A Comparative Analysis of Transcription Networks Active in Juvenile and Mature Wood in Populus

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Abstract

**Background:** Juvenile wood (JW) and mature wood (MW) have distinct physical and chemical characters, reflecting the different wood formation over the tree life-span. However, the regulatory mechanisms that distinguish or modulate the characteristics of JW and MW in relation to each other have not been mapped. Using RNA sequencing (RNA-seq) and whole genome bisulfite sequencing (WGBS), we analyzed wood properties associated with JW and MW forming tissue from *Populus* trees with an identical genetic background.

**Results:** JW and MW of *Populus* displayed different wood properties as the result of significant differences in transcriptional programs and patterns of DNA methylation. Differences were concentrated in gene networks involved in regulating hormonal signaling pathways responsible for auxin distribution and brassinosteroids biosynthesis as well as genes active in regulating cell expansion and secondary cell wall biosynthesis. An observed correlation between gene expression profiling and DNA methylation indicated that DNA methylation affected expression of the genes related to auxin distribution and brassinosteroids signal transduction, cell expansion in JW and MW formation.

**Conclusions:** Auxin distribution, brassinosteroids biosynthesis and signaling play critical roles in formation of JW and MW. DNA methylation is involved in formatting the transcriptional programs in different development phases which contribute to JW and MW formation. The study sheds light to better understand the molecular networks underlying regulation of wood properties which could inform improvement of wood formation.

**Background**

Perennial woody plants are characterized by large size and a long lifespan, in which a long non-flowering period of juvenile phase can last years to decades, for example, 3–5 years in *Populus* and 10–15 years in *Pinus* [1, 2]. Wood produced during juvenile phase is called juvenile wood (JW) which is followed by a mature phase during which trees start flowering and producing mature wood (MW) at outside of JW [3]. Compared to JW, MW is characterized with longer xylem cells, thicker secondary cell walls, lower density of vessels, higher crystallinity of cellulose in fibers and smaller microfibril angles [4]. Thus, MW is more desirable from a processing and utilization perspective for construction wood, wood pulping, and fiber material production. As a matter of fact, to meet the increasing demand for raw wood material, artificial forest plantation aims to reduce the rotation length and enhance productivity, which makes JW with lower wood quality as a major source for wood industry [5]. This seriously affects the utilization and processing of wood. How to make wood to be matured quickly and the proportion of JW to be reduced has become an important aspect of improving wood properties.

Wood formation starts with the cell divisions at vascular cambium and subsequent differentiation into secondary xylem through cell expansion, secondary cell wall thickening and programmed cell death [6]. Plant hormones, such as auxin, brassinosteroids and gibberellin, participate in regulation of wood
formation [7–9]. The size of wood cells depend on cell expansion process while mechanical and chemical properties of wood are largely determined by secondary cell wall thickening [10]. Cell expansion is controlled by extension of the primary cell wall, which is composed of 20–30% cellulose, 30–50% pectins, 20–25% hemicelluloses and 10% glycoproteins [11]. Following cell expansion, wall thickening is initiated with transcriptional programs for secondary cell wall biosynthesis [12]. Secondary cell walls are composed of 40–80% cellulose, 10–40% hemicellulose, 5–25% lignin and glycoproteins [13]. As JW and MW display distinct wood properties, regulation of the secondary cell formation in JW and MW is most likely through different ways.

DNA methylation, a critical epigenetic mechanism among eukaryotes, affects many biological processes. In plant, most of DNA methylation occurs at the fifth carbon of cytosine (including three cytosine contexts, CG, CHG and CHH, where H represents A, C or T) to form 5'-methylcytosine by DNA methyltransferase [14–16]. Evidence indicates that DNA methylation can regulate gene expression in numerous biological processes including response to abiotic stresses [17–21], plant development and morphogenesis [22] and wood formation [23]. The degree of DNA methylation is also related to plant development phases. The degree of DNA methylation at mature phase was significantly higher than that at juvenile phase in Pinus radiata [24]. DNA methylation increases along with the age extension in some species [25]. It is unclear how DNA methylation is involved in regulation of JW and MW formation.

