemental Figure. S3a shows an MS/MS
spectrum of an acetylated peptide obtained in this study. Further analysis found these acetylated proteins approximately possessed 59.49% of the total quantified proteins and the average acetylation degree was 2.93. A distribution analysis of the acetylation sites in the Paulownia acetylome showed that around 57.45% of lys-acetylated proteins had only one acetylation site, 21.43% had two sites, 9.96% had three sites, and the rest had four or more than four sites (Fig. 4a). 64 lys-acetylation sites were found on histone proteins, including 11 sites on H1, 17 sites on H2A, 25 sites on H2B and 11 sites on H3.

For the succinylation events, 1970 lysine-succinylation sites of 1271 succinylated proteins were identified with an average succinylation degree of 1.55 (Supplemental Table S5-2). Supplemental Figure. S3b shows an MS/MS spectrum of a succinylated peptide obtained in this study. Among the succinylated proteins, 797 have one unique succinylation site. Eighteen had more than five succinylation sites (Fig. 4b), including the 40S ribosomal protein S6, adenosylhomocysteinase, beta-glucosidase, plasma membrane ATPase. Further analysis revealed that 787 proteins were both acetylated and succinylated (Fig. 4c). At the same time, 533 sites on 425 protein substrates could be regulated simultaneously by these two types of modifications (Fig. 4d). These results provide a comprehensive overview of the acetylation and succinylation events in Paulownia.

**Functional annotation of total acetylated and succinylated proteins in Paulownia**

To clarify the functions of these acetylated and succinylated proteins in Paulownia, all the identified acetylated or succinylated proteins were subjected to subcellular localization, GO functional classification, KEGG pathway and protein domain analysis (Supplemental Table S5-1, Supplemental Table S5-2). Within the three GO categories (biological process, molecular function and cellular component), multiple acetylated proteins were functionally assigned to a wide range of biological process subcategories, including oxidation-reduction process, carbohydrate metabolic process, ribosome,
membrane, and structural constituent of ribosome, suggesting of lysine acetylation has wide-ranging effects in Paulownia. In the subcellular localization analysis, most of the identified acetylated proteins were assigned to the chloroplast, cytoplasm, and nucleus. A domain analysis of these acetylation proteins showed that they were mainly associated with AAA+ ATPase, aldolase-type TIM barrel, DEAD/DEAH box helicase, and protein kinase domain. Notably, the AAA+ family and DEAD/DEAH box helicases belong to the P-loop NTPase superfamily, whose members regulate multiple biological processes, such as DNA replication and RNA metabolism, indicating the vital role of acetylation proteins in Paulownia. Notably, in this study, we also identified a large proportion of acetylation sites enriched in the H2A domain, histone-fold domain, and histone H2A/H2B/H3 domain (Supplemental Table S5-1), suggesting that these acetylated proteins may be involved in regulating gene expression and transcription initiation and elongation.

The GO functional annotation of succinylated proteins was not as complete as that of acetylated proteins. The GO functional annotation analysis suggested that succinylated proteins mainly participated in peptide metabolism process, protein folding, structural constituent of ribosome, ATP binding, membrane, and ribosome. They were distributed mainly in the chloroplast, cytoplasm, nucleus, and plasma membrane, suggesting that there were some similarities and differences between succinylated and acetylated proteins in Paulownia. At the same time, protein domain analysis revealed that a large proportion of lys-succinylated proteins were enriched in alpha/beta hydrolase fold, heat shock protein 70kD, and NAD(P)-binding domains (Supplemental Table S5-2). The alpha/beta hydrolase fold family contains many enzymes such as proteases, lipases, esterases, dehalogenases, peroxidases, and epoxide hydrolases, which are widely distributed among plant tissues and catalyze multiple reactions in diverse metabolic processes. Together, our results demonstrated that acetylation and succinylation may play
needful roles in regulating biological processes in Paulownia.

