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Transcriptome Analysis Reveals Gene Expression Changes during Repair from Mechanical Wounding in *Aquilaria sinensis*

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Abstract: Plants repair their mechanical wounds by reprogramming secondary metabolism. However, which genes are reprogrammed during this repair process in *Aquilaria sinensis* has rarely been studied. Here, we used high-throughput RNA sequencing to explore the changes in the transcriptome of *Aquilaria*'s xylem, six months after the stem was subjected to mechanical wounding. In total, 1165 transcripts were differentially accumulated, of which 1002 transcripts were increased and 163 were decreased in their abundances ($|\log_2$ (fold change)$| \geq 1$ and FDR ≤ 0.05). The majority of these genes encode products involved in plant secondary metabolism, transcription regulation, and phytohormone metabolism and signaling. The up-regulated genes were classified into 15 significantly enriched GO terms and were involved in 83 pathways, whereas the down-regulated genes were classified into 5 significantly enriched GO terms and represented 43 pathways. Gene annotation demonstrated that 100 transcripts could encode transcription factors (TFs), such as WRKY, AP2, MYB, and Helix-loop-helix (HLH) TFs. We inferred that the differential expression of TFs, genes associated with plant hormones, phenylpropanoid biosynthesis, and sesquiterpenoid biosynthesis may contribute to the repair of the stem after mechanical wounding in *A. sinensis*. Using co-expression analysis and prediction of TF binding sites, a TF–gene regulatory network for *Aquilaria* lignin biosynthesis was constructed. This included the MYB, HLH, WRKY, and AP2 TFs, and the *COMT1*, *4CLL7*, and *CCR1* genes. The changes in 10 candidate genes were validated by quantitative reverse-transcription PCR, indicating significant differences between the treated and untreated areas. Our study provides global gene expression patterns under mechanical wounding and would be valuable to further studies on the molecular mechanisms of plant repair in *A. sinensis*.

Keywords: *Aquilaria sinensis*; mechanical wounding; transcriptome; sesquiterpene biosynthesis; phenylpropanoid biosynthesis

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1. Introduction

Mechanical wounding is a common abiotic stress for plants, which can activate specific genes and metabolic pathways, and finally influence the production of secondary metabolites [1]. Plant secondary metabolites are used for producing functional foods, dietary supplements, and pharmaceuticals [2,3]. Their wound-induced production could potentially be exploited as a strategy for generating high levels of bioactive compounds [4–6]. Manipulating a single gene encoding an enzyme or a transcription factor (TF) might be insufficient to maintain the productivity under mechanical wounding [7]. An understanding of the transcriptome changes during mechanical wounding could contribute to artificially inducing the production of secondary metabolites.

One of the important steps in the process of repair from mechanical wounding is the reprogramming of the transcriptome, and it involves several genes including those encoding TFs such as WRKY and AP2 [8–11]. The 'repair' genes encode proteins that function in signaling, phytohormone regulation, transcription regulation, and the synthesis of secondary metabolites [8]. Previous studies in *Dalbergia odorifera* and *Dracaena cochinchinensis* showed that wounding up-regulated *CHS* (*chalcone synthase*), *CHI* (*chalcone isomerase*), *ANS* (*anthocyanidin synthase*), *PAL* (phenylalanine ammonia-lyase), *C4H* (*cinnamate 4-hydroxylase*), *4CL* (*4-coumarate-CoA ligase*) and other gene families related to the phenylpropanoid and the flavonoid pathways ultimately promoted flavonoid biosynthesis [4,12]. Further, a study of the mechanical wounding of the stems of Scots pine seedlings showed that the MYB and NAC TFs were up-regulated and highly correlated to phenylpropanoid biosynthesis. Here, the concentration of stilbenes, resin acids, and lignans were increased in the xylem that was next to the wound [13].

Aquilaria sinensis (Lour.) Gilg (Thymelaeaceae) is an agarwood tree, the resin portion of which is the product of its external injuries [14]. Agarwood is widely used in traditional medicines, perfumes, and incense across China, Japan, India, and some Southeast Asian countries [15,16]. Research on *A. sinensis* has been mainly focused on understanding the methods of agarwood formation and the identification of its chemical components [17–19]. Only a few reports are available about the composition of its transcriptome and the regulation of gene expression in *A. sinensis* under mechanical wounding [20]. Genes related to sesquiterpene synthesis, such as *HMGR* (*hydroxymethylglutaryl-CoA reductase*), *DXPS* (*1-deoxy-D-xylulose-5-phosphate synthase*), *FPS* (*farnesyl diphosphate synthase*), and *ASS* (*sesquiterpene synthases*), were differentially expressed. Further, genes related to plant defense and stress response, such as the AP2, WRKY, and MYC TFs, were also differentially expressed [20].

The repair process of *A. sinensis* is not well understood. We hypothesize that wound repair would entail a complex reprogramming of metabolic processes, which would exhibit a polygenic character and involve a substantial part of the transcriptome. Therefore, we deployed a deep sequencing technique to monitor a possible large-scale transcriptomic reconfiguration. Here, we provide insight into how *Aquilaria* plants repair themselves after mechanical wounding by a comparative analysis of the transcriptomes of the treated and untreated areas in the xylem of the *Aquilaria* stem. We deployed high-throughput RNA sequencing (RNA-Seq) as it now enables comprehensive and cost-effective analyses of gene expression patterns in many non-model plant species [21]. By reporting genome-wide changes in *A. sinensis* during repair after mechanical wounding, we identified the genes that might function in the signal-transduction and metabolic pathways. This study would further enable investigations on improvements in agarwood quality and yield while protecting the endangered wild *A. sinensis*. These results would also assist in developing a holistic understanding of the repair mechanism of woody plants to mechanical wounding.

