Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020

doi:10.20944/preprints202005.0454.v1

Increasing Lignin Accumulation in Arabidopsis and Poplar by Overexpressing a *CCoAOMT* Gene from Dove Tree (*Davidia involucrata* Baill.)

Jian Li[1, 2], Xiaomin Ji[1, 2], Xujie Dong[1, 2], Fuxiang Cao[2, 3] and Meng Li[1, 2, *]

1. College of Life Science and Technology, Central South University of Forestry and Technology, Changsha 410004, China

2. Hunan Research Center of Engineering Technology for Utilization of Environmental and Resources Plant, Changsha 410004, China

3. College of Horticulture and Landscape, Hunan Agricultural University, Changsha 410004, China

*Corresponding author: Meng Li (limeng0422@foxmail.com)

© 2020 by the author(s). Distributed under a Creative Commons CC BY license.

Abstract: Rapid lignification occurring in the endocarp of dove tree results in the formation of a rigid and compact structure, which seriously hinders seed germination. A gene named *DiCCoAOMT1*, which encodes a hyperactive O-methyltransferase, was identified and thought to play a critical role in the process of endocarp lignification. In this study, the *DiCCoAOMT1* gene was introduced into *A. thaliana* and poplar, respectively, to further verify its function. The lignin content was increased by 45% and 20% in the stems of transgenic *A. thaliana* and poplar lines, respectively. There was a positive correlation between the expression levels of *DiCCoAOMT1* and lignin amount in transgenic lines. Furthermore, the shifts of lignin composition was indicated by the elevated S/G ratio in transgenic poplar lines. Lignin accumulation was promoted specifically in the phloem cells, and the cells in secondary xylem was thickened in transgenic plants. In addition, lengthened pods and elevated plant height, and elongated petioles and internodes were observed in transgenic *A. thaliana* and poplar lines, respectively. Taken together, our data indicated that an endocarp-specific *DiCCoAOMT1* gene could effectively increase lignin accumulation and alter lignin composition in both herbs and woody plants, which provides new insights to understand the regulatory mechanism of lignin biosynthesis and the biological significance of lignification in specific tissues.

Keywords: *CCoAOMT*; lignin; poplar (*Populus tomentosa*); *Arabidopsis thaliana*; dove tree (*Davidia involucrata* Baill.)

1. Introduction

Lignin is one of the most important macromolecular organics in plants with multiple biological functions [1]. Lignin enhances the mechanical hardness and strength of plant body by filling the cellulose framework in cells. Besides the supporting function, lignin also play a role in defense against biotic and abiotic stress, such as avoiding biological invasion and water erosion, anti-bacterial, anti-oxidation, anti UV absorption and flame-retardant, which enhanced the adaptability of plants to diverse environment [2,3]. The lignin biosynthesis pathway has been extensively studied, and the genes and enzymes involved into this process have also been well identified. Lignin biosynthesis starts from the monolignols, which were derived from Phe, and the monolignols was converted into lignin monomer or units by a series of enzymes. The units resulting from the monolignols, when incorporated into the lignin polymer, are called guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) units [4]. During this process, PAL (phenylalanine ammonia-lyase), C4H (cinnamate 4-hydroxylase), C3H (p-coumarate 3-hydroxylase), F5H (ferulate 5-hydroxylase), CAD (cinnamyl alcohol dehydrogenase), 4CL (4-coumarate: CoA ligase), CCR (cinnamoyl-CoA reductase), COMT (caffeic-acid-methyltransferase) and CCoAOMT (caffeoyl-CoA-methyltransferase) are considered as key enzymes, and their encoding genes were often used as targets of genetic modification for regulating lignin amount or alter the lignin composition in different species [5].