Characterization of sequence motifs and local secondary structures

Amino acid residues in particular positions surrounding the acetylated or succinylated lysines have specific patterns and preferences in both eukaryotes and prokaryotes (29). To determine the characteristics of acetylated or succinylated lysine sequence motifs and identify possible common sequences motif exists nearly to the acylated site, the sequence motif around the specific acylated site were analyzed. The results showed that the amino acid from the −10 to +10 positions surrounding the acylated site had a certain sequence preference. Sixteen conserved acetylation site motifs from 5558 unique modified sites were significantly enriched; namely AKacK, K********KacK, K******KacK, K*****KacK, KacR, KacAK, KacS, KacK, Kac*K, Kac**K, KacN, KacR, KacT, KacH, Kac*D, Kac*E (Kac: acetylated lysine; *: residue of a random amino acid; Fig. 5a). Furthermore, a detail analysis indicated these motifs represent at least two different types: one type contained a charged residue, for instance histidine (H), arginine (R), lysine (K), glutamic acid (E), or aspartic acid (D); and the other type contained an uncharged residue such as tyrosine (Y), serine (S), threonine (T), or asparagine (N). Among these motifs, most amino acid residues located at the −6/ +1/ +2/ +3 position relative to the Kac site were also identified in other plants (30, 45, 46), suggesting that some acetylated lysine motifs are conserved in both plants and microbes. Moreover, amino acid residue of K in S. roseosporus motifs (47) was also highly conserved in Paulownia, expect it was located in different positions. In addition, we also identified two motifs, including alanine (A) at −1 position with K at +1, and A at +1 with K at +2 position were first reported in plants. The presence of K at the positions from −10 to −6 and from +1 to +10, particularly at −7, −6, +1, +2 and +3 positions, and arginine (R) at −7, +1, and +2 position were prior to be acetylated. Besides, residues of serine (S) at +1 and histidine (H) at +1
also showed a higher frequencies around the lys-acetylated sites in Paulownia. While residues of E, D, G and leucine (L) at +1 position showed the lowest frequency. These results were verified by the intensity map analysis (Fig. 5b).

Similarly, a conserved motif analysis of lys-succinylated peptides was also conducted for Paulownia. In total, nine motifs were obtained: KsucP, Ksuc*E, Ksuc*D, KsucK, Ksuc, RKsuc, KsucE, KsucR, and KsucE (where K represents succinylated lysine, and * represent a residue of a random amino acid) (Fig. 5c). Moreover, some motifs (KsucP, Ksuc*D, KsucK, Ksuc, RKsuc, and KsucE,) have been reported previously in Mycobacterium tuberculosis H37Rv or in mice (48, 49), manifesting that some motifs are shared by plants, animals and microbes. The intensity map showed that site with K at –2, –1, +1, +7 positions, R at –2, –1 and E at –3, +2 position and D at –1, +1, +2 were more readily succinylated. As well, a preference of Y at +1 and proline (P) at +1 position was also observed. However, A at +1/–1, S at 1/+1 position, and L and T at –1 position showed the lowest frequencies (Fig. 5d). These results showed that there was a preference for heterocyclic imino acids, aromatic amino acids and aliphatic amino acids around succinylation sites.

Additionally, motif analysis of 533 common sites revealed that these sites had K*D, KK, KR, and K*E (Supplemental Table S6) motifs. These results demonstrated that amino acid residues with long side chains were more readily acylation.

The distribution of acylation sites has been reported to be related to the secondary structure of proteins. To investigate the secondary structure features of acetylated and succinylated proteins surrounding the modification sites, a secondary structure analysis was conducted. In this study, acetylation sites tend to be distributed in unstructured regions (32.2% α-helix, 5.6% β-strand structures and 62.2% unstructured regions) (Fig. 5e), as did succinylation sites (30.9% α-helix, 5.8% β-strand
structures and 63.3% unstructured regions) (Fig. 5f). These results suggested that PTMs in Paulownia tended to occur at coil regions, consistent with the results in other research (50). However, when we compared the distribution pattern of acetylated or succinylated lysine residues with all lysine residues, we found that there was no preference of acetylation or succinylation locations in Paulownia proteins. A surface accessibility analysis revealed only a small decrease in accessibility of acetylated or succinylated lysine residues compared with all lysine residues (Fig. 5g, 5h). That is, lysine acetylation or succinylation did not alter the surface accessibility of proteins in Paulownia.

