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Transcriptome Analysis Provides Insights into Lignin Biosynthesis in *Styrax tonkinensis* Branches

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Abstract: Approximately 12% of China's papermaking raw materials are derived from wood, while the majority are sourced from branches. *Styrax tonkinensis* is a more desirable species for pulpwood in subtropical ultra-short rotations, whose branches are prone to breakage. Lignin has a significant impact on wood quality and pulping yield, and the growth process influences lignin biosynthesis. To explore the lignin biosynthesis pathway in *S. tonkinensis*, we determined the lignin content in the current-year and biennial branches on 20 July, 20 September, and 20 October and analyzed the transcriptome sequencing results. It was concluded that the lignin content showed an increasing trend in the current-year branches (182.26, 206.17, and 213.47 mg/g, respectively), while that in the biennial branches showed a decrease in the samples taken in October, without significant difference (221.77, 264.43, and 261.83 mg/g, respectively). The transcriptome sequencing results showed that 91,513 unigenes were spliced with a total length of 92,961,618 bp. KEGG pathway analysis indicated that the upregulated DEGs were mainly enriched in the phenylpropanoid biosynthesis pathway. Our study suggested that CCoAOMT, COMT, peroxidase, and F5H may serve as key enzymes regulating lignin synthesis in branches of *S. tonkinensis*, thereby influencing the lignin content.

Keywords: lignin; branch; differentially expressed genes; biosynthesis

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1. Introduction

Wood is the primary carbon-rich biomass found on our planet and serves as a significant provider of eco-friendly fiber; it also plays a crucial role in meeting the increasing need for eco-friendly tissue, paper, packaging, textiles, and other products derived from fibers, including structural materials [1]. However, the current capacity to supply wood resources in the country is inadequate. According to the annual report of the China Paper Association, wood accounts for about 12% of China's papermaking raw materials, while the majority is sourced from branches. Branch thickness is a key wood characteristic for selecting trees with improved glucose yield and biomass production, and it also facilitates the exploration of wood formation mechanisms [2]. Histological investigations in forest tree branches have been the subject of several studies, especially in timber species such as *Populus tomentosa*, *Ulmus pumila*, *Betula platyphylla* × *Betula pendula*, and *Salix matsudana* [2–6]. However, research on branches or wood formation in *Styrax tonkinensis* has not been carried out.

Styrax tonkinensis (Pierre) Craib ex Hartw is a fast-growing tropical and subtropical tree species native to Southeast Asia and South China in the genus *Styracaceae* [7]. It is a great biomass energy tree with oil, medicinal, and wood benefits. *S. tonkinensis* stands 6 to 30 m tall and has a diameter at breast height (dbh) ranging from 8 to 60 cm [8]. Its wood is loose-holed, it has a straight trunk, a solid structure, and a fluffy substance, and it may be used to make matchsticks, furniture, and panels [8]. Initially, *S. tonkinensis* was cultivated in Yen Bai, Vietnam, in 1960, and then supplied pulp with short fibers to the Vinh Phu Province pulp and paper mill. It is utilized as a construction material in Laos' highlands

and employed in laminates because it peels easily and produces a durable veneer [9,10]. Previous studies have suggested that *S. tonkinensis* is a more desirable species for pulpwood in the subtropical ultra-short rotation period, and it holds significant value due to its fiber length of 1317~1657 μm, the aspect ratio of 43.94~56.78, the wall-to-cavity ratio of 0.32~0.53, the basic density of 0.3739 g/cm^3, the syntaxylated cellulose content of 78.618%, the α-cellulose content of 41.694%, the 10 g/L sodium hydroxide extractives of 25.328%, and the lignin content of 20.314% [11]. For *S. tonkinensis*, its branches are prone to breakage, which is what we observed in the forest management areas and the field. In addition, its wood-to-pulp ratio during the pulping process appears to be high (8.6 m^3/ton) [9].

Lignin largely influences the use of wood as a building material and the production of pulp and paper [12,13]. Stiffness, density, strength, and the amount of reaction wood are the primary characteristics of wood quality in construction lumber, while in the pulp and paper sectors, as well as in biofuel production, the length of the fibers and their chemical composition will play a more crucial role [14]. Lignin is a complex group of organic polymers that, together with cellulose and hemicellulose, form the backbone of plants. Lignin is formed predominately from three major monolignols: 4-coumaryl alcohol (H-subunit), coniferyl alcohol (G-subunit), and sinapyl alcohol (S-subunit), among which the ratio of S/G is a key factor that influences pulp yield [15,16]. In addition, a kind of natural lignin (called C-lignin) was discovered in the seed coats of vanilla orchids and most Cactoidae genera [17,18]. The lignin polymer is made up of monolignols obtained from the phenylpropanoid and monolignol biosynthetic pathways through a sequence of enzyme processes, the first of which is phenylalanine deamination [19]. It can be seen that the mastery of the lignin biosynthetic pathway is quite important for the efficient use of wood fiber biomass and the improvement of wood lumber properties. In the past, mutations in plants that occurred spontaneously and changed lignification patterns have been used to identify genes involved in the lignin production pathway. Stable genetic transformation is then used to characterize these genes functionally [20]. The abundant biological genome sequences now accessible for woody plants, along with the extensive genomics and transcriptomics resources available for poplar trees in particular, serve as a valuable foundation for conducting functional studies [21]. These ever-emerging molecular and genomic tools have strongly contributed to the achievement of transcriptional regulatory integrity in lignin synthesis.