Lignin is widely distributed in plant cells, while mainly accumulated in the secondary wall of wood fiber cells. Wood fiber cells are concentrated in the xylem of plants, thus most related studies have been focused on the changes of lignin amount and composition in xylem. However, lignification is not limited just in xylem. In many woody plants, lignification can also be observed in roots, endocarps and pulps [5]. Different with that in xylem, the lignification process in these organs has to be completed in a limited time to form a specific structure, such as the cases in the shell of walnut or the endocarp of almond. The enzymes and genes involved into this process need to be tissue-specific and more efficient due to the spatiotemporal constrains. Therefore, the regulatory mechanism of lignin biosynthesis in fruit is

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020 doi:10.20944/preprints202005.0454.v1

supposed to be different with that in xylem. However, the studies regarding lignification in specific tissues is scarce.

Dove tree (*Davidia involucrata* Baill., *Davidia* hereafter) is a deciduous tree and the sole member of Davidiaceae. It is a relic species of Paleotropical flora in Tertiary period with many ancient and unique characteristics [6]. *Davidia* is an endangered species because its seed fertility is very low. The long-term seed dormancy of *Davidia* is believed to be one of the most important reasons for the low fecundity [7]. The seed dormancy of *Davidia* is determined by the unique structure of its endocarp. Rapid lignification occurring during the development process of *Davidia* endocarp, resulting in a thick, rigid and compact structure, which seriously hindered the seed respiration and material exchange [8,9]. In order to reveal the molecular mechanism of rapid lignification in endocarp, we performed transcriptome analysis and identified a gene named *DiCCoAOMT1*, which encodes a hyperactive O-methyltransferase. The expression of *DiCCoAOMT1* is endocarp-specific, and its expression level was increasing along with the development of endocarp, thus *DiCCoAOMT1* was speculated to play a critical role in regulating the lignification in *Davidia* endocarp [10].

CCoAOMT is an S-adenosyl-L-methionine (SAM) methyltransferase. It can transfer the methyl group on S-adenosylmethionine to the benzene ring carbon 3 position of coffee acyl-CoA, to form ferryl-CoA [4]. The *CCoAOMT* genes have been identified in *Arabidopsis thaliana*, rice (*Oryza sativa*), poplar (*Populus tomentosa*) and sorghum (*Sorghum bicolor*) etc., and were proved to be a key enzyme in lignin biosynthesis [11-14]. Inhibition of the expression level of *CCoAOMT* gene in tobacco (*Nicotiana benthamiana*) through expressing the antisense sequence of an *Ah CCoAOMT* gene from *Acacia farnesiana* resulted in the decrease of lignin content and the alternation of lignin components [15]. Similar results were also reported on the *CCoAOMT* genes from *Pinus radiate* [16] and *Populus deltoids* [17]. In maize (*Zea mays*), the lignin content decreased by 22.4% in the transgenic lines with reduced expression level of *CCoAOMT* gene through RNAi [18]. In Arabidopsis, the *comt* and *ccoaomt* mutants also showed significantly reduced lignin amount [14]. On the

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020 doi:10.20944/preprints202005.0454.v1

contrary, overexpression of the *CCoAOMT* gene *PaCCoAOMT1* and *PaCCoAOMT2* from *Polypodiodes amoena* resulted in increased lignin content in transgenic Arabidopsis [19]. While overexpression of a *CcCCoAOMT1* gene from jute (*Corchorus capsularis* L.) [20], and a *PmCCoAOMT* gene from Japanese apricot (*Prunus mume* Sieb.) led to an increase of lignin amount in the reproductive organs of transgenic plants. These findings indicated that although all *CCoAOMT* genes encode similar enzymes, their tissue-specific expression result in different functions.

We have verified *in vitro* that DiCCoAOMT1 is a hyperactive O-methyltransferase, which might promote the lignification in *Davidia* endocarp. However, the expression of *DiCCoAOMT1* is endocarp-specific, whether it can ectopically improve lignin biosynthesis is unknown. In this study, we introduced the *DiCCoAOMT1* gene into Arabidopsis and poplar, respectively, by genetic transformation. Through overexpression in both herbs and woody plants, the gene function on lignin accumulation, lignin monomer composition, organ development and cell structure was further investigated.