**Sequence homology analysis of acetylated and succinylated proteins**

Lysine acetylation and succinylation are conserved PTMs in prokaryotes and eukaryotes. To explore the potential function of these conserved acetylated or succinylated proteins in Paulownia and other plant species, we performed BLAST sequence homology analyses to compare the identified acetylated or succinylated proteins among six plant species, including *Oryza sativa*, *Populus trichocarpa*, *Arabidopsis thaliana*, *Solanum lycopersicum*, *Vitis vinifera* and *Glycine max*. The majority of acetylated proteins in Paulownia (2671, 92.6%) were also detected in the other six plant acetylproteomes (Supplemental Table S7-1). Among them, 2198, 1918, 2248, 2299, 2327, and 2254 proteins had highly conserved orthologs in *A. thaliana*, *O. sativa*, *G. max*, *V. vinifera*, *S. lycopersicum*, and *P. trichocarpa*, respectively. Similarly, 1115 succinylated proteins in Paulownia were also found in the other six plants succinylome (Supplemental Table S7-2). According to our analysis, 734 succinylated proteins were coexisted among these six species. All these results suggested that these lysine acetylation or succinylation might regulate functionally conserved proteins in different species.

**Changes in acetylome and succinylome profiles in response to phytoplasma infection**

Phytoplasma infection can alter multiple metabolic processes in host plants, including primary and
secondary metabolism. Previous studies have shown that PTMs play significant roles in plant-pathogen interactions (10). In this study, 3992 acetylated sites in 2210 proteins and 1723 succinylation sites in 1085 proteins were quantified, respectively. To explore the role of PTMs in the process of PaWB occurring, we analyzed the acetylated and succinylated DEPs in the four samples. Based on the standard of difference, differentially expressed acetylated or succinylated proteins in different comparison were obtained as shown in Table 2. Accordingly, 741 sites in 546 acetylated proteins and five sites in five succinylated proteins as being associated with the occurrence of PaWB (Supplemental Table S8-1; Supplemental Table S8-2). These results suggested that lysine acetylation may be more important than lysine succinylation in response to phytoplasma infection.

To clarify the function of these PaWB related acetylated proteins under phytoplasma infection, GO and KEGG pathway enrichment analysis were performed. The results showed that these proteins were enriched in 29 GO terms (Supplemental Table S9-1). Briefly, in the molecular function category, acetylated proteins associated with tRNA binding, S-acyltransferase activity and aminoacyl-tRNA ligase activity were highly enriched, suggesting acetylation may be involved in RNA processing, modification, and translation during phytoplasma infection in Paulownia. In the cellular component category, multiple proteins were enriched in DNA packing complex, nucleosome, chromatin and protein-DNA complex, indicating that lysine acetylation plays important roles in transcriptional regulation and DNA repair. While in biological process category, proteins related to oxoacid metabolic process, organic acid metabolic process and carboxylic acid metabolic process were significantly enriched, implying that lysine acetylation participates in an extensive range of biological processes in Paulownia. KEGG pathway analysis showed that these proteins were mainly involved in fatty acid metabolism, glyoxylate and dicarboxylate metabolism, peroxisome, carbon fixation in photosynthetic organisms, and the
pentose phosphate pathway (Supplemental Table S9-2). These results demonstrated that lysine acetylation may be related to protein processes, secondary metabolism and oxidative stress. Previous studies have proved that phytoplasma infection results in a burst of reactive oxygen species (ROS), which activates the hypersensitive response in Paulownia. Moreover, H₂O₂ has a direct anti-pathogen effect (51). However, excess ROS may damage the cellular structure. Peroxisomes contain antioxidant enzymes that detoxify ROS, specifically H₂O₂ (52). In all, the role of acetylated proteins in peroxisomes may be to regulate the ROS concentration in Paulownia cell during phytoplasma infection.