Despite studies which have shown physicochemical difference of wood properties between JW and MW, the molecular regulatory networks underlying formation of the different wood properties is not fully elucidated. In this study, by employing Populus trees with an identical genetic background, we analyzed different physical and chemical characters in association with the transcriptomic profiles and DNA methylation during formation JW and MW. Correlation analysis revealed the transcriptional networks and DNA methylation that are involved in regulation of wood formation with different wood properties. This study provides an array of mechanistic information for understanding of JW and MW formation as well as new clues for genetic manipulation for improvement of wood properties.

**Results**

**Properties of JW and MW in Populus**

To examine the properties of the JW and MW produced in Populus, plantation-grown trees that were propagated from a single clone were sampled. Three trees at two-years-old and eight-years-old were collected with trunk at breast height, respectively (Additional file 1). In wood anatomical section, difference in the ratio of fiber/vessel, the length and size of fibers and vessels was observed between JW and MW (Fig. 1a-d). MW contained higher ratio of fiber/vessel, lower density of vessel cell in wood section, longer and larger fiber and vessel than those in JW (Fig. 1e-j). Chemical analysis indicated that MW contained higher content of crystalline cellulose and lower content of lignin compared to JW (Table 1). Sugar composition in hemicelluloses also showed difference between JW and MW. MW contained higher xylose, mannose, glucose, arabinose but lower galactose compared to JW (Table 1).
These results indicated that JW and MW in *Populus* displayed different cellular structure and chemical composition.

| Chemical composition (µg/mg AIR) | JW         | MW         |
|----------------------------------|------------|------------|
| Cellulose                        | 400.3 ± 24.4 | 432.4 ± 27.0* |
| Lignin                           | 223.1 ± 9.7  | 206.7 ± 18.9* |
| Hemicellulose                    | Xylose     | 49.2 ± 3.5  |
|                                  | Mannose    | 9.2 ± 1.4   |
|                                  | Galactose  | 4.2 ± 0.3   |
|                                  | Glucose    | 34.0 ± 3.3  |
|                                  | Arabinose  | 1.3 ± 0.1   |

Significance was determined by Student’s t-test (*p* < 0.05 and **p** < 0.01).

**Dna Methylation In Formation Of Jw And Mw**

The differential gene expression in different growth phases prompted us to examine the whole genome bisulfite sequencing (WGBS). The bisulfite sequencing showed that 87.6–91.9% of the reads were qualified for methylation assay against to the *Populus* genome (http://phytozome.jgi.doe.gov/) (Additional file 10). Overall, the methylation level was different within cytosine methylation contexts (CG, CHG and CHH). The context of CG had higher methylation level, while CHG and CHH was lower (Additional file 11). The DNA methylation context patterns displayed a similarity with those previous observed in *Populus* [18, 59]. PCA showed that JW and MW had distinct DNA methylation (Fig. 5a).

Comparison of the DNA methylation in JW and MW revealed 12176 differentially methylated regions (DMRs) (with methylation difference ≥ 10, Q-value < 0.05). Majority of DMRs were in the contexts of CG sites (10303) and CHG sites (1663) (Additional file 12 and Additional file 13), Among them, 10237 DMRs were located in gene body and/or flanking regions (± 2 kb), named differentially methylated genes (DMGs). In MW, 5414 DMGs showed higher methylation while JW contained 4849 DMGs with higher methylation (Fig. 5b and Additional file 14), suggesting that different DNA methylations occurred in formation of JW and MW. Analysis of the correlation between DMGs and DEGs indicated that DMRs in gene promoter region were more likely to affect gene expression (Additional file 12). About 20% DEGs (802) displayed different methylation (Additional file 15). These DEGs were closely related to plant hormones signaling and response, cell wall formation and modification, metabolic process, transcription and translation etc. (Additional file 12 and Additional file 16). For example, the homologs of *ARFs*, *BAK1*, *BSK1* and *BZR1*, which are involved in auxin and BR signaling, were differential methylated in their different gene region in JW and MW (Fig. 5c and Additional file 15). Furthermore, several genes in related
to cell wall formation such as XTH30, PAEs, WND1B, CESA4, CESA7 and CESA8 (Fig. 5c and Additional file 15) showed differential methylation in JW and MW. In addition, several DMRs in intergenic region were neighbored to the homologs of PILS2, AUX1, PIN7, WND2A, MYBs and PAL1, which are involved in auxin distribution and cell wall biosynthesis (Additional file 15). In summary, the results revealed that DNA methylation displayed a clear difference in formation of JW and MW, which may play a role in regulating gene expression in different growth phases, particularly for the genes involved in hormone signal transduction, cell division and cell wall formation in wood formation.