Many factors affect lignin biosynthesis (e.g., light, plant hormones, amount of sugar, etc.), and the growth process is one of them. To understand the changes in the lignin content of *S. tonkinensis* branches during growth and to identify the relevant regulated genes in the lignin biosynthesis pathway, we collected current-year and biennial branches on 20 July, 20 September, and 20 October, measured the lignin content, and performed the transcriptome sequencing. We expect that our study could give a theoretical framework for a deeper comprehension of the *S. tonkinensis* lignin biosynthesis mechanism and the improvement of its wood properties. Additionally, we aim to contribute to the optimization of pulp production efficiency and the comprehensive utilization of branch wood.

2. Materials and Methods

2.1. Sampling Location and Materials

The material was collected from a five-year-old *S. tonkinensis* plantation (32°7′21.60″ N, 119°13′2.54″ E, 93 m altitude), located in the Xiashu Forestry Farm of Nanjing Forestry University (Jurong City, Jiangsu Province, China). This area is characterized by a north subtropical monsoon climate, showcasing four well-defined seasons with plentiful sunshine, an average annual temperature of 15.2 °C, a frost-free period lasting around 233 days, an average annual precipitation of 1055.6 mm, and an average annual relative humidity of 79%. The environment here is highly conducive to the growth and development of forestry production [22]. The *S. tonkinensis* plantation has a density of 112 plants/mu, with a spacing of 2.15 m × 2 m between plants.

The fast-growing period of *S. tonkinensis* was considered to be from late May to mid-September [23]. Numerous studies have shown that xylem formation in tree trunks during the growing season is an "S" curve, i.e., xylem grows slowly at the beginning of the growing season, and as it continues to warm up, xylem enters a period of rapid growth and then slows down and enters winter dormancy [24]. Therefore, we collected the internode portion of the apical current-year branches and biennial branches of the six sample trees (Figure 1). We washed them rapidly with sterile, enzyme-free water, dried them, and stored them in a −80 °C refrigerator.

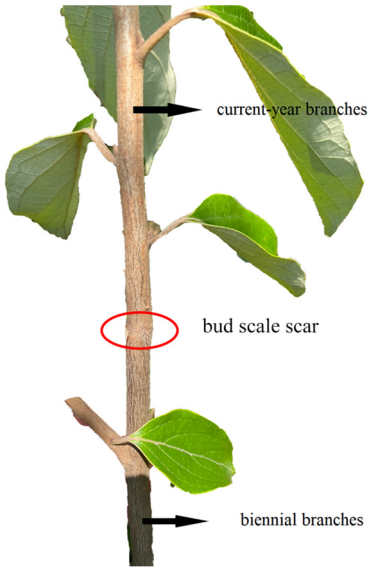

Figure 1. Collection sites of the branches.

2.2. Determination of Lignin Content

The determination of lignin involved utilizing the acetylation method [25]. The branches were dried at 80 °C until constant weight, crushed through a 40-mesh sieve, and weighed about 5 mg in a 10 mL glass test tube. The absorbance value at 280 nm after acetylation of the phenolic hydroxyl groups in lignin exhibits a direct correlation with the lignin content. We used the kit provided by Aoqing Biotechnology Co., Nanjing, China. Calculations were performed according to the formula Lignin (mg/g) = $0.0294 \times (\Delta A - 0.0068) \div 0.002 \times 50$, with ΔA being the difference between the absorbance values of the assay tube and the blank tube.

2.3. RNA Extraction, cDNA Library Construction and Sequencing

The materials were current-year and biennial branches of five-year-old *S. tonkinensis* under three-time points. Three biological replicates were sampled at each time, for a total of 18 samples. The total RNA was isolated using the mirVana miRNA Isolation Kit (OE Biotech Co., Ltd, Shanghai, China) [26]. The following apparatus was used next: Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA); TruSeq Stranded mRNA LTSample Prep Kit (Illumina, San Diego, CA, USA); Illumina HiSeqTM 2500 platform (generating 125 bp/150 bp paired-end reads).

2.4. Quality Control and De Novo Assembly

The transcriptome sequencing and analysis were carried out by OE Biotech Co., Ltd., Shanghai, China. Initially, we used Trimmomatic (version 0.39) to generate clean reads [27]. Subsequently, pure reads were assembled into clusters of expressed sequence tags (contigs) and de novo assembled into a transcript using Trinity (version: 2.4) [28].

2.5. Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment

Differentially expressed genes (DEGs) were detected using the DESeq (2012) functions. A significance threshold was set at p value < 0.05 and fold Change > 2 or fold Change < 0.5 for identifying significantly differential expression. This enrichment was carried out on the DEGs using R, based on the hypergeometric distribution.