An InterPro domain analysis showed most of the PaWB related acetylated proteins had a nucleotide-binding alpha-beta plait domain, RNA recognition motif domain, or 2-oxoacid dehydrogenase acyltransferase, catalytic domain (Supplemental Table S9-3). Proteins with these domains are important in nucleic acid binding. Analysis of the subcellular localization of acetylated proteins showed that those associated with PaWB were mainly located in the chloroplast, cytoplasm, and nucleus (Supplemental Table S9-4). Together, these results suggested that lysine acetylation has a series of biological functions in Paulownia.

**Lysine acetylated enzymes in starch and chlorophyll biosynthesis**

Plants infected with phytoplasma had a higher starch content in leaves, which can disrupt the structure of the chloroplast cell and inhibit photosynthesis. Our results showed that the RuBisCO large subunit-binding protein subunit (RubL), which is involved in starch biosynthesis, were acetylated in phytoplasma-infected seedlings. At the same time, six enzymes in the porphyrin and chlorophyll metabolism pathway were also acetylated, including magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase, glutamate-1-semialdehyde 2,1-aminomutase, POR, delta-aminolevulinic acid dehydratase 1, geranylgeranyl diphosphate reductase and glutamate--tRNA ligase, suggesting a possible
role of lysine acetylation in regulating porphyrin and chlorophyll metabolism during phytoplasma infection.

RuBisCO and POR are key enzymes catalyzing the major steps in starch or chlorophyll biosynthesis, respectively (31, 53). In this study, we identified four acetylation sites in the following three proteins: two RebLs (RcbL1 and RcbL2) and one POR proteins. Among them, the POR protein had two acetylation sites: K339 and K78. To investigate the effect of site-specific modifications on the functions of these proteins, we conducted a conserved domains analysis (Fig. 6a). The results showed that the specific modified sites K339 and K78 in POR were located in PLN00015 domain; and the sites K55 in RebL1, and K764 in RcbL2 were located in the GroEL and PLN03167 domain, respectively. Notably, all these modified sites were distributed in the functional domain of the proteins. Modification in the functional domain may alter protein structure and/or function, suggested that acetylation is an important PTM during PaWB infection.

**Lysine acetylation affects the activities of RuBisCO and POR**

Lysine acylation can alter enzymes activity in plants (27, 54). In this study, we observed that lysine acetylation was much more important than lysine succinylation in phytoplasma-infected Paulownia seedlings, suggesting that acetylation of proteins might be important than succinylation in response to phytoplasma stress. Since the point that PaWB, risen from phytoplasma infection, is a lethal disease of Paulownia has been widely known, which often has the characteristic of leaf yellowing and stunting symptoms. We suggest that the acetylated proteins identified in our research might participate in the development of PaWB. To validate this hypothesis, we tested whether and how lysine acetylation of proteins affect PaWB. We detected several acetylation sites on two RuBisCO large subunits (K55 on RubL1 and K764 on RubL2) and one POR (K339 and K78), which play prominent roles in starch and
chlorophyll synthesis. To further gain a deep understanding of the potential function of acetylation on them, the site-directed mutations of their acetylation sites were performed.

To assess the effects of different lysine acetylation events on the activity of these enzymes, we individually mutated K55Q of RubL1, K764Q of RubL2, and K339Q and K78Q of POR. Each of these sites was mutated to glutamine to mimic acetylated lysine, or to arginine to mimic no acetylation. The results showed that all the point mutations led to a reduced activities of these enzymes compared to the WT (Fig. 6b). Furthermore, the activity of RuBisCO with the mutation of K55Q on RubL1 was lower than that of K55R, and a similar result was also observed for K764Q in RubL2. Moreover, POR with mutation of K339Q and K78Q had an enzyme activities lower than that of K339R and 78R, respectively, suggesting acetylation on RubL and POR might be a native regulation modification on RuBisCO and POR activity.

