

Ethylene-Enhanced Latex Proteome Is Involved in Stimulation of Natural Rubber Production in the Hevea Rubber Tree

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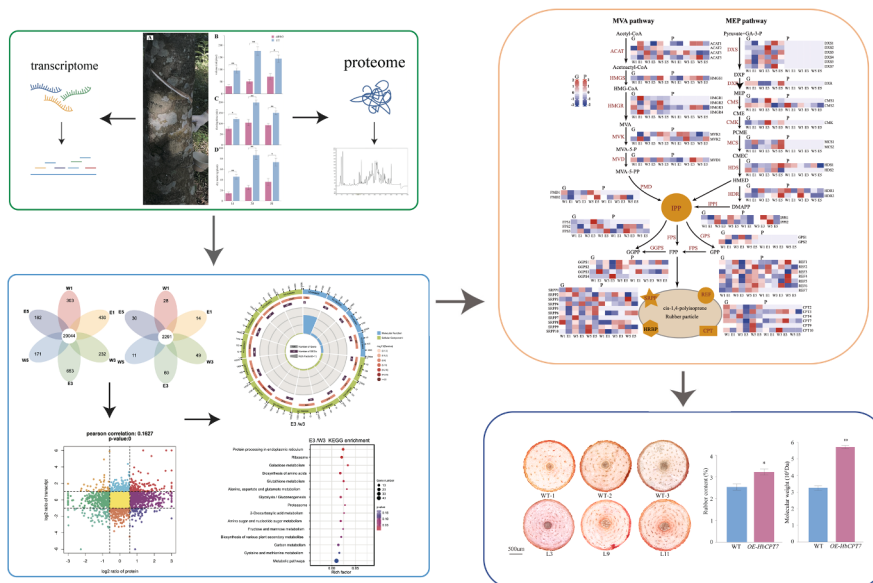
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Graphical Abstract

In Brief

Integrative transcriptomic and proteomic analyses of ethylene-stimulated rubber latex in *Hevea brasiliensis* revealed that ethylene-stimulated rubber yield increase primarily involves translational-level regulation. *H. brasiliensis* cis-prenyl transferase (*HbCPT7*) as a key regulator of natural rubber biosynthesis. Functional validation via *HbCPT7* overexpression in *Taraxacum kok-saghyz* increased rubber content by 27.3% and molecular weight by 1.8-fold, highlighting its pivotal role in natural rubber biosynthesis. This study provides a multiomics framework for understanding ethylene-induced rubber production and identifies *HbCPT7* as a potential target for genetic improvement in rubber crops.



Highlights

- The gene and protein profiles of ethylene-stimulated rubber latex were provided.
- A total of 35,306 genes and 3620 proteins are identified from rubber latex.
- Ethylene-stimulated rubber yield increase primarily involves translational-level regulation.
- Cis-prenyl transferase 7 is the key component for natural rubber production.

Ethylene-Enhanced Latex Proteome Is Involved in Stimulation of Natural Rubber Production in the Hevea Rubber Tree

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The *Hevea brasiliensis* is the only commercial source of natural rubber. In natural rubber production, exogenous ethylene is widely used as a stimulant for increasing rubber latex yield. To reveal the potential regulation mechanisms for ethylene stimulation of natural rubber production in *H. brasiliensis*, we performed an integrative analysis of transcriptomics and proteomics for ethylene-stimulated rubber latex. A total of 35,306 genes and 3620 proteins were successfully identified from the different latex samples upon ethylene stimulation. Gene Ontology analysis revealed that these genes are mainly involved in cytoplasm and cytoplasmic and catalytic activity. Kyoto Encyclopedia of Genes and Genomes analysis demonstrated that their pathways are mainly enriched in alanine and glutamate metabolism, carbon metabolism, and carbon fixation. Ethylene stimulation played a key regulatory role at the translational/post-translational modification level to promote natural rubber synthesis. Notably, 64 genes and 35 proteins are directly involved in natural rubber biosynthesis. Among them, several family members of 3-hydroxy-3-methylglutaryl coenzyme A reductase, small rubber particle protein, and *cis*-prenyl transferase (CPT) are ethylene-responsive ones. It is noteworthy that accumulation of CPT7 was significantly increased after ethylene application. Overexpression of *HbCPT7* in a rubber-producing model plant, *Taraxacum Kok-saghyz*, resulted in a significant increase in rubber content in the transgenic *Taraxacum Kok-saghyz* roots.

Natural rubber, also known as *cis*-1,4-polyisoprene, is an indispensable biopolymer with unique physicochemical properties, making it irreplaceable in industries such as automotive, aerospace, and medical sectors (1). Despite the identification of over 2500 rubber-producing plant species, *Hevea brasiliensis* remains the sole commercial source of natural rubber because of its high yield and superior rubber

quality (2). The increasing global demand for natural rubber, coupled with the limitations of synthetic rubber in meeting high-performance requirements, underscores the necessity to enhance rubber production efficiency in *H. brasiliensis* (3, 4). One of the most effective agronomic practices to increase latex yield in rubber plantations is the application of ethylene (ethephon, an ethylene releaser), which can stimulate latex flow and prolong latex regeneration between successive tappings (5, 6). Ethylene treatment enhances latex production by modulating multiple physiological and biochemical processes, including increasing the metabolic flux toward rubber biosynthesis by elevating acetyl-CoA and ATP availability (7), promoting sucrose hydrolysis and assimilate transport to laticifers (8, 9), regulating water and nutrient uptake by modulating aquaporin activity (10), enhancing the stability of rubber particles by altering membrane permeability and calcium signaling (11), and delaying latex coagulation by reducing luteoid bursting and oxidative stress (12). Despite these well-documented physiological effects, the molecular mechanisms underlying ethylene-induced latex yield enhancement remain incompletely elucidated.

Natural rubber biosynthesis (NRB) takes place in the latex cytosol of specialized laticifer cells. Isopentenyl pyrophosphate (IPP), the key precursor, is synthesized through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway and the mevalonate (MVA) pathway that is catalyzed by a big number of enzymes and cofactors (13). IPP undergoes isomerization and subsequent polymerization to form allyl pyrophosphate, the direct precursor for polyisoprene synthesis. The concentrations of IPP and allyl pyrophosphate critically influence both the yield and quality of natural rubber (14). The elongation of rubber molecules is primarily mediated by four key enzymes, including rubber elongation factor (REF), *cis*-prenyltransferase (CPT), HRT1-REF bridging protein, and small

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rubber particle protein (SRPP) (15). Ethylene has been shown to upregulate several NRB-related genes or proteins, and previous studies have primarily relied on either transcriptomic or proteomic approaches, which do not comprehensively capture their combined response at both the gene and protein levels (16). DNA microarray of latex-expressed sequence tags revealed 163 ethylene-responsive genes (17). In seedlings of *H. brasiliensis*, 3270 differential genes were identified in the ethephon-treated stems (6). Proteomics studies found 143 ethylene-responsive proteins (12) and 144 glycosylated proteins in ethylene-treated rubber latex (18). Comparative proteomics of small rubber particles resulted in 79 and 226 ethylene-responsive proteins by the gel-based and gel-free methods, respectively (19). These ethylene-responsive genes/proteins are related to sucrose metabolism in laticifer cells and post-translational modifications of enzymes (12, 15, 18). While these studies have provided valuable insights, discrepancies between mRNA and protein levels suggest that integrative multiomics approaches are essential to unravel the complex regulatory networks governing ethylene-induced latex production (15).

Transcriptome–proteome integration has emerged as a powerful strategy to bridge the gap between gene expression and functional protein dynamics (13). Therefore, we integrated high-throughput transcriptomics and proteomics methods for the Hevea rubber latex at various stages after ethylene stimulation. Correlation analysis was performed to screen out core genes or proteins, and the key gene was functionally verified through transgenic methods. Our study aimed to provide transcriptomic and proteomic profiles of ethylene-stimulated rubber latex, compare the changed patterns of genes and proteins in rubber latex upon ethylene stimulation, and determine the ethylene-responsive members for genes and proteins in NRB. Among them, *cis*-prenyl transferase 7 (CPT7) is crucial for NRB in *H. brasiliensis*.