2. Materials and Methods

2. 1. Plant materials

The seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia-0 and the tissue culture seedlings of poplar (*Populus tomentosa* Carr.) are preserved in our lab. The tissue culture materials were grown at 22 °C under a 16 h light / 8 h dark photoperiod. The illumination was from cool-white fluorescent lights (100-150 μmol m^{-2} s^{-1}). Wild type (WT) and transgenic Arabidopsis plants, and WT and transgenic poplar plants were grown in pots containing mixed medium (vermiculite: high-quality soil = 1: 3). All plants were grown in controlled environment chambers at 20 °C under a 16 h light / 8 h dark photoperiod.

2. 2. DNA extraction, RNA extraction and cDNA synthesis

The frozen samples were rapidly ground into powder in liquid nitrogen for nucleic acid extraction. The genome DNA was extracted by the CTAB method. The total RNA was extracted with an RNAPrep Pure Plant Kit (TianGen). RNA degradation and contamination were monitored on 1 % agarose gels. RNA purity was checked with the NanoPhotometer spectrophotometer (IMPLEN). The first strand of cDNA was synthesised by reverse transcription with the Evo M-MLV reverse transcription kit (Takara). The cDNA was then diluted and used as a template for gene cloning and qPCR analysis, respectively.

2. 3. Genetic transformation in Arabidopsis and poplar

The DNA fragment of target gene was inserted into the pRI101-AN vector by double digestion using *Bam*HI and *Sal*I and ligation using T_4 DNA ligase. Then the vector was transferred into *Escherichia coli* DH5α for sequencing. The successfully constructed vector was transferred into *Agrobacterium tumefaciens* strain EHA105 for genetic transformation.

The floral dip method was used for genetic information of Arabidopsis [20]. Transformed Arabidopsis seeds were sowed on the MS medium [21] containing 50 mg/L kanamycin and 200 mg/L cefotaxime and the seedlings with resistance to the antibiotics were selected. The homozygous transgenic lines were obtained by serial

passage until no trait separation was observed in the progeny. More than 20 individual transgenic lines confirmed by PCR using gene-specific primers were generated, and the lines using for further study were selected according to the expression level of the target gene detected by qPCR.

The leaf disk method was used for genetic transformation of poplar [22]. The leaves using for transformation were collected from the 3-week-old tissue culture seedlings. Transformed poplar seedlings were transferred on the WPM medium [23] containing 25 mg/L kanamycin and 400 mg/L cefotaxime. The seedlings were grown under tissue culture conditions and the plants with resistance to the antibiotics were selected. More than 10 individual transgenic lines were generated, which were confirmed by PCR using gene-specific primers, and the lines using for further study were selected according to the expression level of the target gene detected by qPCR. The information of primers used in this study were listed in Table S1.

2. 4. Detecting gene expression levels by qPCR

The cDNA samples of the leaves collected from 15-day-old Arabidopsis plants and the leaves collected from 60-day-old poplar plants were used as templates for qPCR analysis, respectively. An Arabidopsis gene, *AtUBQ5* and a poplar gene, *PtActin* were used as reference genes for data normalisation. Real-time PCR was performed with the 2× SYBR Green Pro Taq HS Premix (Biotools, USA) and the ABI StepOne Plus-Type qPCR instrument (Applied Biosystems, USA). The 20.0 μL qPCR reaction system contained 10.0 μL of 2× SYBR Green Pro Taq HS Premix (Biotools, USA) , 2.0 μL cDNA, 0.5 μL of the forward primer (10 μM), 0.5 μL of the reverse primer (10 μM), 0.4 μL of ROX Reference Dye (Biotools, USA) (4 μM) and 6.5 μL of RNase-free water. The PCR reaction program was as follows: predenaturation at 95 °C for 30 s, then a cycle of denaturation at 95 °C for 15 s and annealing at 60 °C for 40 s, repeated for 40 cycles. The relative expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ value [24]. Three independent biological replicates of each sample and three technical replicates of each biological replicate were used for qPCR analysis.

2. 5. Analysis of lignin content and lignin monomer composition

Lignin content was detected using the acetyl bromide method described by Fukushima and Hatfield [25] with adjustments. The stems of 30-day-old Arabidopsis plants and 90-day-old poplar plants were collected for lignin content measuring. The lignin content was calculated according to the absorbance at 280 nm. Three biological replicates of each sample were measured with technical repeats.