**Crosstalk among proteome, acetylome, and succinylome in Paulownia**

There is a growing body of evidences that crosstalk between proteome and PTMs plays significant roles in regulating the function of non-histone proteins in plants and bacteria (30, 45). In the present study, a protein-protein interaction (PPI) analysis was conducted to better understand the cellular processes regulated by PaWB-related proteins, acetylated and succinylated proteins. The network had 220 proteins, 194 succinylated proteins and 338 acetylated proteins as nodes, which were connected by 4278 direct physical interactions with the combined score higher than 0.90 (Supplemental Table S10). The proteins were assigned to groups based on their potential function and the cellular processes they were involved in. Five groups containing non-acylated proteins, acetylated proteins and succinylated proteins are shown in Fig. 7. In the largest group, 51 acetylated proteins, 33 succinylated proteins, and 17 non-acylated proteins participated in the ribosome, suggesting that they play key roles in protein
synthesis. The second largest group participated in the proteasome, and comprised 12 acetylated proteins, 12 succinylated proteins and 4 non-acylated proteins. These proteins may interact closely with unnecessary or damaged proteins. Carbon metabolism, the main way of energy production, was also important in this network. Significantly, ribosomal proteins were most closely associated with other proteins in this network. Overall, these results indicated that a complicated interaction among non-acylated proteins, acetylated proteins, succinylated proteins might control events related to the disease response or resistance during infection by phytoplasma.

**Discussion**

Pathogen infection can regulate biological processes of the host plants at various levels, including the transcriptional, post-transcriptional, translational, and post-translational levels (5, 6, 8, 51). Such regulation can lead to major changes in the proteome. As two important PTMs, Kac and Ksu play center roles in regulating metabolic processes. In the present study, proteomics and PTMomics approaches were performed to gain a great insight into the molecular mechanism of PaWB caused by phytoplasma infection. We conducted comparative analyses of four *P. tomentosa* samples, and select out 276 proteins, 546 acetylated proteins, and 5 succinylated proteins that were closely related to the occurrence of PaWB, respectively. Moreover, our results also suggested that acetylation may be more important than succinylation during phytoplasma infection. At the same time, functional analysis showed that some PaWB-related proteins and PaWB-related acetylated proteins participated in porphyrin and chlorophyll metabolism and carbon fixation. Additionally, our results showed that lysine acetylation affected the activities of the key enzymes POR and RuBisCO. All these results suggested that acetylation of certain proteins can explain the low photosynthesis rates in phytoplasma-infected *P. tomentosa*. This study, for the first time, combined proteomics and PTM-omics approaches to explore the roles of acetylation and
succinylation in Paulownia and provide comprehensive insights into the function of proteins in various processes involved in the development PaWB.

Plants infected with phytoplasma usually undergo a series of physiological and biochemical changes at the cellular and molecular levels. Previous study about changes on proteins profile in *P. tomentosa* challenged with phytoplasma have been analyzed (6). However, the molecular mechanism of PaWB remains unclear. To identify key proteins involved in PaWB, we compared a previously reported proteome of phytoplasma-infected Paulownia with the dataset obtained in this study. The results showed that more PaWB-related proteins were identified in this study than the research conducted by Cao et al. (6), and that the overlap was relatively low between our proteome data and previous dataset. These conclusions are not surprising. First, the experimental designs differed between two studies. In the previous study, different concentrations of MMS were used to treat PIS and only one concentration of MMS was used to screen proteins related to PaWB in the present study. Second, the technological progress of mass spectrometry, peptide affinity enrichment and labeling method maybe also the main reasons for more proteins identified in this study. Also, we conducted the present study based on the reference genome, which might be more accurate for gene identification. Nevertheless, further comparative analysis showed that many of the PaWB-related proteins identified in the two studies were involved in photosynthesis, which may explain the yellowing leaves and low photosynthetic rate of phytoplasma-infected seedlings.