2.6. Quantitative Real-Time PCR (qRT-PCR) Analysis

We chose to validate 8 genes associated with lignin biosynthesis through qRT-PCR. The primer sequences were developed by NCBI (https://www.ncbi.nlm.nih.gov/). StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and SYBR Green Premix Pro Taq HS qPCR Kits (AG11701, Accurate Biotechnology, Hunan, Co., Ltd., Changsha, Hunan, China) were used. The expression levels of the genes were determined using the $2^{-\Delta\Delta Ct}$ technique, with 18S ribosomal RNA serving as a normalization control. The primer details can be found in Supplementary Table S1.

2.7. Statistics Analysis

The data were analyzed and plotted using Origin 2023b. Tukey was chosen for mean comparison methods, and a p-value of less than 0.05 indicates a significant difference. PCA analysis was performed using the OECloud tools at http://cloud.oebiotech.com.

3. Results

3.1. Changes in Lignin Content in Current-Year and Biennial Branches at Different Periods

The lignin content of the branches collected in the three periods was determined. It was found that in the current-year branches, the lignin content showed an increasing trend, reaching 213.47 mg/g on 20 October, but there was no significant difference (Figure 2A). The lignin content in biennial branches showed an increase followed by a decrease (Figure 2B). The highest lignin content of 264.43 mg/g was found in the biennial branches on 20 September. A small decrease was observed on 20 October at 261.83 mg/g. Both the samples from 20 September and 20 October showed significant differences from 20 July ($p < 0.01$).

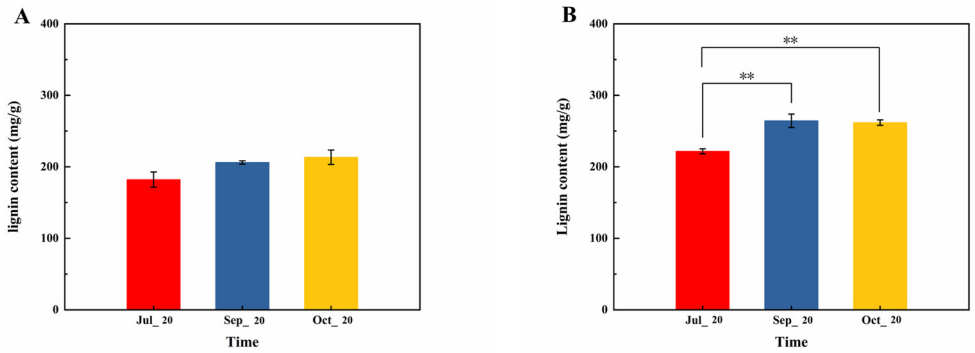

Figure 2. Analysis of lignin content in current-year and biennial branches under three sampling periods. (**A**) Lignin content in current-year branches. (**B**) Lignin content in biennial branches. "**" indicates significant differences ($p < 0.01$) between groups.

3.2. De Novo Assembly and Quality Control

A combined total of 124.78 G of clean data was acquired, with each sample containing an effective data volume ranging from 6.63 to 7.22 G. The Q30 base distribution varied from 93.63 to 94.19%, while the average GC content was recorded at 46.84%. Furthermore, the comparison of reads to the unigene database revealed a comparison rate falling within the range of 87.53 to 91.6%. 91,513 unigenes were spliced, and the total length was 92,961,618 bp (Table S2).

3.3. PCA Analysis

A PCA analysis showed that PC1 and PC2 contributed 48.36% and 16.28%, respectively. Significant differences were found between groups for both current-year and biennial branches, and there was good repeatability of samples within groups (Figure 3).

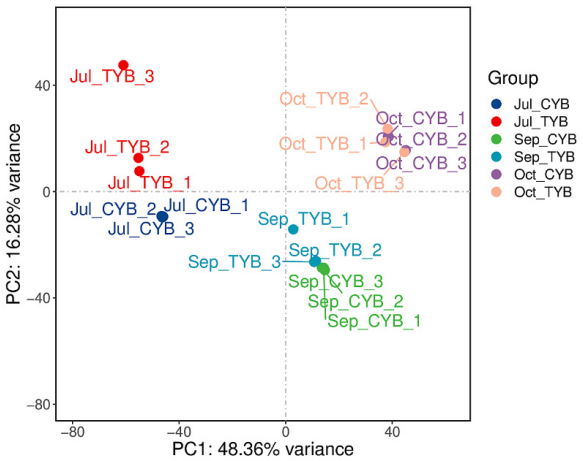

Figure 3. PCA analysis of six samples. "CYB" and "TYB" denote current-year branches and biennial branches, respectively; abbreviations are used for months; the same applies below.

3.4. Analysis of DEGs

It was found that in both current-year branches and biennial branches, the largest number of DEGs were found in the October and July samples, and the lowest DEGs were found in the October and September samples (Figure 4).