EXPERIMENTAL PROCEDURES

Plant Materials

The 60 rubber trees (*H. brasiliensis* Mull. Arg., clone RY 7-33-97) about 10 year old, planted in Tunchang County, Hainan Province, China, were selected for further study. These young trees were divided into two groups and treated with 3% (v/v) ethephon dissolved in ddH₂O, and ddH₂O was used as a control as described (12). According to the S/2 d3 (tapping half of the spiral once in 3 days frequency) harvesting system for the rubber trees used in Hainan province of China, we tapped these young rubber trees as described in our previous reports (12, 18, 19). Fresh rubber latex was collected from these rubber trees after 1, 3, and 5 days of ethylene (E1, E3, and E5) or ddH₂O (W1, W3, and W5) application, respectively. The latex of every 10 trees was pooled as one biological replicate, and each treatment comprised three independent biological replicates. The collected latex was used for subsequent physiological parameters: determination, transcriptome sequencing, proteome profiling, and quantitative real-time PCR (qRT-PCR) verification.

Determination of Latex Physiological Parameters

The rubber tree bark was sliced with a rubber cutter. The fresh latex was collected, and the fresh yield, flowing time, dry rubber content (DRC), and dry matter (DM) were determined. The flowing timing commenced with the emergence of the first droplet of latex, and the flow of latex was considered to have ceased when no further droplets were observed for a continuous period of 10 s. Concurrently, all collected latex from each tree was transferred into a measuring cylinder to ascertain the total volume of latex harvested, which represented the total fresh yield (FY) of latex. Subsequently, the latex was placed in an oven set at 60 °C for drying until a constant weight was reached, a process that typically spanned approximately 72 h. The DM at this stage was recorded. The DRC was then calculated using the following formula: $DRC = (DM/FY) \times 100\%$ (12).

Transcriptomics Analysis

The RNA from the six treatments (W1, W3, W5 and E1, E3, E5) was extracted by the RNeasy Pure Plant Plus Kit (DP441; Qiagen Biotech), with three independent biological replicates for each treatment. The extracted RNA samples underwent quality assessment and then were sent to Wuhan Benagen Technology for the construction of complementary DNA libraries. Clean reads obtained from each sample were mapped on the reference genome of *H. brasiliensis* (accession code: PRJNA587314) by using Star (version 2.7.9a) software. RNA-Seq by Expectation-Maximization (version 1.3.3) was used to obtain the number of reads aligned to each transcript for each sample and then converted to Fragments Per Kilobase per Million bases values. Paired-end reads originating from the same fragment were counted as one fragment, thereby determining the gene expression levels. DESeq2 was utilized to identify differentially expressed genes (DEGs) based on the criteria of $|\log_2 \text{fold change } [FC]| > 1$ and $p < 0.05$. Functional annotations of genes and proteins were crossreferenced with various public databases, including the nonredundant protein sequences database (Nr), universal protein database (UniProt), protein family (Pfam), cluster of orthologous groups for eukaryotic complete genomes (KOG), Kyoto Encyclopedia of Genes and Genomes (KEGG), Pathway, and Gene Ontology (GO) databases, using a threshold E value of 10^{-5} .

Proteomics Analysis

Three independent biological replicates were used for each of the six treatment groups (W1, W3, W5 and E1, E3, E5), and latex proteins from the six treatments were isolated using an optimized phenol extraction method as described in our previous report (12). The Bradford assay was used for protein concentration determination, with bovine serum albumin as the protein standard. For enzymatic digestion, trypsin was introduced at an enzyme-to-substrate ratio of 1:50 (w/w), corresponding to 50 µg trypsin for the given protein quantity, followed by overnight incubation at 37°C. The resulting peptide mixture was then lyophilized using a vacuum concentrator. These peptides were separated using the Ultimate 3000 HPLC instrument (Thermo Fisher Scientific). The peptides were initially trapped on the trap column (PepMap C18, 100 µm × 20 mm, 5 µm; Thermo Scientific) and subsequently separated on the analytical column (PepMap C18, 75 µm × 25 mm, 2 µm; Thermo Scientific). The mobile phase consisted of buffer A (20 mM ammonium formate), buffer B (80% acetonitrile and 10% 20 mM ammonium formate), buffer C (100% HPLC water), and buffer D (100% acetonitrile). Peptides were separated at a flow rate of 0.8 ml/min using a 69-min gradient. The eluent was collected in 50 tubes and finally merged into 15 fractions. Fractions were analyzed by using Thermo Scientific Orbitrap Exploris 480 with the analytical column (PepMap C18, 75 µm × 15 mm, 3 µm; Thermo Scientific). The mobile phases

consisted of buffer A (0.1% formic acid, 2% acetonitrile, and 97.9% HPLC water) and buffer B (0.1% formic acid, 80% acetonitrile, and 19.9% HPLC water). A linear gradient elution was programmed as follows: 0 to 2 min from 3% to 7% buffer B, 2 to 42 min from 7% to 25% buffer B, 42 to 50 min from 25% to 38% buffer B, 50 to 56 min from 38% to 100% buffer B, and 100% buffer B for 4 min. The mass spectrometry 1 (MS1) scan range was set to 350 to 1500 m/z with an accumulation time of 250 ms, whereas MS/MS spectra were acquired in high-sensitivity mode covering 100 to 1500 m/z with a 50 ms accumulation time. The top 40 most intense precursor ions from each full scan were selected for fragmentation. The raw data were then imported into Proteome Discoverer 2.4 software for analysis and were searched against the *H. brasiliensis* protein database (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_010458925.1/, downloaded from the National Center for Biotechnology Information on March 15, 2021) containing 44,146 entries. Trypsin was set as the digestion enzyme (allowing up to two missed cleavages), fixed modifications selected carbamidomethyl of cysteine, variable modifications selected oxidation of methionine, and precursor and fragment ion mass accuracies were set to 10 ppm and 0.05 Da, respectively. Proteins that had at least two unique peptides with a confidence level of $\geq 95\%$ and a false discovery rate of $< 1\%$ were considered as positively identified. The hypergeometric test was used to determine differentially expressed proteins (DEPs) with $FC > 1.5$ ($q < 0.05$) or < 0.67 ($q < 0.05$).

Experimental Design and Statistical Rationale

For label-free quantitative proteomic analysis, equal amounts of proteins from each treatment were separately subjected to enzymatic digestion and peptide enrichment, followed by LC-MS/MS analysis. For each of the six treatments (W1, W3, W5 and E1, E3, E5), three independent biological replicates were analyzed. The peptides were identified and quantified using Proteome Discoverer 2.4. Proteins that had at least two unique peptides with a confidence level of $\geq 95\%$ and a false discovery rate of $< 1\%$ were considered as positively identified. For remaining quantitative assessments, results are expressed as mean $\pm$ SD relative to corresponding control groups. Statistical significance (p value) was determined using a two-tailed Student's t test; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. All experimental procedures were replicated at least three times, with representative data shown.

Integrated Analysis of Transcriptome and Proteome Data

The gene/protein expression FCs of the E1/W1, E3/W3, and E5/W5 values were transformed into $\log_2$ format. Pearson's correlation coefficient between the E1/W1, E3/W3, and E5/W5 values obtained from the transcriptome and proteome data was calculated, and nine quadrant diagrams were used to visually show their distribution patterns.

Functional Analysis of HbCPT7

HbCPT7 was overexpressed in a rubber-producing model plant, *Taraxacum Kok-saghyz* (TKS). In this study, TKS germplasms were originally obtained from the Tekes River Basin (81.53°E, 43.13°N), Yili Kazak Autonomous Prefecture, Xinjiang, China, and subsequently cultivated under controlled greenhouse conditions. The *HbCPT7* gene was cloned into the pSuper1300 expression vector downstream of the CaMV35S constitutive promoter following established protocols (2). The resulting constructs were subsequently transformed into *Agrobacterium rhizogenes* EHA105. Stable transgenic plants were generated through the leaf disc method according to the published methodology (20). Transgenic lines were initially identified through genomic DNA PCR, followed by qRT-PCR analysis to determine relative expression levels of the *HbCPT7* gene. For

observation of the laticifer cells in TKS roots, 3-month-old TKS root specimens were first preserved in 80% ethanol solution for 24 h before being processed into 100 μ m slices by vibrating microtome (Leica VT1200S). These slices were then treated with histological stain (Oil Red O; Sigma-Aldrich) and mounted in 60% (v/v) glycerol. Laticifer cells were observed through microscopic SZX16 (Olympus). Then the rubber yield of the *HbCPT7*-overexpressed transgenic TKS and the WT TKS (used as a control) was evaluated as described (18), and the molecular weight of *cis*-1,4-polyisoprene was determined by using gel permeation chromatography. For each construct, three independent lines with high expression levels were selected for analyses.

qRT-PCR Analysis

To confirm the candidate genes identified from transcriptome data, we conducted qRT-PCR analysis using the ABI StepOnePlus real-time PCR instrument (Thermo Fisher). The ChamQ SYBR qPCR Master Mix was utilized for the amplification reaction. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method, with the actin gene serving as the reference gene.