Discussion

At a given point of tree development, wood can be differentiated into juvenile wood and mature wood which have distinct properties [3, 4]. In the present study, we profiled the transcriptome and DNA methylation patterns in JW and MW derived from an identical genetic background in order to uncover the paths involved in wood formation at different developmental phases. Different transcription profiles and DNA methylation were identified in formation of JW and MW. Differences in gene expression were primarily associated with plant hormones including auxin and BR signaling and response, cell wall formation and modification, cell organization and biogenesis, transcription regulation processes. Different patterns of DNA methylation were also detected in genes involved in auxin transport, BR signaling, and cell expansion which suggest a role for the epigenetic regulation of JW and MW formation.

Different expression of auxin transport genes was observed in JW and MW. Relative to JW, we observed the genes related to auxin influx (homolog of AUX1) [26] were down-regulated in MW, while the genes related to auxin efflux (homologs of PINs, PILSs, ABCB19) [26, 27] were up-regulated in MW. Meanwhile, that different members of the AUX1/LAX3 family were expressed in JW and MW implies a possibility that formation of JW and MW involves distinct auxin molecule formats, as AUX1/LAX members correspond with different auxin formats [60]. Further characterization of PIN, ABCB, AUX1 and LAX3 proteins in association with auxin in the JW-/MW-forming tissues would be able to provide mechanistic evidence for verification of the findings. However, current results indicate that auxin transport play a role in regulating formation of JW and MW.

Furthermore, we also found that homologs of DIM which is a key gene for BR biosynthesis [28] was up-regulated in MW, while homologs of BAK1, BSK1, BZR1 which are marker genes for BR signaling [61–64] showed down-regulated expression in MW, suggesting that BR signaling play a role in regulating MW formation. Studies have showed that BR promotes wood formation [65]. It is worthy of studying whether BR manipulates wood properties in wood formation because the properties JW and MW are different.

DNA methylation act as an epigenetic mechanism to regulate gene expression in plants [21, 66–68]. In this study, we found that the methylation level of the auxin transport genes PILS2, AUX1, PIN7 was different between JW and MW. In addition, the different degree of DNA methylation was also detected in the BR signaling genes BAK1, BSK1, BZR1. It is likely that the different expression of these genes in JW and MW may be related to their DNA methylation changes over the developmental process. Further
investigation of the DNA methylation effect on the transcription activities of the auxin and BR genes would help understanding the regulation of the hormones signaling during different development phases in perennial trees. In summary, the present results suggest that auxin distribution and transportation, BR biosynthesis and signaling are involved in regulating the wood formation at juvenile and mature phase. DNA methylation plays an important role in regulating the expression of the auxin and BR genes at different development phases.

In consistent with the hormones signaling changes, the downstream biological processes in response to auxin and BR also showed alternation in JW and MW. For instance, TFs such as ARFs, SRSs, AP2, MYB3R4 and genes related cell loosing and cell expansion such as HA11, XTHs, FUC1, PAEs, PMEs, PMRs, PIPs, TIPs, of which the expression is responding to auxin signaling [48, 69], showed differential expression in formation of JW and MW. These transcription regulations are in agreement with the MW properties that have significantly longer and larger fiber cells and vessel elements.