Protein acetylation can modulate the cellular metabolic status by affecting enzyme activity or protein function has been well known in pathogen-infected plants (10, 55). In phytoplasma-infected Plants, several key enzymes that involved in lower photosynthetic rate and decreased forest production have been reported, for instance, RuBisCO participating in starch biosynthesis (31, 56). However, the
question of what modulate these enzymes status and whether protein actetylation has a crucial role in this process is still unclear. In this study, we found acetylation on these proteins seems to be the major reason by acetylproteomes and site-directed mutagenesis analysis. Based on the results of the previous study and the current study, a model were proposed to elucidate the occurring of yellowing leaves and stunting (Fig. 8).

Stunting is one of the typical symptoms of phytoplasma-infected Paulownia seedlings, which may be mainly result from the lower Pn and CE. RuBisCO is a critical factor limiting photosynthesis and productivity of many plants (34). The activity of RuBisCO is determined by the conformational changes of itself, which is induced by a AAA+-ATPase enzyme RAC (26). RAC β1-isoform acetylation on K438 may increased RAC and lead to high RuBisCO activity, which co-occurred with increased acetylation level at K474 on RuBisCO large subunit in Arabidopsis hda14 mutant (26). In contrast to this result, Finkemeier et al. (35) and Gao et al. (27) suggested that the activity of RuBisCO reduced after acetylation modification on a specific site, suggesting acetylation at different sites on RuBisCO might play vital roles in regulating its activity. In this study, the abundant of RuBisCO not altered at the protein level, while the acetylation level increased in phytoplasma-infected Paulownia seedlings. At the same time, neither K474 on the RuBisCO large subunit nor K438 on RAC were detected in this study. Enzymatic assays showed that the activity is natively regulated by lysine acetylation, which is consistent with the result that in phytoplasma-infected plant the activity of RuBisCO is reduced (56). Moreover, we also found the Pn and CE decreased in phytoplasma-infected seedlings compared with those of healthy seedlings (Fig 1c), suggesting that the activity of the photosynthesis is reduced. All these results above may lead to a reduced carbohydrate biosynthesis. However, this appears to be inconsistent with the accumulation of starch observed in phytoplasma-infected leaves (Fig 1a). Since callose deposits...
have been detected in phytoplasma-infected Paulownia (57), and the transcript levels of the gene encoding for β-1,3-glucanase, which degrades callose, were found to be decreased in phytoplasma-infected seedlings (Data not shown). Accumulation of callose in the sieve tube can impede the transport of organic substances, leading to carbohydrates accumulation in the source leaves. Therefore, the source-sink imbalance means that the sink organ cannot obtain enough nutrients, so plant growth and development are affected, which is observed as stunting.

Yellowing leaves is another prominent symptom of PaWB, which is thought to be caused by a lower chlorophyll content. POR can catalyze protochlorophyllide to chlorophyllide, which is the essential regulatory step of chlorophyll biosynthesis (56). It has been reported that in cyanobacterial POR mutant, a decreased Chl content was observed (58). In our study, though the abundant of POR not changed in phytoplasma-infected seedlings at protein level, the acetylation level has been found to be increased, and acetylation at K78 and K339 reduced POR activity. Meanwhile, lower contents of photosynthetic pigments in infected seedlings was observed compared with the healthy ones (Fig. 1b). In addition, we also found a succinylation site in POR. However whether the reduced activity of POR might be precisely control by these two distinct post-modification is not clear. This result need further confirmation in our future work. Furthermore, an increased carbohydrates content in leaves can also disrupt the source-sink balance, which can affect the transport of inorganic ions that essential for chlorophyll synthesis. Together, all these changes may lead to a decreased chlorophyll content in phytoplasma-infected seedlings (Fig. 1b), causing the typical leaf yellowing symptom.