2. Materials and Methods

2.1. Plant Material and Sample Collection

The three well-developed *Aquilaria* trees (about the age of 5 years) were grown at an artificial nursery in Hainan Province in China (19°38′56″ N, 110°14′29″ E). The trees were approximately 5 to 6 m high, and larger than 8 cm in diameter. We used a chisel to cut a fan-shaped wound at the height of 1 m in the stem, and the depth of the wound was the radius of the stem. The mechanical wounding treatment started at 9 a.m. on 2 July 2020, and samples were collected after 6 months. The mature xylem of the wound was set as the treated area (TA). The mature xylem of the untreated area (UA) located 1 m above the TA was set as the control. The TA and UA samples were isolated from the same individual to enable comparisons in the same genetic background (Figure S1). The collected samples were immediately frozen in liquid nitrogen for RNA isolation.

2.2. RNA Extraction and Qualification

Six samples (TA1, TA2, TA3, UA1, UA2 and UA3) were used for RNA extraction. Total RNA was isolated using the RNAprep pure plant plus kit (Tiangen, China). Agarose gel electrophoresis was performed by DYY-6D electrophoresis apparatus of Beijing Liuyi Biological Technology Co., Ltd. (Beijing, China) to evaluate RNA degradation and contamination. A NanoDrop 2000 (Thermo Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies) were used to assess RNA quality and concentration. Only those RNA samples with 260/280 ratios of 1.9 to 2.2, 260/230 ratios $\geq$ 2.0, and RIN (RNA integrity number) > 7.2 were used for further experiments. Polyadenylated mRNA was enriched from 0.4 µg total RNA by using oligo (dT) magnetic beads.

2.3. Transcriptome Profiling of Xylem of A. sinensis under Mechanical Wounding

For Illumina sequencing, fragmentation buffer was added to produce short mRNA fragments. The cDNA libraries were constructed with the help of NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA). We used random hexamer primers to synthesize single-stranded cDNA from these mRNAs and added dNTPs, buffer, and DNA polymerase I to synthesize double-stranded cDNA. The double-stranded cDNA was purified using AMPure XP system (Beckman Coulter, Beverly, MA, USA). Then, end repair, poly-A tail addition, ligation sequencing adapters, and fragment size selection were performed. Finally, the six cDNA libraries were subjected to PCR enrichment and sequenced on the Illumina NovaSeq 6000 platform.

After sequencing, clean reads were obtained by removing low-quality reads, 3′ ends with base 10 quality score (Q) < 20 ($Q = 10 \log^{error\_ratio}$), reads containing N blur, any adapter sequences, and any sequences with < 20 nucleotides. The clean reads were mapped to the reference genome sequence of *A. sinensis* using HISAT2 (v 2.0.5) and read counts for each gene were obtained [22]. The read counts were converted into FPKM values. Then, we estimated the expression levels of mRNAs through the FPKM values.

Differentially expressed genes (DEGs) were obtained using the following criteria: $|\log_2 (\text{fold change})| \geq 1$ and FDR ≤ 0.05. All DEGs were mapped to GO terms of the Gene Ontology database (http://www.geneontology.org/ accessed on 10 July 2021), and gene numbers were calculated for every term. GO enrichment analysis was conducted by GOseq software to identify significantly enriched terms. The analysis of gene-regulatory pathways was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database (http://www.genome.jp/kegg/pathway.html accessed on 17 July 2021). The functional pathway analysis was conducted using MapMan software (v 3.6.0RC1; http://mapman.gabipd.org accessed on 17 July 2021) [23].

2.4. Construction of the Co-Expression Network

Co-expression among genes and TFs related to phenylpropanoid biosynthesis was assessed with the help of Pearson correlation coefficient, calculated in R (v 4.0.1) (Table S1). *Aquilaria* tree gene promoter sequences (2000 bp upstream of the transcription start site in most cases) were retrieved and analyzed using TBtools (v 1.098696; https://github.com/CJ-Chen/TBtools accessed on 1 November 2021). The promoter analysis and the prediction of TF binding sites were conducted using PlantPAN 3.0 (http://plantpan.itps.ncku.edu.tw/ accessed on 1 November 2021) [24]. The TF–gene interaction networks were visualized using Cytoscape (v 3.7.2) [25].

2.5. Quantitative Reverse-Transcription PCR (qRT−PCR) Validation

cDNA was synthesized by reverse transcription using 1 µg total RNA from the six samples (TA1, TA2, TA3, UA1, UA2 and UA3), respectively. Ten genes were randomly selected from the TA and UA in the xylem of the *Aquilaria* stem. Specific primers for the target genes were designed by using Primer Premier v5 software (Table S2). For the latter, using TB Green Premix Ex Taq II (Tli RNaseH Plus; Takara, Beijing, China) for qRT−PCR analysis. The amplification reaction was incubated at 94 °C for 2 min, followed by 40 cycles

of 95 °C for 5 s and 60°C for 30 s. Melting curve analysis was performed at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The *AcUbiquitin* and *AcHistone* genes served as internal controls for normalization (Table S2). The relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method against the internal control [26]. Three technical replicates were performed for each sample to ensure reproducibility and reliability.

3. Results

3.1. Global Transcriptome Analysis of the RNA-seq Data

The RNA-seq experiment generated a total of 146,892,168 (TA) and 145,971,064 (UA) raw reads. After trimming, 140,828,722 (TA) and 139,775,768 (UA) clean reads remained (Table 1). Among the total clean reads, 127,784,588 (TA) and 129,637,924 (UA) were mapped to the *Aquilaria* tree genome with average mapping ratios of 90.77% (TA) and 92.74% (UA) (Table 1). The analysis identified a total of 1165 differentially expressed transcripts ($|\log_2$ (fold change)$| \geq 1$ and FDR ≤ 0.05; Table S3) in TA: the accumulation of 163 transcripts decreased and the abundances of 1002 transcripts increased (Figure 1a and Figure S2). These transcripts were used for subsequent analysis.

Table 1. Summary of RNA-seq data from treated area (TA) and untreated area (UA).