The lignin monomer composition was analysed using the method described by Lapierre *et al*. [26]. The stems were collected from 90-day-old transgenic and WT poplar plants. The stem samples were dried at 55 °C for 24 h, and then crushed by a crusher for measurement. For each sample, 10 mg powder was measured by adding 10 mL of derivatization solution (1 mL ethanethiol + 0.25 mL boron trifluoride etherate + 8.75 mL dioxane) and incubating at 100 °C for 1 h. Then the reaction was terminated by cooling to room temperature, and the supernatant was diluted with 30 mL sterile water, and the pH was adjusted to 3.5 with 0.4 M $NaHCO_3$. Extract 30 min with 30 mL CH_2Cl_2, dry the organic phase with nitrogen, reconstitute n-hexane, and measure on an Agilent 7890B Meteorological Chromatography-Mass Spectrometer. The conditions were: inlet temperature 290 °C, sample volume 1 μL, furnace temperature from 5 °C at 35 °C /min to 220 °C, and then at 0.5 °C /min to 230 °C, then heated to 280 °C at 50 °C /min, and stayed for 7 min. The ion source temperature is 280 °C, the quadrupole temperature is 280 °C, and the flow rate is 1 mL/min. The injection method is splitless, and the carrier gas is helium.

2. 6. Microscopic observation

The paraffin sections of plant stems were prepared according to the method described by He *et al*. [27]. The stem segments were collected from 90-day-old transgenic poplar and Arabidopsis thaliana. Then the samples were fixed, dehydrated, transparent, dipped in wax, embedded, sliced, and patched to prepare paraffin sections. After the prepared paraffin sections were stained with 1% TBO (Toluidine Blue O) dye solution for 20-30 s, the dye solution was washed away, and the microscope (Leica DM2000 LED) was observed and photographed after mounting. After staining the prepared paraffin section with 5 % resorcinol ethanol solution for 2-3 min, absorb

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020 doi:10.20944/preprints202005.0454.v1

the excess liquid with absorbent paper, then treat with 50 % hydrochloric acid for 2-3 min, immediately observe and take a picture under the microscope.

2. 7. Statistical analysis

One-way ANOVA was performed on the mean of the experimental results, and one-way analysis of variance was performed on the data by SPSS ver. 25.0 (SPSS Inc., USA) for Windows (SPSS Inc.) using with the honestly significant difference test of l.s.d.LSD, Duncan's test (D), and Dunnett's test (E). The level of significance was set to $P < 0.05$. Pearson method was used for correlation analysis.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020 doi:10.20944/preprints202005.0454.v1

3. Results

3. 1. Overexpression of DiCCoAOMT1 *caused morphological change in transgenic Arabidopsis*

Transgenic Arabidopsis lines with relatively higher expression level of *DiCCoAOMT1* were selected for further analysis. The expression level of *DiCCoAOMT1* was 2291-, 358- and 1170-fold of that in wide type (WT) plants in A5, A7 and A16 lines, respectively (Figure 1e). The transgenic Arabidopsis plants exhibited a significant increase in vitality (Figure 1a). The plant height of transgenic lines was elevated by 12.70% on average compared with the WT plants (Figure 1b). In addition, significantly longer pods were observed in the transgenic plants (Figure 1c). The length of transgenic pods was 1.10-fold on average of that of WT pods (Figure 1d). Correlation analysis indicated that there was a significant positive correlation between plant height and relative expression levels of *DiCCoAOMT1* (Figure 1f).