In addition, effectors secrete by pathogens can induce the acetylation level of non-histione by function as an acetyltransferase, which induce an inappropriate immune response or a suppressor to defense the host immunity has been well characterized (10, 59-62). In this study, expect for the proteins mentioned...
above, we also found some acetylated proteins in response to phytoplasma infection related to transcriptional regulation, like transcription initiation factor TFIID subunit 5 (a component of the transcription factor IID complex), basic transcription factor 3, and histone deacetylase. Previous studies have been documented that transcription factors modified enzymes play important roles in plant defense response (63, 64). Acetylation of these transcriptional regulatory proteins might able to alter their function. In this study, we found the acetylation level of these type proteins were increased in phytoplasma-infected seedling, suggesting that an alteration of transcriptional response triggered by effector molecules has happened, which might able to alter the immune response of Paulownia and lead to a more greater virulence of effectors secrete by phytoplasma. However, this is only a hypothesis, and further testing is required.

In summary, we used proteomics and PTMomics approaches to survey changes in proteins and lysine acylation events in phytoplasma-infected Paulownia seedlings. The results of our study indicate that lysine acetylation and succinylation of proteins occurs extensively in Paulownia seedlings, and that acetylation might be more important than succinylation in answering phytoplasma-stress. Further site-directed mutagenesis and recombinant of RuBisCO and POR proved that acetylation can affect their activities, indicting these enzymes play vital roles in the development of PaWB. Together, our analysis have provided a large-scale dataset of lysine acetylation in phytoplasma-infected Paulownia seedlings. On the basis of our results, we have proposed a relatively detailed model of the functional significance of lysine acetylation in the development of PaWB.
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Data availability

The mass spectrometry proteomics data in this study have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD012701. All the sequencing data from *P. tomentosa* transcriptome used in this study is available from the SRA-Archive (http://www.ncbi.nlm.nih.gov/sra) of NCBI under the study accession SRX1013178, SRX1013200, SRX1013201 and SRR8517664.

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Footnotes
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This article contains supplementary material.

### Table 1 The number of differentially abundant proteins in different comparisons

| Comparison group | Regulated type | Number |
|------------------|----------------|--------|
| PIS_60/PIS       | up-regulated   | 193    |
|                  | down-regulated | 174    |
| PIS/HS           | up-regulated   | 369    |
|                  | down-regulated | 413    |
| HS_60/HS         | up-regulated   | 17     |
|                  | down-regulated | 7      |
### Table 2-1 The number of differentially abundant acetylated site (protein) in different comparisons

| Comparison group | Regulated type   | Fold change >1.5 |
|------------------|------------------|-------------------|
| PIS_60/PIS       | up-regulated     | 0(0)              |
|                  | down-regulated   | 1087(736)         |
| PIS/HS           | up-regulated     | 815(599)          |
|                  | down-regulated   | 19(13)            |
| HS_60/HS         | up-regulated     | 0(0)              |
|                  | down-regulated   | 18(18)            |

### Table 2-2 The number of differentially abundant succinylated site (protein) in different comparisons

| Comparison group | Regulated type   | Fold change >1.5 |
|------------------|------------------|-------------------|
| PIS_60/PIS       | up-regulated     | 1(1)              |
|                  | down-regulated   | 56(46)            |
| PIS/HS           | up-regulated     | 14(13)            |
|                  | down-regulated   | 19(16)            |
| HS_60/HS         | up-regulated     | 0(0)              |
|                  | down-regulated   | 0(0)              |
Figure legends

Figure 1 Measurement of starch, chlorophyll content and photosynthetic activity in healthy and phytoplasma-infected *Paulownia tomentosa* seedlings

a. starch content; b. chlorophyll content; c. photosynthetic activity

Figure 2 Pearson’s correlation analysis for quantitative proteome and QC validation of MS data

a. Pearson’s correlation of protein quantitation; b. Mass delta of all identified peptides; c. Length distribution of all identified peptides.