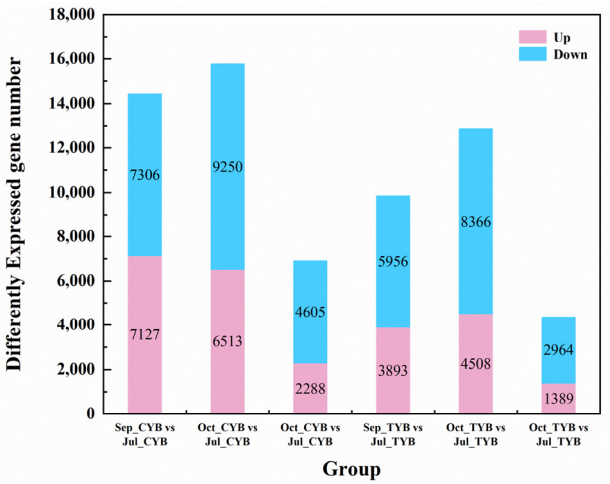

Figure 4. Gene expression in each sample of *Styrax tonkinensis* branches in July, September, and October.

3.5. KEGG Pathway Enrichment Analysis of DEGs

The up-regulated genes showed significant enrichment in various metabolic pathways, including "Phenylpropanoid biosynthesis", "Glutathione metabolism", and "Starch and sucrose metabolism" (Figures 5 and 6), while the down-regulated genes were mainly enriched in metabolic pathways and pathways related to environmental information processing, such as the "Plant hormone signal transduction" and "MAPK Signaling pathway".

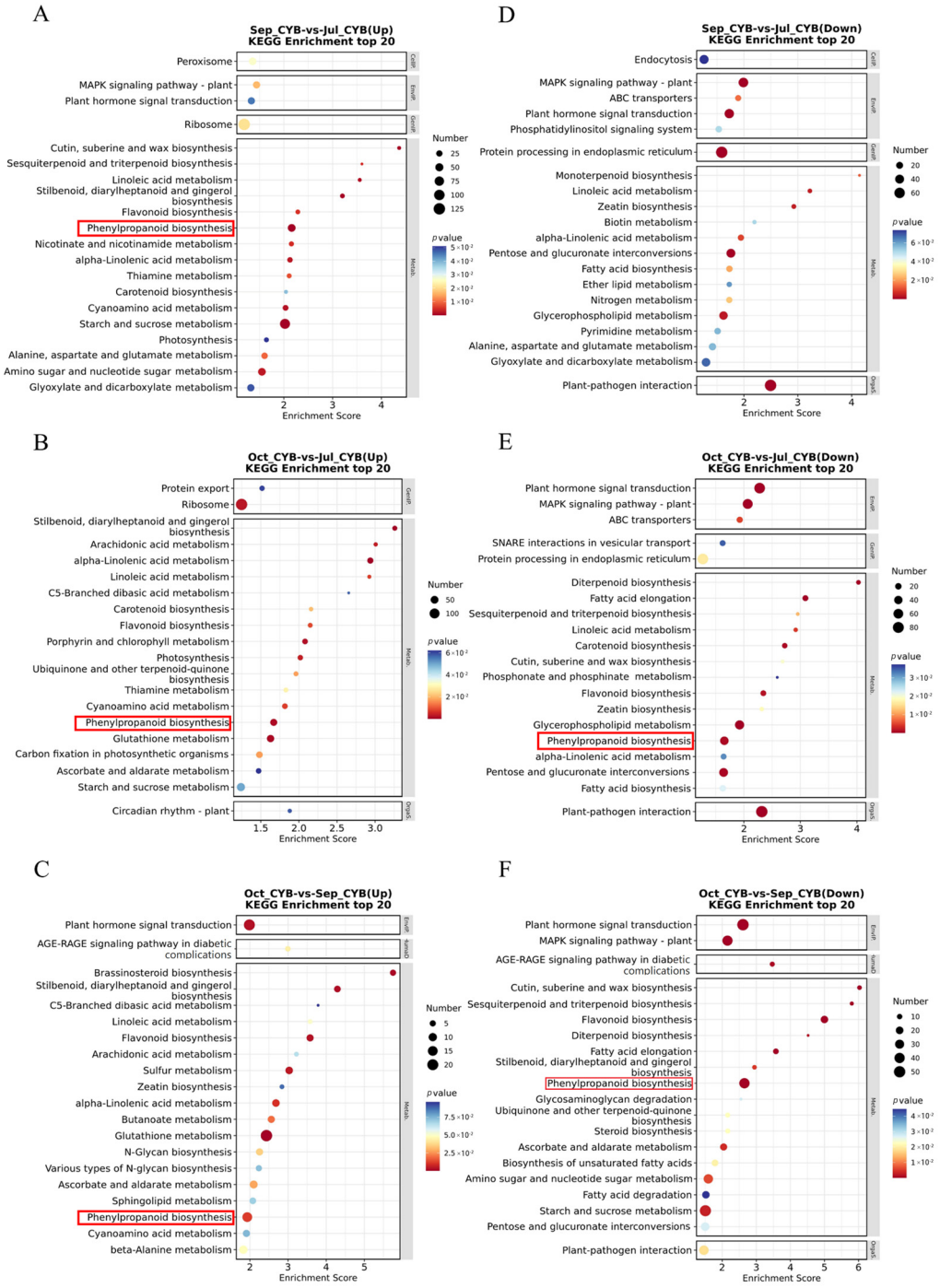

Figure 5. KEGG pathway analysis of up/down DEGs in the current-year branches. (**A**–**C**) or (**D**–**F**) in order of September vs July, October vs. July and October vs September. "Phenylpropanoid biosynthesis" is highlighted with a red border.