Cell wall composition (including lignin, cellulose and hemicellulose) which is closely related to wood properties is rather different in JW and MW (Table 1). Expression of the genes related to lignin biosynthesis were down-regulated and the genes for hemicelluloses biosynthesis were up-regulated in MW, consistent with the result of less lignin content and higher hemicellulose content in MW. Interestingly, expression of the cellulose biosynthesis genes (such as CesA4, CesA7 and CesA8) was down-regulated in MW compared to JW. However, the cellulose content was higher in MW. As this discrepancy requires further verification, regulation of the CesA activity at protein level may be considered. It is known that protein phosphorylation plays a crucial role in regulating CesA catalytic activity and motility [53, 70, 71]. More evidence is needed for elucidation of the different cellulose accumulation in JW and MW.

Conclusions

In this study, we analyzed transcription profiles and genome-wide DNA methylation in association with the wood properties of JW and MW by employing Populus trees with an identical genetic background. Results suggest that auxin distribution and BR signaling may act as major mechanisms to modulate the wood formation in different development phases. In response to the hormones signaling alteration, the transcription activities are modulated, leading to the formation of different wood properties in JW and MW. Furthermore, results also indicate that the transcription changes of hormones-related genes may regulated through their DNA methylation. The study outlines a picture of the main transcription networks related to wood formation in JW and MW and a possible role of DNA methylation in tuning the transcriptional network (Fig. 6). These findings shed light towards a better mechanistic understanding of wood formation in different development phases and new evidence to inform the engineering of wood properties.

Methods

Tissue sampling
Populus trees propagated from the same clone (*Populus deltoides*×*P. euramericana* cv.'Nanlin895') were grown in an experimental plantation of the Nanjing Forestry University located at Siyang, Suqian, Jiangsu, China (33° 47′ N, 118° 22′ E). Wood-forming tissues were sampled from at the trunk in a length of 1 meter at the breast height (1.3 meter from ground) from 2-years-old (formation of JW) and 8-years-old trees (formation of MW). The tissues were directly collected into liquid nitrogen and stored at -80 °C for late analysis [72]. Three biological replicates were sampled from juvenile and mature trees (Additional file 1), respectively. The tree trunks at the breast height were collected and used for wood property analysis.

**Analysis of wood properties**

JW and MW were sampled according to the year-ring of wood. Wood tissue was sectioned into 20-µm in thickness and stained with 0.5% phloroglucinol in 12% HCl. Cross sections were observed under a microscope (Olympus, BX53). Number of bers and vessels and their cross area were counted using Image J. Meanwhile, the wood cells were separated after treatment using acetic acid/ hydrogen peroxide (1:1 v/v) solution at 80°C for 6 hours. Then, the separated wood cells were stained with safranine (1% in water) and the length of fiber cells and vessels was measured under a microscope (Olympus, BX53) using Image J.

**Cell wall composition determination**

Air-dried wood sample was ground into powder and filtered through 60-mesh. According to our previous established protocol [73], alcohol insoluble residues (AIR) was firstly obtained by extracting the wood powder with 70% ethanol, chloroform/methanol (1:1 v/v) and acetone. Amylase and pullulanase in 0.1 M sodium acetate buffer (pH 5.0) were used to treat the extracted AIR overnight. For analysis of the sugar in hemicelluloses, AIR was treated with 2 M trifluoroacetic acid (TFA) at 121°C for 90 minutes. The supernatant was evaporated and incubated in 20 mg/ml fresh sodium borohydride solution at 40° for 90 minutes. The product then was neutralized with acetic acid and mixed with 1-methylimidazole and acetic anhydride for acetylation. After extraction with dichloromethane, the product was mixed with ethyl acetate for GC-MS (6890N GC system and 5975 Mass detector, Agilent Technologies, equipped with a SP-2380 capillary column, Supelco, Sigma-Aldrich) analysis. Meantime, the insoluble precipitate from the AIR treated with TFA was collected for crystalline cellulose content determination. The updegraff reagent (Acetic acid: nitric acid: water, 8:1:2 v/v) was added to the precipitate and incubated at 100 °C for 30 minutes. After washed with H₂O and acetone, the precipitate was incubated with 72% sulfuric acid at room temperature for 1 hour. The content of crystalline cellulose was determined by anthrone assay [74]. For lignin measurement, AIR was incubated with freshly prepared acetyl bromide (25%, acetyl bromide in acetic acid) at 50°C for 3 hours. After cooled, the AIR was mixed with 2M NaOH, 0.5 M fresh hydroxylamine hydrochloride and acetic acid. Lignin content was determined using a microplate reader (Varioskan Flash, Thermo) [75].