Figure 3 Enrichment analysis of Proteins related to PaWB in *Paulownia tomentosa* seedlings

a. GO enrichment analysis; b. KEGG pathway enrichment analysis; c. Domain enrichment analysis

Figure 4 Profile of identified acetylated and succinylated sites and proteins in *Paulownia tomentosa* seedlings.

a. Distribution of Kac peptides in one protein; b. Distribution of Ksuc peptides in one protein; c. The statistic analysis of the overlap between the Kac and Ksuc proteins; d. The statistic of the overlap between the Kac and Ksuc sites.

Figure 5 Bioinformational analysis of lysine acetylation and succinylation sites

a. Probability plot of significantly enriched acetylation proteins motifs. Amino acid frequency plots of 20-residue surrounding the acetylation sites using motif-x; b. The intensity map of acetylation motif shows the relative abundance of ±10 amino acids flanking the acetylated lysine site. The colors in the intensity map means the log10 (the frequencies within acetyl-21-mers/the frequencies within non-acetyl-21-mers) (red: enrichment; green: depletion); c. Probability plot of significantly enriched succinylated proteins motifs. Amino acid
frequency plots of 20-residue surrounding the succinylation sites using motif-x; d. The intensity map of succinylation motif shows the relative abundance of ±10 amino acids flanking the succinylated site. The colors in the intensity map means the log10 (the frequencies within succinyl-21-mers/the frequencies within non-succinyl-21-mers) (red: enrichment; green: depletion); e. Secondary structures of identified acetylated protein. Different secondary structures (α-helix, β-strand and coil) of identified acetylated lysine were compared to the secondary structures of all lysines obtained from proteomics; f. Secondary structures of identified succinylated protein. Different secondary structures (α-helix, β-strand and coil) of identified succinylated lysine were compared to the secondary structures of all lysines obtained from proteomics; g. Surface accessibility of identified acetylated protein; h. Surface accessibility of identified succinylated protein

Figure 6 Functional analysis of RuBisCO and POR

a. Domain architecture of RuBisCO and POR. The conserved domain analysis was performed in NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the lengths of proteins are displayed on the right; b. Effect of site-directed mutagenesis on RuBisCO and POR activity. acetylation levels of the recombinant POR and RuBisCO proteins without or with site-specific acetylation were assessed by Western blotting using the anti-acetyllysine antibody.

Figure 7 Protein-protein interaction network of the PaWB-related proteins, acetylated-proteins and succinylated proteins

Figure 8 A model for clarifying the possible molecular mechanism of malformations symptom
Fig 4

(a) Percent of proteins (%) by acetylation site:
- 1: 57.45%
- 2: 21.43%
- 3: 9.96%
- ≥4: 11.16%

(b) Number of proteins by succinylation site:
- 1: 797
- 2: 240
- 3: 103
- 4: 39
- 5: 19
- >5: 18

(c) Venn diagram showing overlap of Kac and Ksu proteins:
- Kac proteins: 2106
- Ksu proteins: 787
- Shared: 430

(d) Venn diagram showing overlap of Kac and Ksu sites:
- Kac sites: 5025
- Ksu sites: 533
- Shared: 1437
Fig 6

(a) POR

- **K** PLN00015 domain **K**
- (354 aa)

- **K** GroEL domain
- (591 aa)

- **K** PLN03167 domain
- (922 aa)

(b) RuBisCO 1

- bars and graphs showing enzyme activity (umols/min) for different conditions

- **His** WT K55Q K55R

RuBisCO 2

- bars and graphs showing enzyme activity (umols/min) for different conditions

- **His** WT K764Q K764R

POR

- bars and graphs showing enzyme activity (umols/min) for different conditions

- **His** WT K339Q K339R K78Q K78R
Fig 8

[Diagram of photosynthesis showing chloroplast, light, CO₂, H₂O, ATP, NADPH, Calvin Cycle, Rubisco, COH, Chla, chl, Pro, Mg-PIX, chlH, PIX, LGA, starch, TP, sieve tube, protein]