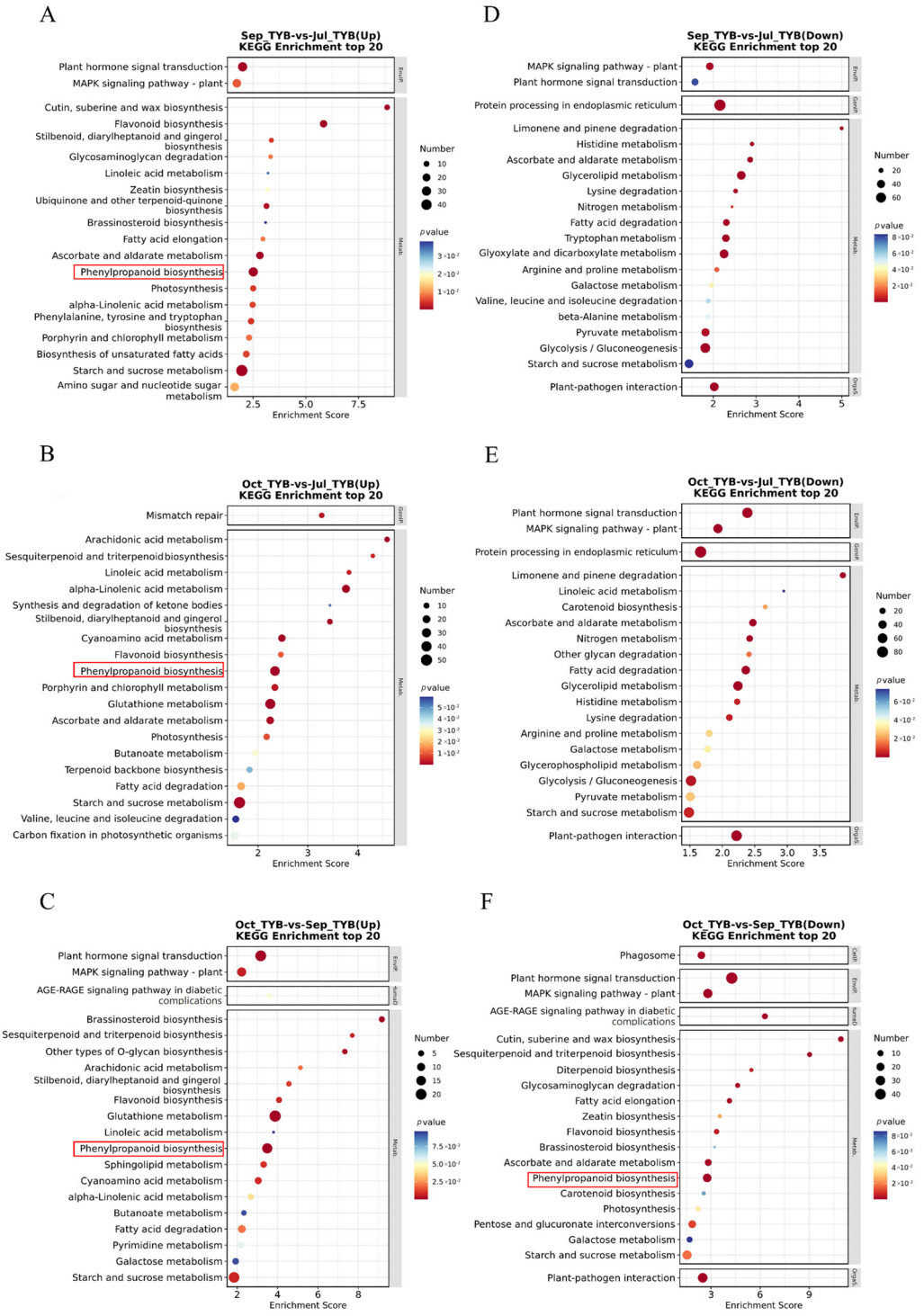

Figure 6. KEGG pathway analysis of up/down DEGs in the biennial branches. (**A**–**C**) or (**D**–**F**) in order of September vs July, October vs. July and October vs September. "Phenylpropanoid biosynthesis" is highlighted with a red border.

3.6. Identification of DEGs Associated with Lignin Biosynthesis

DEGs on the phenylpropanoid biosynthesis pathway (ko00940) were screened in the current-year and biennial branches, and further, DEGs with a GO annotation of the lignin biosynthesis process (GO: 0009809) were selected (Table S3).