Sample	Raw Reads	Clean Reads	Mapped to Genome	Mapping Ratio (%)
TA1	50,104,244	49,039,422	44,415,691	90.57%
TA2	50,548,442	47,978,998	43,143,945	89.92%
TA3	46,239,482	43,810,302	40,224,952	91.82%
UA1	48,359,462	46,624,834	43,266,564	92.80%
UA2	46,146,082	44,122,496	40,868,251	92.62%
UA3	51,465,520	49,028,438	45,503,109	92.81%

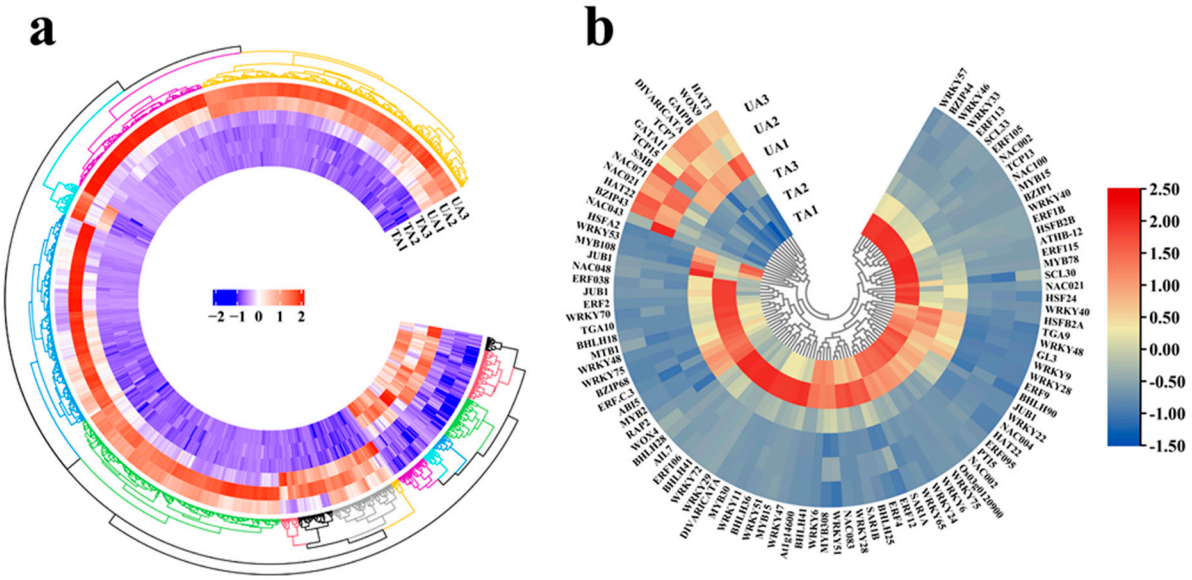

Figure 1. Expression profiles of mRNAs in *A. sinensis*. (**a**) Hierarchical clustering of all differentially expressed transcripts. (**b**) Hierarchical clustering of differentially expressed transcription factors.

3.2. GO and KEGG Enrichment Analyses of DEGs

To evaluate their potential functions, GO categories were assigned to 1165 transcripts that were differentially expressed during the repair from mechanical wounding. For genes with increased transcript abundance, 15 significantly enriched GO terms were identified. All of the terms related to molecular functions included 'iron ion binding', 'DNA binding transcription factor activity', 'transcription regulator activity', and 'terpene synthase activity' (Figure S3a, Table S4). Among the genes with a decreased transcript abundance, only five significant GO terms were identified in molecular function, which included

'nucleoside-triphosphatase activity', 'pyrophosphatase activity', and 'ATPase activity' (Figure S3b, Table S4).

The KEGG pathway enrichment analysis was subsequently conducted for DEGs in *A. sinensis* to gain further insights. Eighty-three pathways were related to the set of genes with increased transcript abundances. Six of the most significantly enriched pathways (Table S5) corresponded to phenylpropanoid biosynthesis (19), plant–pathogen interaction (18), MAPK-signaling pathway (15), biosynthesis of amino acids (15), carbon metabolism (14), and plant hormone signal transduction (12). Similarly, forty-three pathways were annotated for genes with decreased transcript abundances. The four most significant of these (Table S5) corresponded to cysteine and methionine metabolism (3), plant hormone signal transduction (3), carbon metabolism (3), and ABC transporters (2).

3.3. Transcription Factor Response to Mechanical Wounding

TFs play a vital role in molecular switches that control the expression of specific genes and regulate plant growth and development as well as the response to changes in environmental conditions. Fourteen TF families, corresponding to 100 transcripts, were identified (Table S6). Of these, the abundances of 87 were increased and 13 were decreased, respectively (Figure 1b). The transcript abundances of most TF genes from the WRKY, AP2, NAM, MYB, HLH, bZIP_1, and B3 families increased, whereas some TF genes in the TCP and HD-ZIP families had decreased abundances in the TA as compared to the UA (Figure 2).

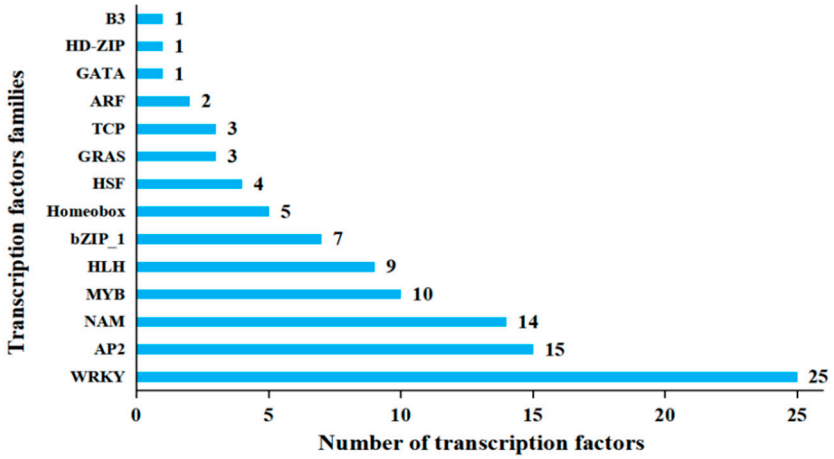

Figure 2. Transcription factors differentially expressed under mechanical wounding in *A. sinensis*.