RESULTS

Physiological Analysis of Rubber Latex in Response to Ethylene Stimulation

Latex physiological parameters can reflect the metabolic status of the rubber laticifer system; therefore, we collected the latex after treating with ethylene or ddH₂O for 1, 3, and 5 days in the virgin rubber trees (Fig. 1A) and determined the fresh yield, flowing time, DRC, and DM (Fig. 1, B–E) for the latex. After ethylene treatment, latex FY was sharply increased than the control. Among different sampling days, the average amount of latex FY was highest after 3 days of stimulation, reaching 176.5 ± 28.8 ml per tree (Fig. 1B). The latex flowing time was significantly prolonged from 75.9 ± 14.1 , 104.6 ± 20.3 , and 92.5 ± 13.9 min to 121.2 ± 15.3 , 199 ± 19.6 , and 150 ± 15.3 min after treatment with ethylene for 1, 3, and 5 days, respectively (Fig. 1C). Prolonging treatment, rubber flowing time was longer than the control, and the longest time reached nearly 200 min on the third day after ethylene stimulation. In terms of DRC, there was a gradual increase with the prolongation of treatment. The DRC of ethylene treatment was significantly lower than that of the control group (Fig. 1D). Ethylene stimulation significantly increased the DM of rubber latex compared with ddH₂O treatment (Fig. 1E).

Transcriptomics Analysis

Functional Annotation of Genes in the Sequencing Data—Following a rigorous quality assessment and data filtration, 108.58 Gb of clean bases (730.57 million clean reads) were contained, ranging from 5.88 to 6.55 Gb per sample. These clean data with Q20 rate 96.93% to 98.06%, Q30 rate 91.61% to 94.07%, and GC content 44.12% to 44.89% after filtration (Supplemental Table S1). Clean reads obtained from each sample were aligned to the reference Hevea rubber tree genome, and the comparison efficiency ranged from 75.08% to 77.65%. This resulted in the identification of 35,306 genes;

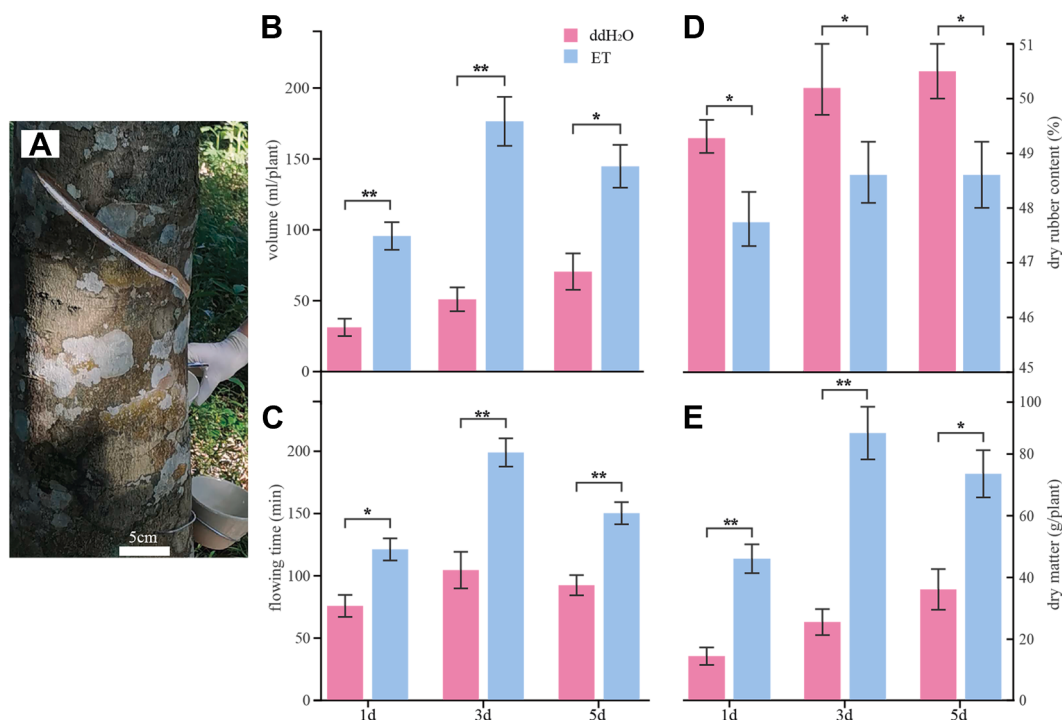


FIG. 1. Changes of latex physiological parameters after ethylene treatments. A, the virgin rubber tree. B, fresh yield. C, flowing time. D, dry rubber content. E, dry matter.

among them, 6151 were determined to be novel genes (Supplemental Table S1). A total of 24,363 (W1), 24,291 (E1), 23,959 (W3), 25,202 (E3), 23,365 (W5), and 23,528 (E5) shared genes were positively recognized from the three biological replicates, and these genes were determined as the genes expressed in these latex samples and used for further analysis (Supplemental Table S1). Furthermore, 20,044 genes were found to be shared among all samples (Fig. 2A).

Functional annotations of the detected 35,306 genes were performed in seven public databases, including Nr, UniProt, KOG, KEGG, GO, Pfam, and Pathway databases, and the number of genes in each database accounted for 30.15%, 30.70%, 71.83%, 1.20%, 23.30%, 23.30%, and 67.46% of total genes, respectively. A total of 23,817 genes were categorized into 53 functional subclasses by GO classification (Supplemental Fig. S1A and Supplemental Table S2). The majority was observed in the cellular component category, with significant GO terms related to intact cells or cell components. In terms of biological processes, the most prominent group was cellular processes. In addition, 8266 latex genes were categorized into various biological pathways, with the most prevalent pathway being carbohydrate metabolism (Supplemental Fig. S1B and Supplemental Table S2).

Determination of Differential Genes and Their Expression Patterns—The DEGs were characterized according to the filter conditions. After ethylene stimulation for 1 day, 5647 DEGs were determined, comprising 3074 upregulated and 2573 downregulated ones. After 3 days, 6234 DEGs were detected,

and with the prolongation of treated time to 5 days, many fewer (only 3834) DEGs were observed, including 1817 upregulated and 2017 downregulated ones (Fig. 2, B and C and Supplemental Table S3). Among these DEGs, 1439 genes were shared in all the detected latex samples. Hierarchical clustering revealed that 1439 DEGs in W1, W3, and W5 latex samples were grouped into one cluster; on the other hand, the E1, E3, and E5 samples were clustered separately in another cluster (Fig. 2C and Supplemental Table S3). By analyzing the heatmap of DEG expression levels in response to ethylene stimulation, it was clear that latex gene expression changes over time. Based on hierarchical clustering analysis, the DEPs could be categorized into three distinct expression patterns: one group exhibited consistent upregulation over the course of 1, 3, and 5 days of ethylene exposure; another group showed sustained downregulation across the same time points; and a small subset displayed irregular or non-monotonic expression changes (Fig. 2D and Supplemental Table S3).

GO classification of these DEGs revealed that the principal classification was binding in the molecular function category. In the cellular component category, the DEGs were primarily classified as being intact with or as parts of cells (Supplemental Fig. S2). To further explore the biological functions, GO enrichment analysis was conducted. Among the detected GO terms, the most enriched one was small molecule metabolic process, followed by proteasome complex (Fig. 2E and Supplemental Table S3). KEGG enrichment