In the current-year branches, 17 DEGs in total were identified. A further analysis was conducted on the up-regulated genes. In the comparison of Jul_CYB vs. Sep_CYB, four up-regulated genes were identified, including two encoding caffeic acid 3-O-methyltransferase

[EC:2.1.1.68], one encoding caffeoyl-CoA O-methyltransferase [EC:2.1.1.104], and one encoding peroxidase [EC:1.11.1.7]. When comparing Jul_CYB vs. Oct_CYB, a total of seven genes were up-regulated, with three encoding caffeoyl-CoA O-methyltransferase [EC:2.1.1.104], two encoding caffeic acid 3-O-methyltransferase [EC:2.1.1.68], and two encoding beta-glucosidase [EC:3.2.1.21]. In the comparison of Sep_CYB vs. Oct_CYB, three genes showed up-regulation, with two encoding beta-glucosidase [EC:3.2.1.21] and one encoding peroxidase [EC:1.11.1.7].

There were a total of 12 DEGs in the biennial branches, which were also screened in the current-year branches. Between Jul_TYB and Sep_TYB, two genes with increased expression levels were detected, encoding caffeoyl-CoA O-methyltransferase [EC:2.1.1.104] and caffeic acid 3-O-methyltransferase [EC:2.1.1.68], respectively. There were six up-regulated genes in Jul_TYB vs. Oct_TYB, of which two were encoding caffeoyl-CoA O-methyltransferase [EC:2.1.1.104], two were encoding caffeic acid 3-O-methyltransferase [EC:2.1.1.68], and two were encoding beta-glucosidase [EC:3.2.1.21]. Additionally, in the Sep_TYB vs. Oct_TYB comparison, five down-regulated genes were identified, with two encoding caffeic acid 3-O-methyltransferase [EC:2.1.1.68], two encoding ferulate-5-hydroxylase [EC:1.14.-.-], and one encoding peroxidase [EC:1.11.1.7]. Notably, the key enzymes identified from our analyses—caffeoyl-CoA O-methyltransferase (CCoAOMT), caffeic acid 3-O-methyltransferase (COMT), peroxidase, and ferulate-5-hydroxylase (F5H)—may play crucial roles in regulating lignin synthesis in branches of *S. tonkinensis* (Figure 7).

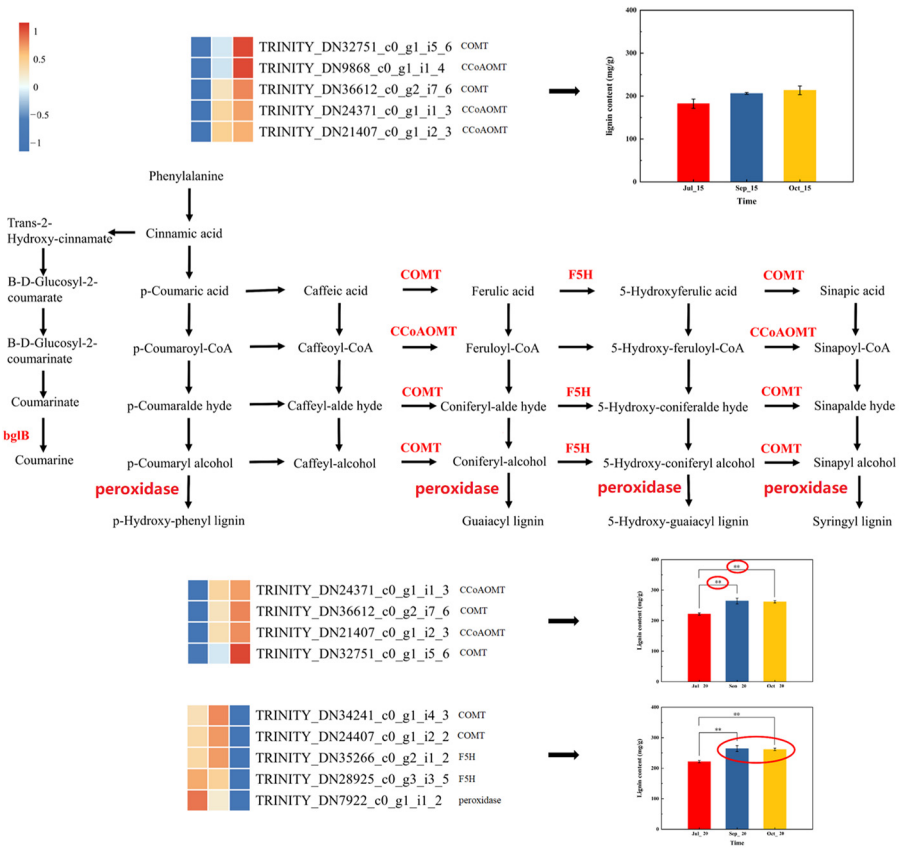

Figure 7. Heat map of the pathway of several important DEGs in the lignin biosynthesis pathway of *S. tonkinensis*. The squares in the heat map represent July, September, and October, from left to right. Red squares indicate high expression and blue indicates low expression. "**" indicates significant differences ($p < 0.01$) between groups.

By plotting the expression levels of the eight DEGs obtained via qRT-PCR and the FPKM values, the results showed consistent expression trends (Figure S1).