We noted that the transcript abundances of the genes encoding heat shock factor protein, such as *HSFA2*, *HSFB2A*, *HSF24*, and *HSFB2B*, were increased. In addition, the *SAR1A* (*secretion-associated Ras-related GTPase 1A*) and *SAR1B*, were increased in abundance. The *SCL14* (*scarecrow-like protein 14*), which functions in activating stress-induced promoters, was increased in abundance. Additionally, the transcript abundance of the members of the homeobox family, *ATHB-12* (*Arabidopsis thaliana homeobox 12*) and *WOX4* (*WUSCHEL-related homeobox 4*), which act as regulators of plant growth, were increased. In contrast, the transcript abundance of *GATA11*, which is involved in cell differentiation and possessed a type-IV zinc finger and a DNA-binding domain, was decreased.

3.4. Differentially Expressed Transcripts Related to Plant Hormones

Fifteen genes involved in plant hormone signal transduction (hormone synthesis, transport, and response), including jasmonates (6), ethylene (2), salicylic acid (2), auxin (2), indoleacetic acid (1), ABA (1), and gibberellic acid (1), were identified (Table S3). Almost all of the genes related to JA, ethylene, SA, ABA, and auxin signaling were up-regulated (Table S3). Among these genes, the transcripts of *ERF1B* (*ethylene-responsive transcription factor 1B*), *GH3.1* (*glycoside-hydrolase-family-3-like protein 1*), and *TGA10* (*TGACG*

motif-binding protein 10), were significantly increased by more than 200 fold, 400 fold, and 300 fold, respectively (Table S3). On the other hand, the transcripts of gibberellic-acid-insensitive phloem protein B (*GAIPB*), TIFY domain/Divergent CCT motif family protein 6B (*TIFY6B*), and indoleacetic-acid-induced protein (*IAA18*) were decreased in abundances after mechanical wounding (Table S3).

The cytochrome p450 proteins are involved in the phytohormone metabolism and regulate plant growth. We found 55 transcripts related to the p450 family: *CYP71A1*, *CYP71D55*, *CYP83B1*, *CYP71B34*, *CYP75B2*, *CYP76A2*, *CYP81Q32*, *CYP82A3*, *CYP90D1*, *CYP736A12*, *CYP92C6*, and *CYP749A20* (Table S3). Among these transcripts, *CYP71D55*, *CYP75B2*, and *CYP83B1*, encoding premnaspirodiene oxygenase, flavonoid 3′-monooxygenase, and beta-amyrin 6-beta-monooxygenase-like, were significantly increased. They are involved in sesquiterpenoid biosynthesis, flavonoid biosynthesis, and auxin biosynthesis, respectively.

3.5. Differentially Expressed Transcripts Related to Secondary Metabolites

The functions of fifteen transcripts could be related to terpene synthase activity and terpenoid backbone biosynthesis. In the 'terpenoid backbone biosynthesis' pathway, the transcripts, which were annotated to encode hydroxymethylglutaryl-CoA synthase (HMGS), HMGR, and phosphomevalonate kinase (PMK), related to the mevalonate (MVA) pathway enzymes, and were more accumulated after wounding. For instance, the HMGR transcripts rapidly increased by more than 40 fold (Table S3). *CHLP* has functions related to geranylgeranyl reductase, and its transcript abundances were decreased (Table S3). Additionally, the abundances of five genes related to terpene synthase were increased, such as *TPS1* (*terpene synthase 1*), *TPS9*, and *TPS32* (Table S3).

The KEGG enrichment analysis indicated that the genes related to phenylpropanoid biosynthesis were differentially expressed. A total of 21 transcripts of 13 genes were associated with lignin synthesis (Table S3). The transcript of the *BBE-like 13* gene, encoding for berberine bridge enzyme, was decreased in abundance (Figure 3). This may imply that the *BBE-like 13* conversion step was suppressed in lignin synthesis in *A. sinensis* during mechanical wounding. We also noted that the genes encoding 4-coumarate-CoA ligase (4CL), cinnamoyl-CoA reductase (CCR1), caffeate O-methyltransferase (COMT), glucosyltransferase (GT5), scopoletin glucosyltransferase (TOGT1), glycosyl hydrolase (GH3B), beta-glucosidase 11 (BGLU11), and aldehyde dehydrogenase (ALDH2C4) appeared in phenylpropanoid biosynthesis. Most of the transcripts of these genes were increased (Figure 3). Additionally, transcripts of *PER55*, *Prx15*, and *PNC1* (peroxisomal adenine nucleotide carrier 1), encoding peroxidase were also increased (Figure 3). Among these genes, the transcript expression levels of *BGLU11*, *COMT*, and *ALDH2C4* were increased by more than 20 fold (Table S3). These results suggest that lignin synthesis in *A. sinensis* was promoted during mechanical wounding.

In order to discover potential transcriptional regulatory networks in phenylpropanoid biosynthesis, DEGs encoding TFs were identified (Figure 2). Significantly co-expressed TF–gene pairs were selected using Pearson correlation coefficients ($|\mathrm{cor}| \geq 0.9$ and $p < 0.05$). Meanwhile, we also evaluated the promoter sequences of genes co-expressed with the TFs to determine potential TF-binding sites (Table S7). We found that nine TF families could regulate phenylpropanoid-biosynthesis-related DEGs (Figure 4). *COMT1*, *4CLL7*, *BBE-like 13*, *BGLU11*, and *PER55* were potentially regulated by 9, 8, 7, 7, and 7 TF families, respectively. Notably, the MYB, WRKY, and AP2 families could regulate 12, 12, and 12 DEGs, respectively, which were involved in phenylpropanoid biosynthesis. Similarly, the NAM, HLH, GRAS, TCP, B3, and GATA families could regulate the 11, 10, 6, 5, 4, and 2 DEGs, respectively (Figure 4). We infer that these TF families are likely to play important roles in the phenylpropanoid biosynthesis of *A. sinensis* by regulating numerous downstream genes.