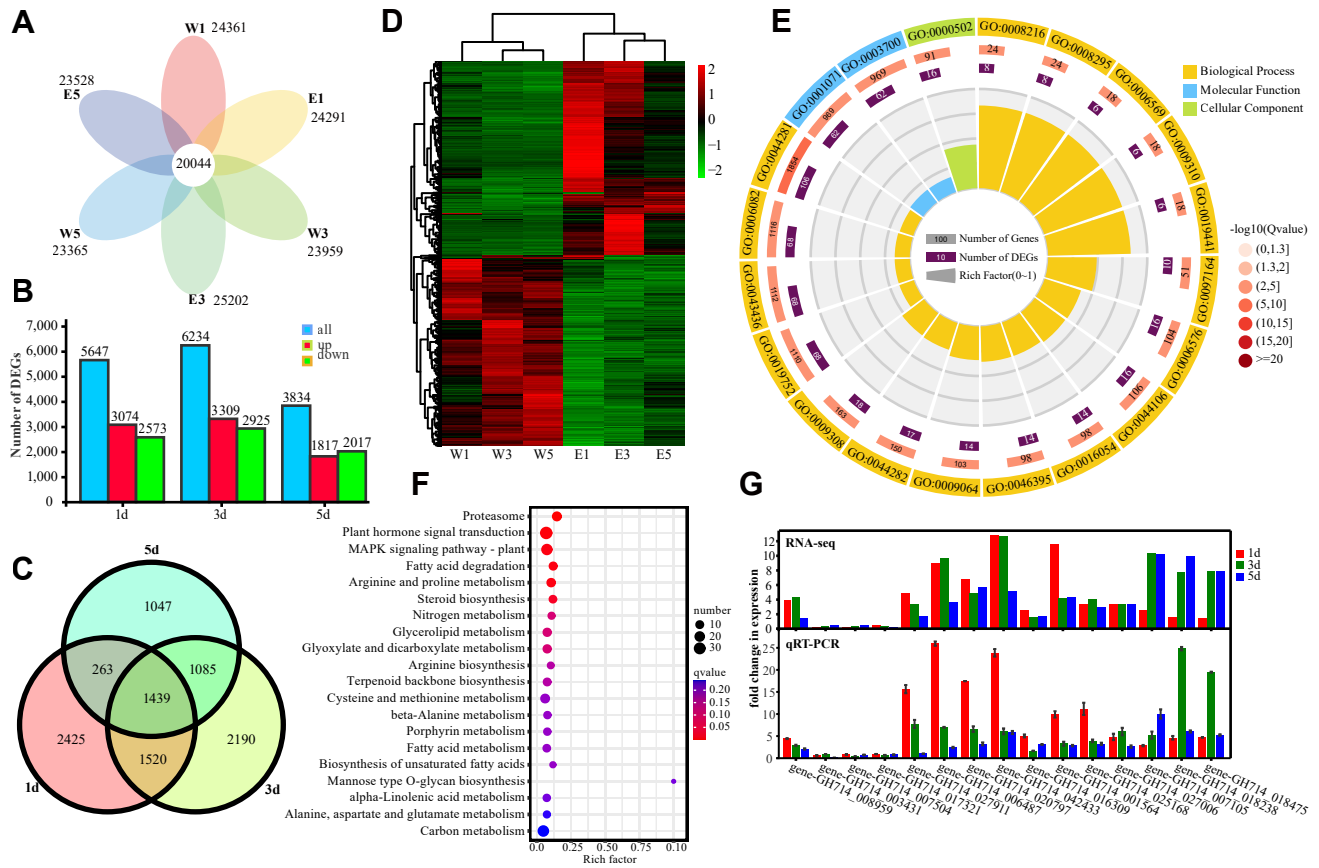


FIG. 2. Genes identified from the rubber latex and their expression patterns after different treatments. A, Venn diagram of the shared and unique genes. B, number of DEGs in each comparison. C, Venn diagram of DEGs was highlighted. D, hierarchical clustering of the DEGs. E, GO enrichment of the DEGs. F, KEGG enrichment of the DEGs. G, the qRT-PCR verification of the gene expression profiles. DEG, differentially expressed gene; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT-PCR, quantitative RT-PCR.

analysis of the DEGs revealed their involvement in various pathways, including proteasome, plant hormone signal transduction, mitogen-activated protein kinase (MAPK) signaling pathway (plant), steroid biosynthesis, fatty acid degradation, and proline metabolism, which are important for the response to ethylene stimulation (Fig. 2F and Supplemental Table S3).

qRT-PCR Analysis of Gene Expression Patterns—The qRT-PCR experiments were conducted for the selected 15 DEGs that were associated with the response to ethylene stimulation (Supplemental Table S7). Among them, 10 candidates involved in ethylene biosynthesis and signaling showed a consistent upward trend in expression. Furthermore, two of the five candidate genes involved in NRB also showed a consistent upward trend, whereas three DEGs exhibited a consistent decrease in expression, consistent with the corresponding RNA-Seq data (Fig. 2G and Supplemental Table S7).

Proteomics Analysis

Identification of Proteins in the Latex—High-throughput shotgun proteomics was conducted to investigate the

proteins accumulated in the latex from the six treatments (W1, W3, W5 and E1, E3, E5). In this study, only proteins detected from at least two repetitions were considered as positively identified ones. Finally, 3620 proteins were identified from the proteome (Supplemental Table S4). In the three biological replicates of the rubber latex samples, a total of 2610, 2360, 2817, 2806, 2543, and 2842 proteins were detected for W1, E1, W3, E3, W5, and E5, respectively (Supplemental Table S4). In addition, 2291 shared proteins were determined in all samples (Fig. 3A).

Comparison of Protein Accumulation Patterns Upon Ethylene Stimulation—The DEPs were characterized according to the filter conditions. A total of 1835 DEPs were detected from these latex samples. Compared with W1, 517 proteins were determined as DEPs in the E1 treatment, including 200 induced proteins. For the E3/W3 group, 863 DEPs were detected, including 528 upregulated and 335 downregulated proteins. After ethylene treatment for 5 days, more than 1000 DEPs were detected; among them, 759 upregulated and 301 downregulated proteins were observed. A total of 72 DEPs were shared in three pairwise comparisons. In addition, 12 upregulated and six downregulated DEPs were shared by the

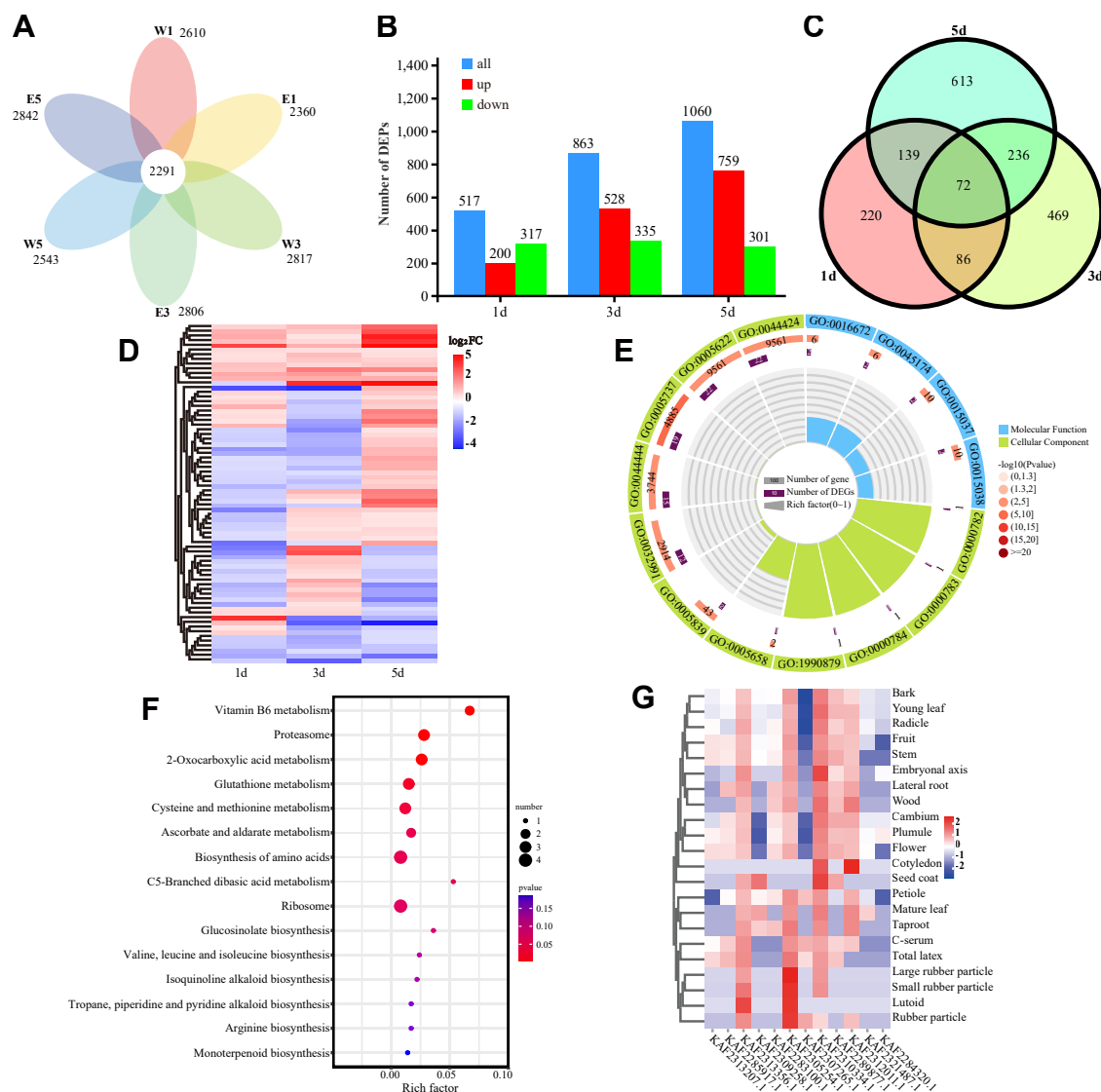


FIG. 3. Proteins identified from the rubber latex and their expression patterns after different treatments. *A*, Venn diagram of the shared and unique proteins. *B*, number of DEPs in each comparison. *C*, Venn diagram of DEPs was highlighted. *D*, hierarchical clustering of the DEPs. *E*, GO enrichment of DEPs. *F*, KEGG enrichment of DEPs. *G*, verification of protein expression profiles in the 22 tissues of *Hevea brasiliensis*. DEP, differentially expressed protein; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

E1/W1, E3/W3, and E5/W5 pairs. The remaining 54 shared DEPs, however, did not exhibit a consistent expression trend over the course of ethylene treatment (Fig. 3, B and C and Supplemental Table S5). The results from the hierarchical clustering analysis showed that these DEPs could be found in almost all clusters, although their changed values were moderate (Fig. 3D and Supplemental Table S5).