4. Discussion

During tree growth, we can collect secondary xylem at different developmental stages, and then select key groups with significant differences for histological sequencing based on anatomical observation or determination of content [29]. In our study, the lignin content of the current-year branches showed a gradual increase over the three sampling times, while the lignin content of the biennial branches had a slight decrease in October. The results of the annual shoots of the two peonies stained with mesquite showed a gradual increase in lignin accumulation, as did our study [24]. A more comprehensive study was carried out in bamboo, and the results showed that both total lignin and acid-insoluble lignin content increased with age while acid-soluble lignin content decreased, and the authors speculated that this might be due to impurities such as proteins and furfural that affected the absorbance at 240 nm [25].

Many researchers agree that the lignin synthesis process can be divided into three stages. The first stage is the mangiferic acid pathway, which involves the conversion of plant assimilation products after photosynthesis into the aromatic amino acids phenylalanine, tyrosine, and tryptophan; the second stage is the phenylalanine pathway, which leads from phenylalanine to hydroxycinnamic acid (HCA) and its coenzyme A lipids; and the third stage is the lignin synthesis-specific pathway, which proceeds from hydroxycinnamic acid coenzyme A esters to the synthesis of lignin monomers and their polymers [30]. The phenylpropanoid pathway involves a series of enzymes governing various sequential steps. These enzymes belong to an assembly of genes and gene families, including phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), *p*-coumaroyl-shikimate/quinate 3-hydroxylase (C3H), caffeoyl-CoA O-methyltransferase (CCoAOMT), 5-hydroxyconiferyl aldehyde O-methyltransferase (AldOMT), coniferyl aldehyde/ferulate 5-hydroxylase (CAld5H/F5H), cinnamyl alcohol dehydrogenase (CAD), cinnamoyl-CoA reductase (CCR), caffeoyl shikimate esterase (CSE), and caffeic acid O-methyltransferase (COMT) [21]. Subsequently, laccases and peroxidases catalyze the oxidative polymerization of monolignols, with laccases utilizing molecular oxygen and peroxidases utilizing hydrogen peroxide [31]. Through transcriptome analysis, we suggested that CCoAOMT, COMT, peroxidase, and F5H might be the four key enzymes involved in the variation of lignin content in branches of *S. tonkinensis*. However, the key enzymes screened varied in different plant stems. For example, 4CL, COMT, CCoAOMT, CAD, POD, PAL, and CCR were screened in *Platycladus orientalis*, while PAL, C4H, 4CL2, CCR, and COMT were screened in *Paeonia* [32,33]. Such differences may be due to genetic variations, environmental adaptations, and other factors. The enzymes we screened were discussed primarily in the next paragraphs.

CCoAOMT is a methyltransferase involved in monolignol synthesis that provides precursors for coniferyl and sinapyl alcohols, which is one of the key enzymes regulating G lignin synthesis, and it is also essential in providing substrates for the synthesis of syringyl lignin [34,35]. Research has indicated a direct relationship between the amount of lignin found in ramie stems and the activity levels of CCoAOMT genes [36]. The antisense approach in transgenic poplar plants has been shown to be effective in reducing the total lignin content. This reduction is primarily due to decreased levels of guaiacyl and syringyl lignins, which are key components of lignin. The repression of CCoAOMT expression plays a crucial role in this process [35]. Conversely, the increased accumulation of lignin in Arabidopsis and Poplar was observed when a CCoAOMT gene from the Dove Tree was overexpressed [37]. Similar results were also obtained in studies of flax, jute, tea plants, and *Paeonia ostii* [38–41].

COMT is another important methyltransferase in the lignin synthesis pathway. It has been discovered that it essentially regulates the biosynthesis of syringyl lignin units [42]. In 6-month-old transgenic poplars (*Populus tremula* × *Poppulus alba*) with almost zero COMT activity, lignin levels were significantly reduced by 17%, and syringyl units were almost completely absent [43]. Zhao et al. [44] obtained similar results in tobacco and found that

COMT and CCoAOMT acted synergistically and that inhibition of CCoAOMT caused a greater reduction in lignin content than inhibition of COMT.

The final catalytic step in the synthesis of lignin is the oxidation of cinnamyl alcohols, catalyzed by a peroxidase and/or laccase enzyme [45,46]. This enzyme also participates in the regulation of lignin polymerization and the generation of reactive oxygen species, contributing to plant defense mechanisms. Moreover, Prx has been recognized for its involvement in lignin modification and remodeling during plant growth and development. Prx activity is positively correlated with lignin accumulation in loquat pulp tissue [47]. By employing an antisense approach, researchers reduced the expression of a tobacco peroxidase isoenzyme (TP60), leading to genetically modified plants showing a decrease in the lignin content of approximately 40%–50% in comparison to unmodified plants [48]. Transgenic gerbera plants with the Prx gene exhibited a greater lignin content compared to wild-type plants, resulting in reduced stem bending and enhanced cell wall stiffness of cut gerbera flowers [49].

F5H is a crucial enzyme that plays a role in the biosynthesis of sinapyl (S) monolignol in angiosperms [50]. F5H genes in Populus, Arabidopsis, and Flax were cloned to verify their functionality, improve timber properties, or enhance saccharification [50–53]. Studies have also been conducted to regulate the role of F5H genes in plant resistance; for example, knockout of the F5H gene in oilseed rape improved sclerotinia sclerotiorum resistance, and F5H genes were significantly upregulated in oleocellosis-damaged flavedo, which may enhance the pathogen resistance of citrus fruit [54,55].