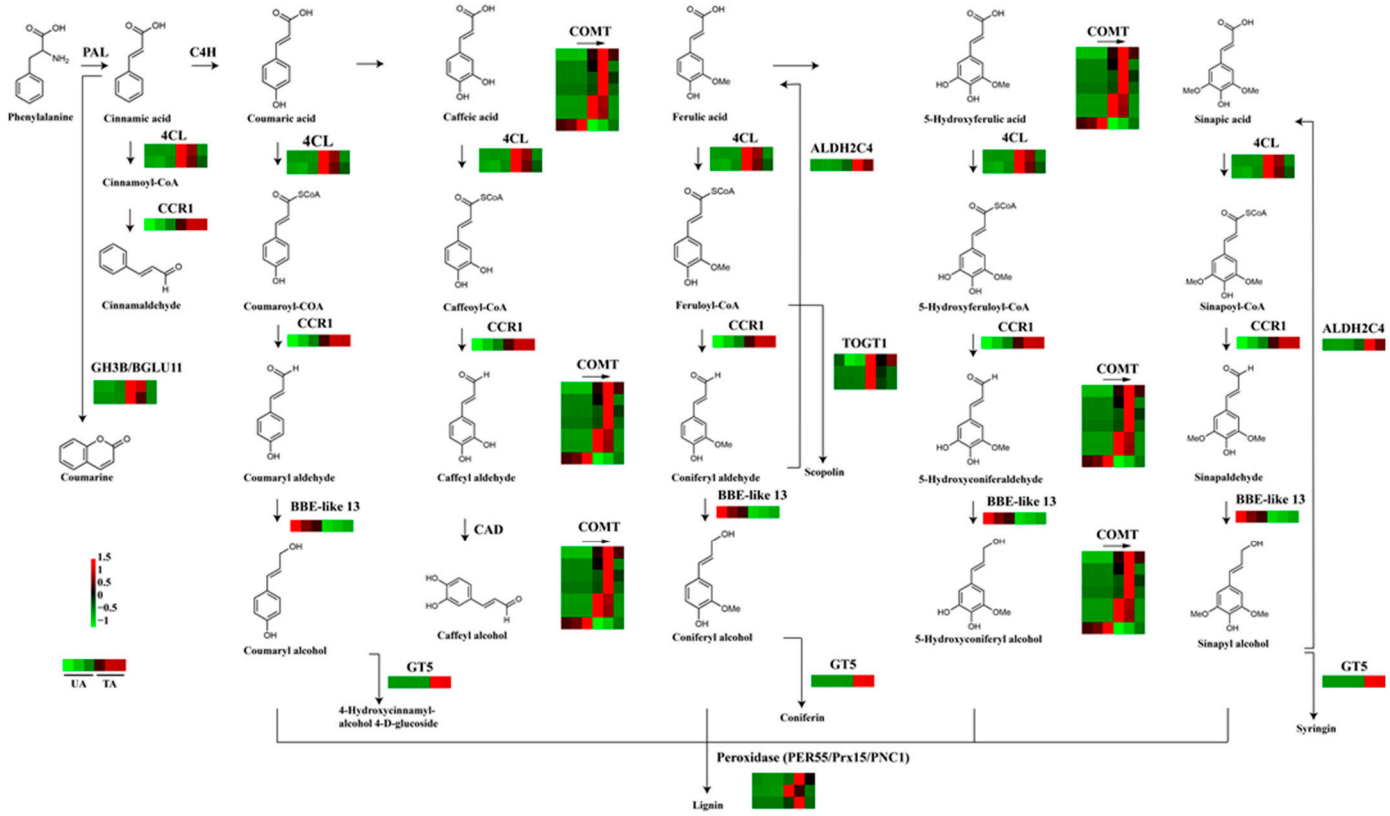

Figure 3. Expression of genes in the phenylpropanoid-biosynthesis pathway. The first three columns represent the three untreated samples, while the next three columns represent the treated samples. Red represents up-regulation and green represents down-regulation. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CCR1, cinnamoyl-CoA reductase 1; GH3B, glycosyl hydrolase family protein 3B; BGLU11, beta-glucosidase 11; COMT, caffeate O-methyltransferase; GT5, glycosyltransferase 5; TOGT1, scopoletin glucosyltransferase; ALDH2C4, aldehyde dehydrogenase family 2 member C4; BBE-like 13, berberine bridge enzyme-like 13; PER55, peroxidase 55; Prx15, peroxidase 15; PNC1, cationic peroxidase 1.

3.6. Validation of RNA-seq Data by qRT−PCR

To quantitatively determine the reliability of the transcriptome data, ten genes were selected for validation by qRT−PCR analysis. The expression patterns of most of the genes in the treated and untreated areas of the *Aquilaria* stem showed similar trends between the high-throughput RNA-seq data and qRT−PCR data (Figure 5 and Figure S4). Although the $\log_2$ (fold change) values calculated by sequencing did not exactly match the expression values detected by qRT−PCR, the expression profiles were consistent for the genes tested. These results validate the reliability of the gene expression values generated from the sequencing data.