Functional Enrichment Analysis of Differential Proteins—First, all DEPs were performed for GO enrichment analysis and revealed that the major GO terms were related to cellular components. In terms of molecular function, the most representative categories were catalytic activity and binding. In the biological process, the dominant terms were cellular and

metabolic processes (Supplemental Fig. S3). Furthermore, GO classification indicated that the represented subclasses were cell part, cell, catalytic activity, binding, cellular, and metabolic process. Then, the 72 shared DEPs performed GO enrichment analysis. The most significant term was cytoplasm, along with other cytoplasmic parts and oxidoreductase activity (Fig. 3E and Supplemental Table S5). The most noteworthy pathway was vitamin B6 metabolism, followed by proteasome, two-oxocarboxylic acid metabolism, and glutathione metabolism (Fig. 3F and Supplemental Table S5).

Validation of Protein Accumulation in Different Tissues—The atlas of *H. brasiliensis* protein was obtained from our public database (<http://biokb.ncpsb.org.cn/hebatlas>), and a total of

22 different tissues from *H. brasiliensis* were selected for the gene expression analysis, aimed at gaining a comprehensive understanding of the molecular responses of *H. brasiliensis* to ethylene stimulation. These tissues included bark, cambium, cotyledon, C-serum, embryonal axis, flower, fruit, lateral root, large rubber particle, lutoid, mature leaf, petiole, plumule, radicle, rubber particle, seed coat, small rubber particle, stem, taproot, total latex, wood, and young leaf tissues. Subsequently, 12 representative DEPs, which are key members involved in ethylene biosynthesis, ethylene signaling, and NRB, were selected to investigate their accumulation profiles in the 22 tissues of *H. brasiliensis* (Fig. 3G and Supplemental Table S7). Protein expression profiles in the 22 tissues of *H. brasiliensis* were highlighted in the heatmap. Among them, all proteins were expressed in the embryonal axis; two proteins were expressed in 21 rubber tree tissues, and three proteins were specifically expressed in small rubber particles and large rubber particles. Five proteins showed high expression in multiple tissues. It was noteworthy that REF 3 (KAF2305254.1) was highly expressed in the rubber particles (Fig. 3G).

Combination Analysis of Gene and Protein Profiles

Comparison of Transcriptomic and Proteomic Data—To compare the gene and protein expression levels at the same tissues, we examined the transcriptome and proteome data. Our results revealed that many more genes than proteins could be identified from rubber latex. More than 23,000 genes were successfully identified, whereas less than 3500 proteins were detected from each sample. For all the 24,363 identified genes in W1, 2850 of their respective proteins were identified. In E1, 24,291 genes were detected, and proteomics analysis revealed that 2573 proteins were produced. A total of 3027 and 3041 genes and their products were simultaneously identified in W3 and E3. Furthermore, in W5 and E5, 2698 and 3021 genes and their respective proteins were detected (Supplemental Fig. S4). The total number of function-disclosed genes detected in all samples was 35,306; for all the identified genes and proteins, 3580 proteins could be matched to genes (Supplemental Fig. S4).

Comparison of Matched DEGs and DEPs—Approximately 99% of the detected proteins could be found in RNA-Seq data. However, many proteins showed different changed patterns upon ethylene stimulation with their transcripts. Based on the Fragments Per Kilobase per Million bases expression ratio values, the relationship between proteins and genes in the transcriptomics and proteomics of the three comparisons (E1/W1, E3/W3, and E5/W5) was investigated. The results showed low correlation values between proteins and genes. Pearson's correlation coefficient analysis resulted in only as low as 0.0213, 0.1627, and 0.098 in the above three comparisons, respectively (Fig. 4A). In the integrated nine-quadrant transcriptomic–proteomic analysis, each quadrant represents distinct relationship patterns between gene and

protein expression changes. We observed that the number of DEGs/DEPs in quadrants 4 and 6 (characterized by significant protein changes without corresponding transcriptional alterations) was consistently the highest across all ethylene treatments and showed a gradual increase with prolonged ethylene treatment (from day 1 to day 5). In E1/W1, quadrant 4 and quadrant 6 together comprised 129 DEPs (77 and 52, respectively). In E3/W3, they totaled 249 (81 and 168), and by E5/W5, they reached 318 (86 and 232) (Fig. 4A and Supplemental Table S6). This pattern indicated that ethylene-induced proteomic remodeling operates largely independently of transcriptional changes in the corresponding genes, underscoring the increasing importance of post-transcriptional regulation in the ethylene response.

Subsequently, we specifically focused on the DEPs located in quadrants 4 and 6 across the E1/W1, E3/W3, and E5/W5 comparisons, which represented proteins with significant changes at the translational or post-translational level independent of corresponding transcriptional alterations. KEGG enrichment analysis revealed distinct temporal patterns of pathway activation in response to ethylene stimulation. In the E1/W1 comparison, carbon metabolism emerged as the most significantly enriched pathway, followed by biosynthesis of amino acids and the proteasome, indicating an early shift in energy metabolism and protein turnover. In the E3/W3 comparison, the most prominently enriched pathway was protein processing in the endoplasmic reticulum, with ribosomes also being highly represented, suggesting enhanced protein synthesis and folding activities at this intermediate stage. By the E5/W5 comparison, the top enriched pathways included phagosome, ribosome, and proteasome, highlighting the increasing importance of degradation processes, sustained protein synthesis, and proteolytic regulation during prolonged ethylene exposure (Fig. 4B). These findings revealed potential functional implications and pathway associations for the identified DEPs across different treatment pairs.

Integrated proteomic and transcriptomic analyses revealed that ethylene treatment triggers a translation-level-dominated regulatory program in the laticifers of rubber trees, characterized by the substantial and progressive accumulation of a specific subset of proteins without corresponding increases in their mRNA levels at days 1, 3, and 5 after treatment (Supplemental Table S6). This post-transcriptional enhancement followed a distinct temporal pattern, initiating with moderate upregulation (FCs predominantly 1.5- to 4.8-fold) at day 1, involving proteins responsible for rapid functional adjustments, such as ubiquitin-like domain-containing proteins, protein-serine/threonine phosphatases, and importin subunit alpha. As the treatment progressed to days 3 and 5, the response escalated into a systematic and widespread amplification, with dramatic increases in protein abundance exemplified by epimerase domain-containing protein (21.74-fold at day 3) and serine O-acetyltransferase (16.39-fold at day 5), alongside numerous other proteins exceeding 8.0 FCs