Precise regulation of lignin synthesis is conducive to the efficient use of wood fiber biomass for energy and wood property improvement. Gui et al. [56] focused on a transcription factor associated with lignin, known as LTF1. Modification of LTF1 for more stable control of lignin synthesis, followed by combination with the promoters of cell type-specific genes, provided precise control of lignin synthesis in vessel and fiber cells, respectively, and the results showed that modulation of lignin synthesis in fiber cells significantly improved the efficiency of lignocellulosic fiber biomass accumulation and utilization, whereas the opposite was true in vessel cells. The researchers identified a set of six director proteins (DPs) in *Arabidopsis thaliana* that are located in the Casparian strip of root endodermal cells and regulate the precise deposition of lignin there, providing a new theory for the molecular design of future crops for the efficient use of water and nutrients and the creation of efficient "carbon sink" plants [57].

Moreover, lignin is active in a wide range of abiotic and biotic stresses. It was reported that stress-related lignin is controlled at several regulatory levels, and changes in cellular redox status caused by the accumulation of reactive oxygen species (ROS) in response to biotic and abiotic stresses are likely to be the first layer of regulation during lignin formation [58]. Regulation of lignin synthesis is favorable to the breeding of varieties better adapted to environmental stresses. For example, tobacco and Arabidopsis plants overexpressing *CsHCTs* showed increased lignin content and resistance to abiotic stresses and bacterial infections [59]. *PuC3H35* could enhance lignin biosynthesis, which conferred drought tolerance in Populus ussuriensis [60].

The widespread presence of AC elements in the promoters of major phenylpropanoid and lignin biosynthesis genes suggests regulation by transcription factors (TFs) [61]. Based on the results of molecular biology studies in Arabidopsis, a hierarchical network of NAC and MYB transcription factors is thought to be the main regulatory mechanism for lignin synthesis, in which NAC transcription factors directly activate *MYB46*/*MYB83*, followed by downstream activation of *MYB58*, *MYB63*, *MYB85,* and lignin biosynthesis genes [62]. Overexpression of lignin-specific MYBs—*MYB58*, *MYB63*, and *MYB85*—led to the activation of lignin-biosynthesis genes and the ectopic deposition of lignin in cells that are usually not lignified [63]. Similarly, not only in model plants, overexpression of *EgNAC141* from *Eucalyptus* positively regulated lignin biosynthesis [64]. Coordinated networks regulating lignin biosynthesis also involve microRNAs (miRNAs) and long

noncoding RNAs (lncRNAs) [65]. The exploration of the transcriptional regulatory network of lignin synthesis is still widely underway.

For *S. tonkinensis*, genome sequencing results are not available, and there is no genetic transformation system or sufficient research base. Therefore, further research will require more time, money, and energy. About the lignin synthesis in *S. tonkinensis*, this is the first exploration, and a great deal of work remains to be done to master and delve into the regulatory mechanisms. We are currently analyzing data on the lignin monomer composition of *S. tonkinensis* stems and the activities of key enzymes involved in lignin biosynthesis to gain further insights. Certainly, anatomical microscopy is also a method that needs to be supplemented and adopted. In the future, attempts will be made to manipulate and regulate key genes in the lignin biosynthesis pathway of *S. tonkinensis* through biotechnological approaches such as gene silencing, antisense RNA technology, and others. This may serve to improve wood properties, optimize the efficient utilization of lignocellulose, and facilitate further research endeavors.

5. Conclusions

To comprehend the lignin synthesis in *S. tonkinensis* branches and its regulatory mechanisms, we determined the lignin content of both the current-year and biennial branches during the months of July, September, and October. Our findings revealed an increasing trend in lignin content in the current-year branches, whereas the lignin content in the biennial branches exhibited a decline in October, with no obvious difference. We also discovered several DEGs related to the lignin biosynthesis process, and it was concluded that CCoAOMT, COMT, peroxidase, and F5H may be the key enzymes regulating lignin biosynthesis in the branches of *S. tonkinensis*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15040601/s1, Figure S1: qRT-PCR verification of eight DEGs; Table S1: Primers used for quantitative real-time PCR (qRT-PCR) analysis; Table S2: Summary of sequencing data quality preprocessing results; Table S3. DEGs selected from lignin biosynthesis process.

Author Contributions: Conceptualization, F.Y. and C.H.; methodology, C.H.; software, C.H. and Q.X.; validation, C.H. and H.C.; formal analysis, C.H.; investigation, C.H. and Q.X.; resources, H.P.; data curation, C.H.; writing—original draft preparation, C.H.; writing—review and editing, F.Y.; visualization, C.H.; supervision, F.Y.; project administration, F.Y.; funding acquisition, C.H. and F.Y. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The transcriptome data used in this article have been uploaded to the National Center for Biotechnology Information (NCBI), the accession number is PRJNA1062279.

Conflicts of Interest: The authors declare no conflicts of interest.

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