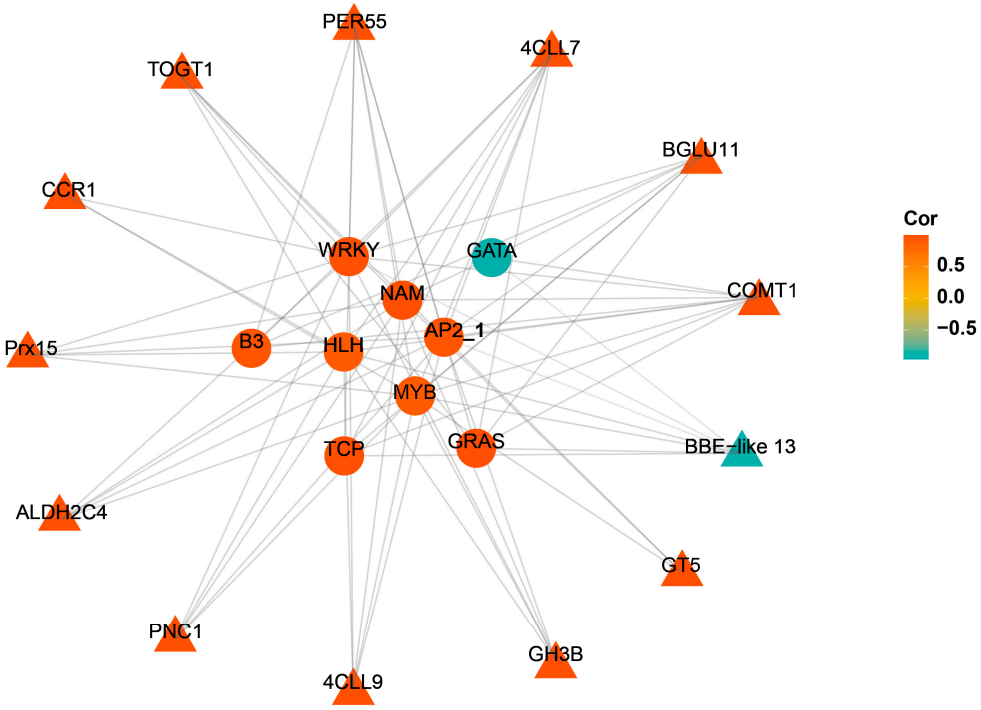

Figure 4. Transcription factor–gene network in *A. sinensis* lignin biosynthesis. Triangles represent genes and circles represent transcription factors. Cor represents Pearson correlation. Orange represents positive correlation and green represents negative correlation.

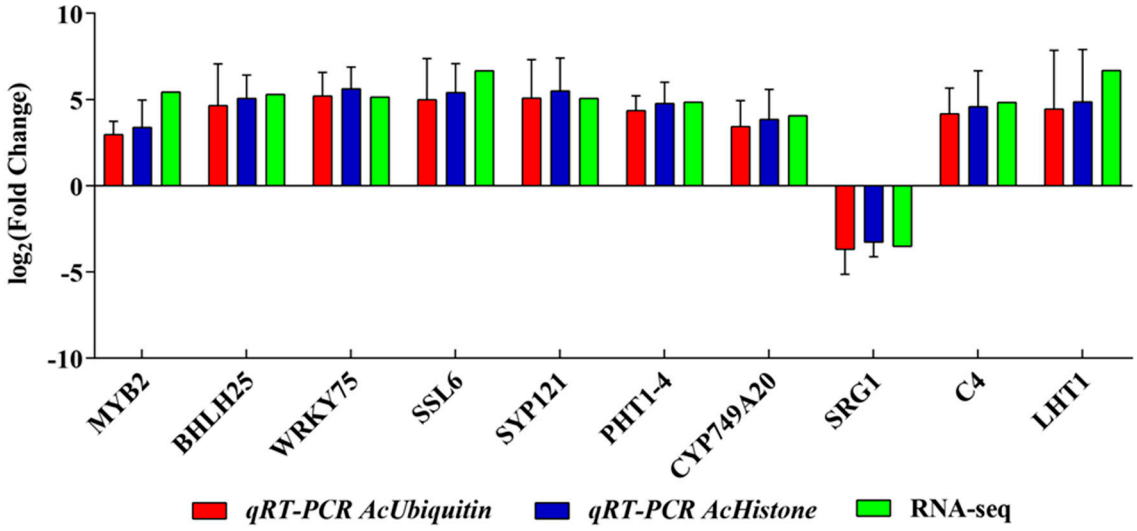

Figure 5. qRT−PCR analysis of the expression levels of ten DEGs in the TA and UA. The *AcUbiquitin* and *AcHistone* genes were used as the internal controls.

4. Discussion

4.1. Transcriptome Differences and Characterization of Transcription Factors under Mechanical Wounding

The repair of plants from mechanical wounding is a complicated process and involves a large number of genes. RNA-seq is a valuable tool that can nearly uncover the complete transcriptome. Therefore, we characterized the changes in the expression of genes during the repair process in *A. sinensis* with the help of RNA-seq. A previous investigation reported that the components of essential oils and total chromones could be clearly determined six months after wounding [27]. Further, a recent histological study on the process of resin formation in the agarwood layers revealed that a large amount of agarwood resin filled up

the inter-xylary phloem six months after wounding [28]. Therefore, these studies prompted us to choose this time point (six months after wounding) for our investigation on the repair of *A. sinensis*. Here, we identified 1165 differentially accumulated transcripts, including TFs, in a mechanical-stress-response network (Table S3). Based on the GO analysis of increased DEGs, several enriched terms related to molecular functions were related to 'DNA binding transcription factor activity' and 'transcription regulator activity' (Table S4). The KEGG enrichment analysis of the DEGs revealed several significantly enriched metabolic and signal-transduction pathways, such as hormone signaling, phenylpropanoid biosynthesis, and sesquiterpenoid biosynthesis (Table S5), suggesting that these might play vital roles in mechanical-stress tolerance in *A. sinensis*. Although we observed some variation within the replicates of the treatment and control samples, these variations are not unexpected. Obtaining high-quality RNA from xylem tissues is challenging. Yet, these variations did not significantly affect the outcomes of the RNA-seq analysis. We found that the majority of the genes were clustered together within a biological group (TA vs. UA; Figure 1). The RNA-seq results were further corroborated by qRT−PCR (Figure 5 and Figure S4).