**RNA isolation and RNA sequencing**
Total RNA was extracted from wood-forming tissues using a mirVana miRNA Isolation Kit (Ambion-1561) following the manufacturer's instruction. After treated by RNase-free DNase I (Sigma, 4716728001), the quality of total RNA was assessed on NanoDrop spectrophotometer (NanoDrop 2000, Thermo Scientific) and on agarose gel electrophoresis. For RNA sequencing (RNA-seq), cDNA library was generated from 5 μg of total RNA with TruSeq Stranded mRNA LTSample Prep Kit (Illumina, RS-122-2101) and Agencourt AMPure XP (BECKMAN COULTER, A63881). cDNA library was qualified through length distribution of fragments using Agilent 2100 (Bioanalyzer). The 150 bp paired-end sequencing was performed using platform of Illumina HiSeq X10. About 5 million reads per samples were generated.

DNA isolation and bisulfite sequencing

Genomic DNA was extracted from wood-forming tissues using QIAamp DNA Mini kit (Cat.51306, Qiagen). DNA quantification and integrity were determined by a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE) and 1% agarose electrophoresis, respectively. Before bisulfite treatment, lambda DNA was added to the purified DNA, which was used as an internal reference to calculate the conversion rate. Then, the mixed DNA was bisulfite treated using a Zymo Research EZ DNA methylaiton-Glod Kit (Zymo, D05005). Bisulfite sequencing (BS-seq) libraries were constructed by TruSeq® DNA Methylation Kit (Illumina, EGMK91396) following the manufacturer's instruction. After libraries were qualified, sequencing was performed on the Illumina HiSeq X Ten platform and 150 bp paired-end reads were generated.

Analysis of transcriptome sequencing data

Raw reads of sequencing were processed using NGS QC Toolkit to remove low-quality reads [76]. The cleaned reads were mapped to *Populus trichocarpa*’s genome ([http://phytozome.jgi.doe.gov/](http://phytozome.jgi.doe.gov/)) using hisat2 with default parameters [77]. Gene expression level was measured as fragments per kilobase per million reads (FPKM) using cufflinks [78, 79]. Read counts for each gene in each sample were obtained using htseq-count and standardized by rlog [80]. Principle component analysis (PCA) was performed by plotPCA of DEseq2 R package with default parameters. Differential expression genes (DEGs) were identified using the DESeq R package by estimation of Size Factors and nbinomTest. Analysis of DEGs with gene ontology (GO) enrichment and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [81] pathway enrichment was performed using R based on the hypergeometric distribution.

Analysis of genome bisulfite sequencing

The raw reads of BS-seq were cleaned using Fastp [82] by removing adapters, ploy-N and low quality reads. The remaining high-quality clean reads were mapped to the *Populus trichocarpa*’s genome ([http://phytozome.jgi.doe.gov/](http://phytozome.jgi.doe.gov/)) using Bismark software with default parameters [83]. Methylcytosine (mC) sites were identified using MethylKit [84]. With default parameters, MethylKit was applied for PCA analysis. Differentially methylated regions (DMRs) were identified using MethylKit software with a Q-value (P-value corrected by FDR method) threshold of 0.05 and an absolute delta cutoff of 10% between
the two groups. Analysis of DMGs with GO enrichment and KEGG pathway enrichment was performed according to the same method used for DEGs analysis.