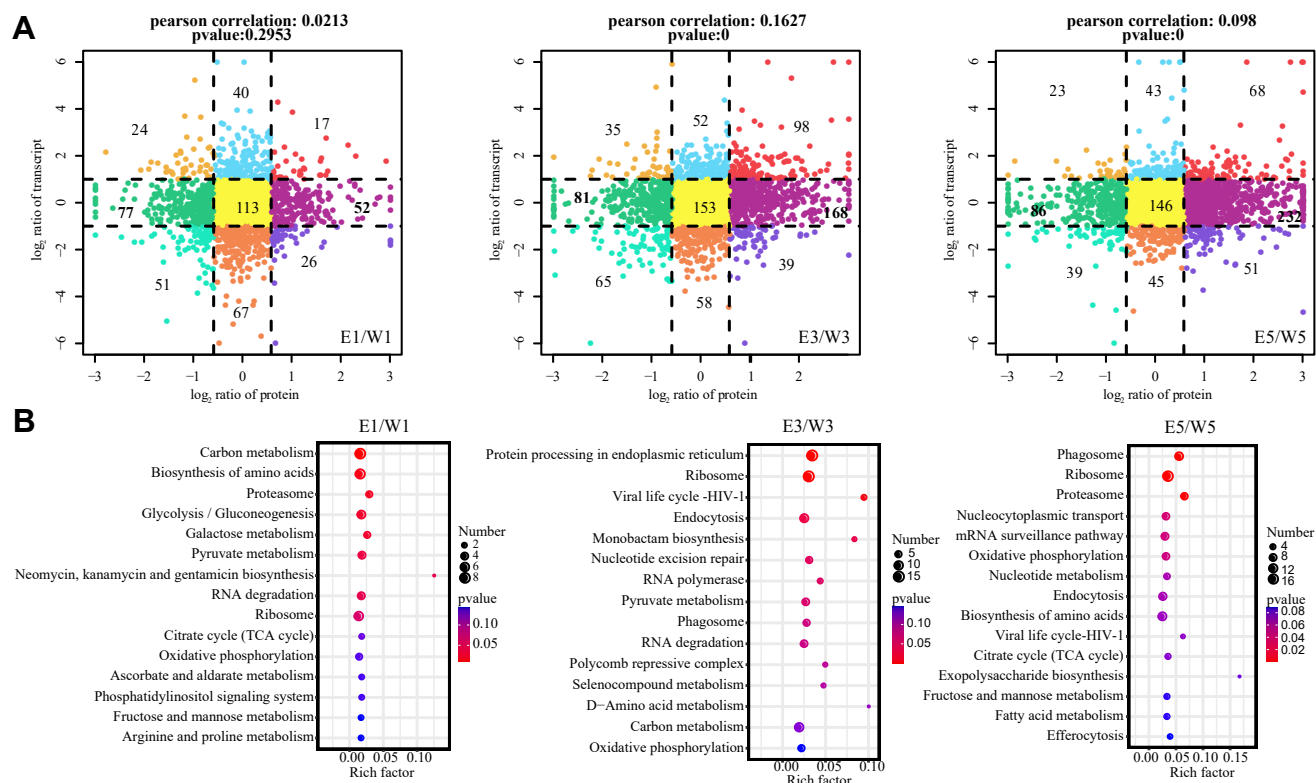


FIG. 4. Integrated analysis of genes and proteins in transcriptome and proteome. A, nine quadrant diagrams were presented: quadrants 1 and 9 represent genes negatively correlated with protein expression; quadrants 2 and 8 represent differential gene expression with no corresponding change in protein expression; quadrants 3 and 7 represent genes positively correlated with protein expression; quadrants 4 and 6 represent differential protein expression with no corresponding change in gene expression; and quadrant 5 represents no significant change in either gene or protein expression. B, KEGG enrichment of DEPs in quadrants 4 and 6 for the E1/W1, E3/W3, and E5/W5 pairs, respectively. DEP, differentially expressed protein; KEGG, Kyoto Encyclopedia of Genes and Genomes.

by day 5 (Supplemental Table S6). The early response (1 day) featured the accelerated accumulation of proteins involved in post-translational modification (e.g., ubiquitin-like domain-containing protein), phosphorylation signaling (e.g., protein-serine/threonine phosphatase), and nucleocytoplasmic trafficking (e.g., importin subunit alpha), suggesting a rapid adjustment of pre-existing protein function. As treatment progressed to 3 and 5 days, this transcription-independent protein enhancement became systematic and extensive. The most salient feature was a concerted, direct fortification of the cell's global protein synthesis and secretory capacity. This was evidenced by the specific upregulation of core components of (i) translation machinery, including subunits for ribosome biogenesis (multiple 40S and 60S ribosomal proteins), translation initiation and elongation (multiple subunits of eukaryotic initiation factor 3, eukaryotic initiation factor 4C, and elongation factor 1-alpha), and tRNA aminoacylation (several aminoacyl-tRNA synthetases); (ii) energy and substrate provision for synthesis (multiple V-type proton ATPase subunits, inorganic diphosphatase); (iii) cotranslational folding and modification (protein disulfide-isomerase, peptidyl-prolyl isomerases); and (iv) vesicular trafficking and secretion (exocyst complex components, reticulon-like

proteins, and coat protein complex I/coat protein complex I coatomer subunits) (Supplemental Table S6). Crucially, the significant upregulation of key factors with established roles in latex production, specifically, endo/exonuclease/phosphatase domain-containing proteins (REF/SRPP family) and multiple V-ATPase subunits, provided a direct molecular link between this global translational boost and the physiological mechanism of ethylene-induced yield increase, namely, the stabilization of rubber particles and the regulation of luteal osmolarity and cytoplasmic pH to prolong latex flow.

Genes and Proteins Involved in NRB—We detected 64 genes and 35 proteins related to NRB in transcriptomic and proteomic data, respectively. Changed patterns of these genes and proteins were compared *via* heatmaps (Fig. 5 and Supplemental Table S7). Almost all members of genes and proteins in the MVA pathway could be detected in our results. However, in the MEP pathway, 14 genes were detected, and just four gene-coding proteins were obtained by proteome. Finally, we obtained 34 DEGs and 27 DEPs involved in NRB, and different DEG and DEP members had different changed patterns. Among them, most members of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), SRPP, and CPT families were ethylene-responsive ones, indicating they

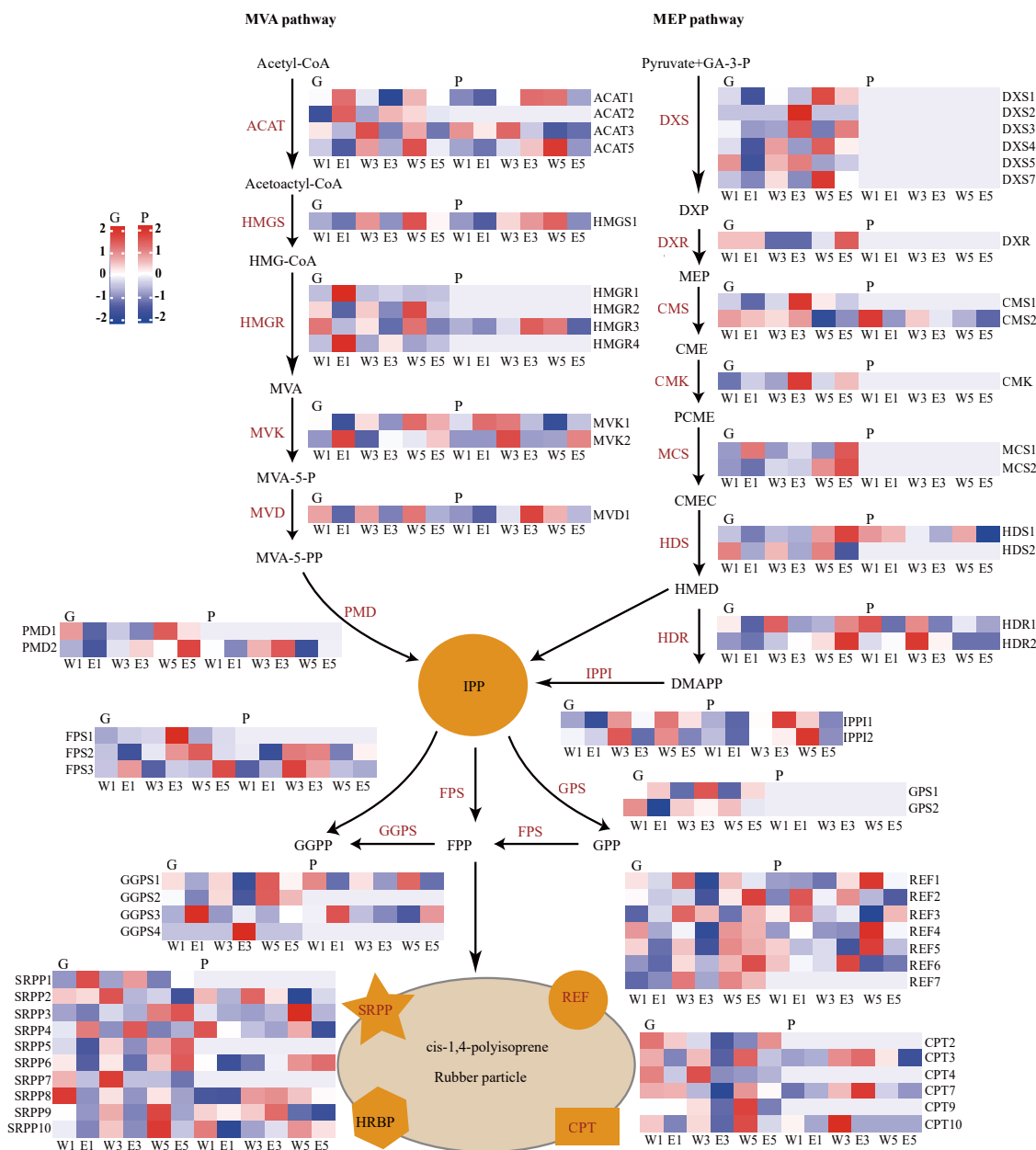


FIG. 5. Expression profile of genes and proteins involved in NRB in rubber latex. The left side depicts the MVA pathway, and the right side depicts the MEP pathway. MEP, 2-C-methyl-d-erythritol 4-phosphate; MVA, mevalonate; NRB, natural rubber biosynthesis.

might have more roles than the other family members for NRB after ethylene application.