TFs constituted one of the largest groups of genes differentially expressed in *A. sinensis* under mechanical wounding. TFs act directly on stress-responsive genes, such as those related to defense or secondary metabolite biosynthesis, and are therefore one of the most crucial components in mounting a tailored response [29]. Numerous TFs, such as members of the AP2/ERF, AP2/EREBP, MYB, WRKY, bHLH, HSF, and NAC families, have been identified and characterized as being important regulators of the abiotic stress response in model [30–33] and non-model plant species [34,35]. In this study, a total of 100 transcripts were identified as potential TFs that belong to 14 families (Figure 1b, Table S6). This indicated that various transcriptional regulatory circuits are involved in the mechanical-stress signal-transduction and metabolic reprogramming in *A. sinensis*. The differentially expressed TFs were enriched in WRKY, AP2, NAM, MYB, and HLH, followed by the bZIP_1, Homeobox, HSF, GRAS, TCP, and ARF families. Among these, the members of the WRKY, AP2, MYB, and HLH families have been well characterized with regard to their roles in regulating stress responses in *Arabidopsis* [8], tobacco [36], sweet potato [37], and sweet orange [38]. The WRKY genes are one of the best-characterized families of plant TFs, many of which (such as *WRKY40*, *WRKY33*, *WRKY53*, *WRKY22*, and *WRKY11*) are involved in the response to mechanical stress in *Arabidopsis* [8]. In our study, 25 WRKY-related transcripts were increased, indicating a vital role for this TF family in the repair process and in *Aquilaria* growth and development (Figure 2). In addition, the MYB TFs play an important role in the defense against wounding [8]. They are common regulators of plant secondary metabolism as they regulate the expression of flavonoid genes [8]. In our study, the transcript abundances of many of these TFs, such as *MYB2*, *MYB30*, *MYB78*, *MYB108*, and *MYB308*, were increased. The transcripts of several AP2s and WRKYs that act as positive regulators of sesquiterpene synthases in different plant species, such as cotton [39], *Artemisia annua* [40,41], *Arabidopsis* [42], and *Actinidia* [43], were increased under mechanical wounding. Further, a large number of transcripts encoding HLH, NAM, and bZIP_1, which are implicated in mechanical-stress tolerance, were increased after wounding. NAC TFs are involved in senescence and nutrient remobilization [44], as well as in responses to biotic and abiotic stresses, such as stripe rust, drought, and salt [45,46]. Functional analyses of these mechanical-stress-inducible TFs may provide more information on the complex regulatory gene networks that are involved in the repairing of wounds in trees.

4.2. Characterization of Genes Related to Plant Hormones under Mechanical Wounding

Plant hormones play vital roles in multiple aspects of plant growth and development [47]. The accumulating molecular evidence has shown a close relationship between changes in plant hormones and various abiotic stresses [48]. Under mechanical wounding, the genes involved in plant growth and development are regulated, and the hormones, such as jasmonates, participate in this adjustment process [49]. Jasmonate ZIM-domain (JAZ) proteins are important transcriptional repressors of jasmonate-responsive genes [50]. In

our study, the transcript abundances of many genes coding JAZ proteins, such as *TIFY7*, *TIFY9*, *TIFY10A*, and *TIFY11B*, were increased under mechanical wounding in *A. sinensis* (Table S3). A gene coding for a JAZ protein in *Senna tora*, which is highly similar to *TIFY10A* of *A. sinensis*, was found to repress JA-regulated responses under mechanical wounding [9]. Further, *GH3* (*glycoside hydrolase 3*) belongs to the auxin-responsive gene families and is tightly associated with the hormone-homeostasis and signaling pathways [51]. Previous studies have demonstrated that *ZmGH3-1* responds to various abiotic stresses, such as cold, heat, and salt [52]. In our study, transcripts of the *GH3.1* gene were increased, indicating that it could also play an important role in the *A. sinensis* tolerance to mechanical wounding (Table S3). The *ERF1B* gene belongs to the ethylene-responsive element-binding factor (ERF) family, and is related to plant defense [53], the regulation of secondary metabolism, and plant senescence; its transcript abundance was also increased in this study. Next, gibberellin is an important plant hormone that has been demonstrated to modulate *Arabidopsis* plant growth and development through the regulation of the levels of the phosphorylated DELLA protein [54]. In our study, the *GAIPB* gene, which encodes the DELLA protein and is a negative regulator of gibberellin signaling [55,56], had decreased transcript abundance under mechanical wounding [57] (Table S3). Abscisic acid (ABA) is an important regulator of numerous flexible traits of plants. ABA also helps in mitigating the detrimental effects of abiotic stress, such as those caused by drought and heat, in plants [35,58,59]. In our study, the *DPBF1* gene, related to ABA signaling, was up-regulated. In addition, jasmonate is also an important regulator of the defense response against insect herbivores. The process of plants being fed upon by insect herbivores is often coupled with mechanical wounding. So, crosstalk between wound repair and insect defense is highly possible [49,60]. Similarly, several genes related to heat stress were significantly differentially expressed in this study, such as *HSFA2*, *HSFB2A*, and *HSF24*, which further indicates crosstalk between wound repair and other abiotic stresses. The molecular response to mechanical wounding and its repair might have complex and pleiotropic interactions with the responses to other abiotic stresses, such as heat stress [35], severe drought [59,61,62], nutrient deficiency [63], and soil toxicity [64]. These studies indicate that the responses to abiotic stresses are polygenetic in nature with a genomic architecture, and that phytohormones play a crucial role in modulating the appropriate adaptive response to these stresses.