**Quantitative real-time PCR**

The first-strand cDNA was synthesized from 2 μg of total RNA using a cDNA Synthesis SuperMix (TransGen Biotech, AT311-03). Using cDNA as template, quantitative real-time PCR (qRT-PCR) was performed using Perfectstart™ Green qPCR SuperMix (TransGen, AQ601) and a Quantstudio™ 3 Real-Time PCR Detection System (Thermo). The primers used for selected genes are listed in Additional file 17 and *TUB9* was used as an internal control to normalize gene expression.

**Abbreviations**

JW  
Juvenile Wood; MW: Mature Wood; RNA-seq: RNA sequencing; WGBS: Whole Genome Bisulfite Sequencing; BS-seq: Bisulfite sequencing; PCA: Principal Component Analysis; FPKM: Fragments Per Kilobase per Million reads; DEGs: Differential expression genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; AIR: Alcohol Insoluble Residues; TFA: Trifluoroacetic Acid; mC: Methylcytosine; DMRs: Differentially methylated regions; DMGs: Differentially Methylated Genes; qRT-PCR: Quantitative real-time PCR; BR: Brassinosteroids; GAs: gibberellins; ER: Endoplasmic Reticulum; TFs: transcription factors; CSC: cellulose synthase complex; UXS: UDP-glucuronic acid decarboxylase; UDP-GlcA: UDP-glucuronic acid

**Declarations**

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**Availability of data and material**

The data sets supporting the results of this article are included within the article and its additional file.

**Author Contributions**
LFL performed experiments, analyzed data and wrote the manuscript. YYZ analyzed data and wrote the manuscript. JSG conducted RNA-seq analysis and wrote the manuscript. TMY prepared the tree samples and analyzed data. WCL and JQL analyzed data and wrote the manuscript. LGL conceived the project, analyzed data and wrote the manuscript. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

All authors have read and approved the final manuscript

**Declarations**

The authors declare that they have no competing interests.

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**Figures**
Figure 1

Properties of the wood produced in juvenile phase (JP) and mature phase (MP). (a) and (b) Wood sections stained with phloroglucinol from juvenile wood (JW) (a) and mature wood (MW) (b). Bars=50 µm. (c) and (d) Fibers and vessels from JW (c) and MW (d). Bars=200 µm. (e) Ratio of numbers of vessel/fiber. (f) Density of vessel elements. (g) and (h) Length and cross area of vessel. The values were means ± SE of 500 and 800 independent vessels from JW and MW, respectively. (i) and (j) Length and cross area of fiber. The values were means ± SE of 1000 independent fibers from JW and MW, respectively. Significance was determined by Student's t-test (* p<0.05 and **p<0.01). f, fiber cell; v, vessel elements.
Figure 2

(a) the functional of the differentially expressed genes (DEGs). (b) The heat map of genes related to plant hormones. (c) The results of quantitative real-time PCR of selected genes related to plant hormones in JW and MW. Significance was determined by Student’s t-test (* p<0.05 and **p<0.01).
Figure 3

Transcriptional factors in DEGs. (a) Number of TF genes in detected DEGs. (b) Functions of the TFs among DEGs. (c) The results of quantitative real-time PCR of selected TFs in JW and MW. Significance was determined by Student's t-test (* p<0.05 and **p<0.01).
Figure 4

Differentially expressed genes involved in cell wall formation in JW and MW. (a) The heat map of genes related to cell wall formation. (c) The results of quantitative real-time PCR of selected genes related to cell wall formation in JW and MW. Significance was determined by Student's t-test (*) p<0.05 and **p<0.01.)
Figure 5

The methylation profile of JW and MW. (a) Principal Component Analysis (PCA) of DNA methylation in each sample. (b) The number of higher methylation genes in DMGs. (c) Methylation and expression of genes related to wood formation.
Figure 6

Outline of the DAN methylation and transcription regulation in formation of JW and MW.

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