Functional Analysis of the HbCPT7

In this study, we observed that HbCPT7 (KAF2310866.1) demonstrated a sustained upregulation at the protein level on both day 1 and day 3 after ethylene treatment with a 2.4-fold and 1.6-fold increase, respectively, whereas other regulated proteins showed upregulation only at isolated time points (Supplemental Table S7). This persistent early phase protein accumulation suggested that HbCPT7 was specifically tuned

by ethylene at a post-transcriptional or translational level. Biologically, HbCPT7 is a member of the CPT family and catalyzes the rate-limiting chain-elongation step in natural rubber (polyisoprene) biosynthesis, strongly linked to high-molecular-weight rubber production. Therefore, we overexpressed its gene, *HbCPT7* (gene-GH714_017856), in the model rubber-producing plant TKS. Finally, 13 positive transgenic lines (L1–L13) were obtained, and the expression level of the *HbCPT7* gene in transgenic TKS was detected by qRT-PCR. Among them, the expression level of *HbCPT7* in transgenic line L3 was the highest, followed by L11 and L9

(Fig. 6A). Therefore, transgenic TKS L3, L9, and L11 were selected for subsequent physiological analyses. Observing and comparing the number of laticifer cells of WT and transgenic TKS roots, we found that at 3 months old, the number of laticifer cells in the transgenic TKS roots was significantly more than WT (Fig. 6B). Furthermore, the rubber content and molecular weight in the *HbCPT7* transgenic TKS and WT were measured. In comparison to WT, the *HbCPT7* transgenic TKS was increased approximately 27.30% for rubber content, and molecular weight was increased 1.8-fold (Fig. 6, C and D), indicating that overexpression of the *HbCPT7* gene in the TKS played a certain role in promoting rubber biosynthesis and finally leading to an increase in rubber content and molecular weight.

DISCUSSION

Ethylene Stimulates Energy Metabolism and Latex Regeneration in the Laticifer Cells

Ethylene is an important plant growth regulator that can regulate many plant growth, development, and metabolic processes, including effects on natural rubber production (12). In natural rubber production, it has been proposed that the highest yield and optimal properties can be achieved by applying ethylene within the S/2 d3 harvesting system (19). Here, we used this system for producing rubber latex. Our newly obtained findings, similar to the previous reports (9, 12),

demonstrated that ethylene stimulation can lead to a notable increase in both the FY and dry yield of rubber latex and can also sharply prolong the flowing time of fresh rubber latex (Fig. 1). Ethylene stimulation has been found to not only improve sucrose uptake and degradation in laticifer cells (15) but also promote water absorption (9) and energy metabolism (7). One of the most striking physiological responses to ethylene stimulation is the significant extension of latex flow time (Fig. 1D). Ethylene upregulates aquaporin activity and facilitates water influx into laticifers, maintaining turgor pressure and sustaining latex flow over an extended period (9). Ethylene delays luteoid bursting and reduces oxidative stress, thereby delaying latex coagulation and preserving the fluidity necessary for continuous exudation (12). Ethylene promotes sucrose hydrolysis and assimilates transport into laticifers, ensuring a sustained supply of substrates for rubber biosynthesis during prolonged tapping (8). This prolonged flow could be a major contributing factor to the increased latex yield. It was reported that ethylene can promote nitrogen assimilation (21) and finally cause a defensive response (22). MAPK in the rubber latex can be induced after ethylene stimulation (12). Our proteomics data also revealed that MAPK can activate phosphatase activity and sucrose metabolism, thus helping to regenerate rubber latex in the ethylene-stimulated rubber trees (Fig. 2F and Supplemental Table S3).

Recent omics studies demonstrated that the detected DEPs are mainly involved in energy and carbohydrate

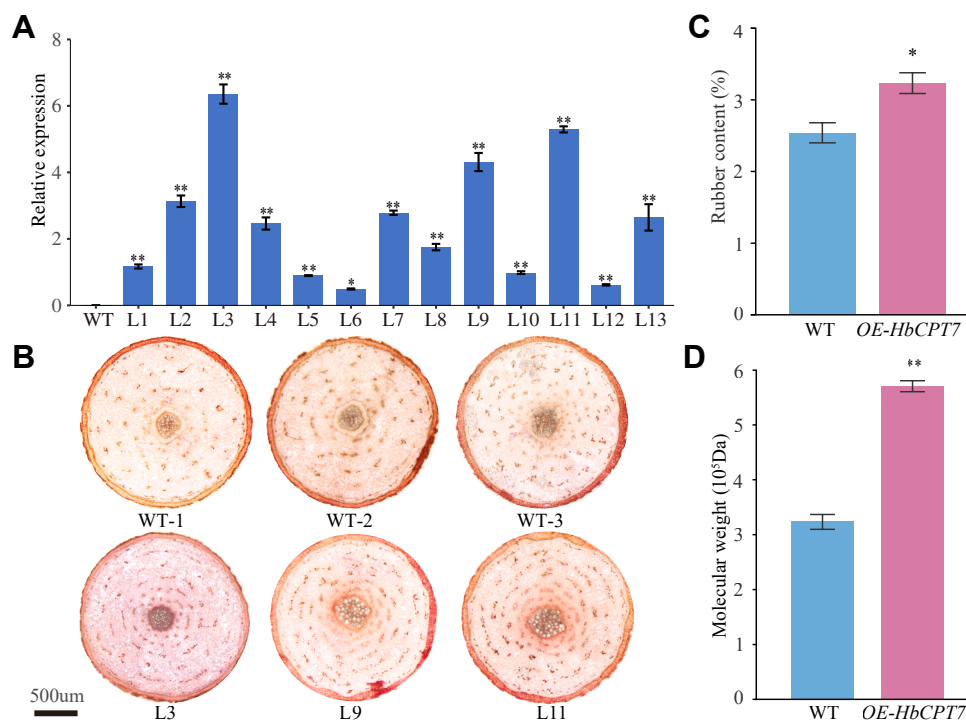


FIG. 6. **Function analysis of *HbCPT7*.** A, expression level of *HbCPT7* gene in positive transgenic plants (L1–L13). B, the number of laticifer cells of transgenic TKS compared with WT for 3 months. Rubber content (C) and rubber molecular weight (D) of WT and transgenic TKS roots for 4 months. *HbCPT7*, *Hevea brasiliensis* cis-prenyl transferase 7; TKS, *Taraxacum Kok-saghyz*.

metabolism (23, 24). Ethylene stimulation can change the activity of enzymes involved in carbohydrate metabolism (23). Our results in this study also revealed that ethylene stimulation can affect energy metabolism, carbon metabolism, pentose phosphorylation, and carbon fixation in photosynthesis (Fig. 4 and Supplemental Table S6), which are consistent with previous reports (3, 6, 11, 12). These results indicated that photosynthetic carbon fixation might play a significant role for latex production by continuously providing carbohydrates for glycolytic transformation on ethylene stimulation and thus finally enhance the sustainable rubber productivity (25, 26). These processes may be an important driving force to induce latex regeneration or discharge after ethylene stimulation.