4.3. Characterization of Genes Related to Plant Secondary Metabolites under Mechanical Wounding

Plant secondary metabolites are vital sources of food additives, pharmaceuticals, and other industrial products [65]. The commercial values of the secondary metabolites of *A. sinensis* have sparked great interest in exploring the possibilities of enhancing their production [18,19,66]. The accumulating evidence demonstrates that plant repair after mechanical wounding influences secondary metabolism production [4,18]. When an *Aquilaria* tree is physically damaged, defensive substances such as sesquiterpene derivatives are produced [17,18,67]. Therefore, we tried to identify the genes and pathways related to sesquiterpene biosynthesis. Sesquiterpenoids are mainly synthesized via the cytoplasmic mevalonate (MVA) pathway, using acetyl-CoA as substrate, and through a series of enzyme-catalyzed reactions to form isopentenyl pyrophosphate (IPP) [68]. *HMGS* and *HMGR* have been recognized as important enzymes in the MVA pathway [69,70]. A positive correlation was found between the sesquiterpene yield and the expression of *HMGS* and *HMGR* [71,72]. PMK is a crucial ATP-dependent enzyme that affects the building blocks of sesquiterpene skeletons. In this study, the expression levels of the MVA-related genes, *HMGS*, *HMGR*, and *PMK*, were increased, indicating that the sesquiterpene biosynthetic process may be enhanced in *A. sinensis* under mechanical wounding (Table S3). Terpene synthases (TPSs) are the enzymes responsible for synthesizing terpenes [73]. Many sesquiterpenes found in the tomato leaf trichomes have been attributed to the activities of TPS9, which encodes a germacrene C synthase [74]. In our study, we found that the accumulation of the TPS gene family was increased, indicating that terpene synthesis may be enhanced (Table S3).

Lignin is a complex polymer of monolignols and strengthens the cell wall to avoid cell collapse. Plants can alter their lignin content in response to various stresses [75,76]. The precursor for lignin biosynthesis is coniferyl alcohol. The supply of coniferyl alcohol starts from phenylalanine under the action of many enzymes in the lignin-biosynthesis pathway (Figure 3). Lignin synthesis is positively associated with the expression levels of *4CL*, *COMT*, *CCR*, and *POD* [77–80]. In this study, the majority of the DEGs related to lignin biosynthesis were up-regulated. These include *GT5*, *4CL7*, *4CL9, CCR1*, *ALDH2C4*, *GH3B*, *BGLU11*, *TOGT1*, *COMT1*, and *peroxidase* genes (PER; Figure 3). This indicates that the lignin biosynthesis process may be enhanced. Previous studies have shown that *BBE-like 13* may be involved in the mobilization and oxidation of the monolignols that are required for the polymerization (such as lignification) process of the plant cell wall [81]. However, *BBE-like 13* was decreased in our study, indicating that the reaction steps involving *BBE-like 13* may be inhibited.

Lignin biosynthesis is an essential yet complex biological process. Important TFs involved in lignin biosynthesis, such as members of the MYB, WRKY, and HLH TF families, have been identified [82–84]. In this study, 100 TFs from 14 TF families were differentially accumulated in the TA (Figure 4). Among them, the MYB, HLH, WRKY, AP2, and NAM families might regulate most of the DEGs involved in phenylpropanoid biosynthesis and could play pivotal roles in mounting responses to mechanical wounding. Previous studies have shown that the overexpression of *PtoMYB74, PtoMYB92*, and *PtoMYB216* could activate the expression of some poplar genes, promote the formation of additional xylem layers, thicken xylem cells, and accelerate ectopic lignin deposition; plants overexpressing these genes accumulated 13%–50% more lignin [85,86]. Similarly, three *Arabidopsis* MYBs (*AtMYB55*, *AtMYB61*, and *AtMYB63*), when expressed in transgenic rice (T1 lines), resulted in culms with a 1.5-fold higher lignin content than that of the control plants [87]. The R2R3-MYB, bHLH, WDR, and MBW complexes (MYB-bHLH-WD40) act as regulators of the phenylpropanoid biosynthesis in plants [83,88–90]. These findings support our TF–gene regulatory network for lignin biosynthesis in *Aquilaria* trees, in which the MYB, HLH, WRKY, AP2, and NAM TFs are likely to play important roles.

5. Conclusions

This study investigated the transcriptional changes that might be related to the process of repair after mechanical wounding in *A. sinensis*. A total of 1165 transcripts were altered in their abundances due to mechanical wounding. Secondary metabolite synthesis and plant hormone signal transduction were involved in wounding repair. Our study has shown that 100 differentially expressed TFs are a part of the complex regulatory gene network that is involved in the repair of wounding. All of these findings would be valuable to further studies on the molecular mechanisms of plant repair. Furthermore, omic technologies in this study would provide an effective method to assess abiotic stress tolerance in tree species, and for sure future studies of mechanical wounding in *Aquilaria* also would benefit from these fresh innovative perspectives.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13081258/s1, Figure S1: Illustration of different *Aquilaria* parts induced by mechanical wounding. (a) The wounded area. (b) The area of the sample collection; Figure S2: Differentially expressed transcripts in wound repair. Orange color is indicative of up-regulated and blue color of down-regulated transcripts; Figure S3: Distribution and analyses of DEGs. (a) Gene Ontology enrichment analysis for DEGs with increased transcript levels. (b) Gene Ontology enrichment analysis for DEGs with decreased transcript levels. The size and color of the dots represent the gene number and the range of the padj value, respectively; Figure S4. Scatter plot of expression levels of ten DEGs by sequencing and qRT−PCR. (a) The *AcHistone* gene was used as the internal control. (b) The *AcUbiquitin* gene was used as the internal control. The Pearson coefficient for the scatter plot was calculated to be 0.9046, indicating that gene expression values generated from the sequencing data and qRT−PCR had a high correlation and were very similar at the quantitative level; Table S1: R code used to calculate the Pearson correlation coefficients; Table S2:

Oligonucleotide primers used in qRT−PCR assays in this study; Table S3: Details of differentially expressed transcripts; Table S4: A summary of significantly enriched GO terms for differentially expressed genes; Table S5: The KEGG pathways related to differentially expressed genes; Table S6: Details of differentially accumulating transcription factors; Table S7: Prediction of the TF binding sites in promoters.

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