Proteins Are Specifically Enhanced by Ethylene Stimulation at the Post-transcriptional Level

Based on integrated multiomics analysis, this study revealed that ethylene-induced yield enhancement in rubber trees was predominantly driven by a sophisticated reprogramming of translational and post-translational regulation within laticifers, rather than through transcriptional activation alone. The strikingly low correlation between protein abundance and corresponding mRNA levels across all time points provided compelling quantitative evidence for a dominant post-transcriptional regulatory layer. This pronounced transcript-protein decoupling indicated that ethylene signaling rapidly and effectively modulates protein output without relying primarily on changes in gene expression, enabling a more immediate and flexible physiological adjustment (27, 28). KEGG enrichment analysis revealed a coherent temporal progression: early phase enrichment in carbon and amino acid metabolism pathways corresponds with ethylene's known role in redirecting metabolic flux toward anabolic processes (29). The subsequent upregulation of ribosomal and endoplasmic reticulum processing pathways reflects a systemic investment in translational infrastructure, consistent with findings in other plant models where growth-promoting hormones enhance ribosome biogenesis (12). The late-phase coenrichment of ribosome, phagosome, and proteasome pathways indicates a shift toward proteostatic rebalancing, critical for maintaining cellular function under sustained hormonal signaling, as previously observed in stress-adapted tissues (8, 9). Notably, the translationally enhanced proteome included specific proteins with established roles in latex physiology. REF/SRPP family proteins, essential for rubber particle stability, and V-ATPase subunits, which regulate luteoid function and turgor-driven latex flow (25). Their selective upregulation *via* translational control provides a direct molecular link between ethylene signaling and yield enhancement, effectively bridging hormone perception and biophysical determinants of latex production (12). Building on the translation-centric regulatory network identified in this study, future endeavors may focus

on the targeted optimization of core components of the translational apparatus, such as ribosome assembly factors and translation initiation complexes, or on enhancing the translational efficiency and stability of key yield-related proteins, including REF/SRPP family proteins and V-ATPase subunits.

Exogenous Ethylene Changes the Expression of NRB-Related Genes and Proteins

Numerous genes associated with MVA and MEP pathways have been recognized (30–33). The first enzyme in the MVA pathway is acetyl-CoA acyltransferase (ACAT); four ACAT genes were obtained from *H. brasiliensis*, and ACAT protein accumulation was induced on ethylene stimulation (12, 31, 34). Here, three ACAT protein members accumulated in the latex, and except for ACAT3, were upregulated after 5 days of ethylene treatment; other DEPs of ACAT were significantly downregulated (Fig. 5 and Supplemental Table S7). A 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGS) has been previously detected from the rubber latex (35). In particular, HMGS2, as a glycosylated isoform of HMGS, was significantly increased on ethylene stimulation (18). In this study, we identified one gene and its corresponding protein belonging to the HMGS family, but interestingly, both the transcription and protein levels of this gene were significantly downregulated (Fig. 5 and Supplemental Table S7), and this observation was supported by our previous report (12). Meanwhile, four HMGR genes, but only one protein, were identified, in which the genes for HMGR1 and HMGR4 were significantly increased after ethylene application, but HMGR2 and HMGR3 were significantly decreased (Fig. 6 and Supplemental Table S7). It was reported that five HMGR genes have been detected in *H. brasiliensis* (25, 31), and these genes are slightly decreased by ethylene treatment (6, 36). Recently, three genes (25) and five protein isoforms of mevalonate kinase (MVK) were determined (19). Here, two MVK genes and their corresponding proteins were identified, and MVK2 was significantly upregulated (Fig. 5 and Supplemental Table S7). Two mevalonate diphosphate decarboxylase genes (25, 31) and proteins (19) were determined. Here, only mevalonate diphosphate decarboxylase 1 was detected; both gene and protein were highly decreased in E5/W5 treatment (Fig. 5 and Supplemental Table S7). Ethylene treatment was ever reported not to stimulate the gene expression in the MVA pathway, and therefore, at least some researchers considered that the contribution of ethylene to the increase of the rubber latex yield is minimal (36). However, our omics results showed that there are many DEGs and DEPs involved in the MVA pathway after ethylene stimulation, and some of them can be significantly increased, indicating that ethylene application can really stimulate the rubber latex production. In the Hevea rubber genome, 22 genes associated with the MEP pathway have been discovered (25, 31). Among them, two genes of 1-deoxy-D-xylulose

5-phosphate synthase were found in rubber latex (25), and expression of 1-deoxy-D-xylulose 5-phosphate synthase genes is significantly enhanced by ethylene treatment (6). Here, 14 genes and 4 proteins associated with the MEP pathway were detected, and most of them showed low abundance levels at both scales in transcription and protein production (Fig. 5 and Supplemental Table S7). These results indicate that genes/proteins in the MEP pathway may not be as important as those in the MVA pathway for NRB.

This study also identified several proteins related to natural rubber elongation, including CPT, REF, and SRPP, which play crucial roles in the final elongation stage of NRB (37). SRPP is an important protein for NRB (34), whose family members have been discovered in the Hevea rubber tree (31, 38, 39). In our study, 10 SRPP genes were detected, and only SRPP1 and SRPP4 were increased, whereas the other members were downregulated upon ethylene (Fig. 5 and Supplemental Table S7). REF was observed in both small rubber particles and large rubber particle (40), which can be phosphorylated after ethylene stimulation (19). Eight REF genes were obtained (2, 25, 31); among them, REF1 plays a key role for coagulation of rubber latex (41) and REF3 leads to dramatic increases in longer latex flowing time and latex yield under ethylene treatment (42). Here, seven REF members for genes and proteins were obtained, and all REF genes were slightly changed after ethylene stimulation (Fig. 5 and Supplemental Table S7) and supported by the previous report (6). Our results suggested that ethylene treatment can sharply improve rubber latex yield and significantly induce the expressions of many genes and proteins related to NRB and cell metabolism.

HbCPT7 Is Crucial for NRB on Ethylene Stimulation

CPT, also known as Hevea rubber transferase (34), exhibits stable membrane binding activity (43), and it can interact with HRT1-REF bridging protein (44). CPT genes have been determined in *H. brasiliensis* (2, 25, 31), TKS (39) and *Parthenium argentatum* (45), and our previous proteomics studies also indicated that CPT protein can be identified in rubber latex (12, 18), but it is expressed predominantly in small rubber particles (35). In addition, CPT protein can be positively identified in the latex of TKS root (14). Another study revealed that the expression of the CPT gene is significantly upregulated after ethylene addition (46), and ethylene can significantly improve CPT2 protein expression (12, 35). In mature roots of TKS, the expression of CPT7 was rapidly induced (14). In this omics-based study, we detected six CPT genes and found that three of their proteins were induced by ethylene stimulation. It is worth noting that CPT7 had been increased 2.4-fold and 1.6-fold after ethylene treatment for 1 day (E1/W1) and 3 days (E3/W3), respectively (Fig. 5 and Supplemental Table S7). Because of the long growth cycle and technical difficulty of genetic engineering for *H. brasiliensis* (47), more researchers used TKS as an ideal model plant to study the NRB mechanism. Compared with

H. brasiliensis, TKS has a more rapid growth cycle and is also easy for genetic manipulation (48, 49). In this study, the HbCPT7 gene was overexpressed in TKS and finally resulted in an increase in rubber content, which indicates that CPT may play an important role in NRB (Fig. 6, C and D). Therefore, these new results show that the CPT7 may be a key member for NRB in both rubber grass TKS and the rubber trees.

DATA AVAILABILITY

The transcriptome data have been submitted to the National Center for Biotechnology Information Sequence Read Archive database under the accession number PRJNA1064042 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1064042>), whereas the proteome data were uploaded to iProX with Project ID IPX0007976001 (<https://www.iprox.cn/page/subproject.html?id=IPX0007976001>, Username: wanglab, Password: wanglab2021).

Supplemental Data—This article contains supplemental data.

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Conflict of interest—The authors declare no competing interests.

Abbreviations—The abbreviations used are: ACAT, acetyl-CoA acyltransferase; CPT, *cis*-prenyl transferase; DEG, differentially expressed gene; DEP, differentially expressed protein; DM, dry matter; DRC, dry rubber content; FC, fold change; FY, fresh yield; GO, Gene Ontology; HbCPT7, *Hevea brasiliensis cis*-prenyl transferase 7; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMGS, 3-hydroxy-3-methylglutaryl coenzyme A synthase; IPP, isopentenyl pyrophosphate; KEGG, Kyoto Encyclopedia of Genes and Genomes; KOG, cluster of orthologous groups for eukaryotic complete genomes; MAPK, mitogen-activated protein kinase; MEP, 2-C-methyl-d-erythritol 4-phosphate; MS, mass spectrometry; MVA, mevalonate; MVK, mevalonate kinase; Nr, nonredundant protein sequences database; NRB, natural rubber biosynthesis; Pfam, protein family; qRT-PCR, quantitative real-time PCR;

REF, rubber elongation factor; SRPP, small rubber particle protein; TKS, *Taraxacum Kok-saghyz*; UniProt, universal protein database.

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