3. 2. Lignin content increased in transgenic Arabidopsis

Lignin content was compared between WT and transgenic Arabidopsis plants. The lignin content of different transgenic lines increased by 23.22%-43.85% compared to that of WT plants (Figure 2a). There was a positive correlation between the relative expression levels of *DiCCoAOMT1* and lignin content, indicating that the high lignin content was determined by the overexpression of *DiCCoAOMT1* (Figure 2b). Then histochemical staining in the cross sections of stems confirmed that lignin accumulation was increased in the xylem of transgenic plants (Figure 2c). Moreover, we observed that the stem diameter was obviously lengthened, and the cortex area was thickened in the transgenic lines. The size of vascular bundle cells increased, and the phloem fibers was lengthened, indicating a faster growth of primary phloem in the transgenic lines. Meanwhile, the proportion of pith area decreased, the size of pith cells significantly increased, whereas the number of pith cells decreased (Figure 2d).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020 doi:10.20944/preprints202005.0454.v1

3. 3. Morphological change in transgenic poplar plants

The *DiCCoAOMT1* gene was then introduced into poplar to reveal its functions in woody plants. Three transgenic lines with different expression levels of *DiCCoAOMT1* were selected for further analysis. The expression level of the target gene was 203-, 4202- and 2868-fold of that in WT plants in P3, P8 and P12 lines, respectively (Figure 3b). Different with the case in Arabidopsis, the plant height was not improved by overexpressing *DiCCoAOMT1* in poplar. Instead, we noticed that the length of internode and petiole was obviously increased in the transgenic plants (Figure 3a). Significantly lengthened internodes and petioles were observed in the fifth true leaf of the transgenic poplar plants (Figure 3c and 3d). There was a positive correlation between internode length and relative expression level of *DiCCoAOMT1*, as well as between petiole length and relative expression level of *DiCCoAOMT1* (Figure 3e).

3. 4. Lignin accumulation increased in transgenic poplar plants

Lignin content was detected in the stems of transgenic poplar lines. The lignin content of P3, P8 and P12 lines increased by 11.47%, 21.54% and 13.63% compared with that of WT plants, respectively (Figure 4a). There was a positive correlation between the relative expression level of *DiCCoAOMT1* and lignin content (Figure 4b). Histochemical staining demonstrated that lignin accumulation was significantly increased in the primary and secondary xylem cells of transgenic plants (Figure 4c). Microscopic observation showed that the intrafascicular cambium and primary xylem were thickened, the pith rays were lengthened, and the pith cells were enlarged in the stems of transgenic lines. There was no obvious change in parenchyma and primary phloem cells of cortex.

3. 5. Lignin monomer composition was changed in transgenic polar plants

In order to further reveal the function of *DiCCoAOMT1*, the lignin monomer composition in transgenic poplar stems was analyzed using GC-MS. The results

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020 doi:10.20944/preprints202005.0454.v1

showed that the G-unit content was significantly increased in P8 and P12, but not in P3. Similarly, the S-unit content was also increased in P8 and P12 but not in P3. Remarkably, the P8 line with highest expression level of *DiCCoAOMT1* showed 7.32-fold and 8.68-fold of G-unit and S-unit content of that in WT plants, respectively. The H-type lignin monomer content was low in both WT and transgenic plants, while there was a slight increase in P3 and P8, but a decrease in P12 (Figure 5a). The S/G ratio was ranged from 0.78 to 1.03 in transgenic lines, which was significantly higher than that in WT plants (0.66), indicating that overexpression of *DiCCoAOMT1* promoted the biosynthesis of S-unit more (Figure 5b).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020 doi:10.20944/preprints202005.0454.v1

4. Discussion

The process of lignin biosynthesis is highly conserved in most plants, and its regulatory mechanism is complicated. As one of the key regulators, the *CCoAOMT* gene family has been a research hotspot for a long time. We have identified 14 *CCoAOMT* gene from the transcriptome data of *Davidia*. However, only one gene, *DiCCoAOMT1*, showed an endocarp-specific expression pattern. This finding indicated that fine division in gene family determines lignin biosynthesis in different tissues. Similar result were reported in other species. Among 7 *CCoAOMT* genes in *A. thaliana*, *AtCCoAOMT1* was expressed in all tissues with the highest expression level. The expression levels of *AtCCoAOMT4*, *AtCCoAOMT5* and *AtCCoAOMT7* increased along with stem development. However, *AtCCoAOMT2*, *AtCCoAOMT3* and *AtCCoAOMT6* were only expressed in the later stages of stem development [28]. In the study in giant bamboo, the expression of *DsCCoAOMT* gene was up-regulated with the development of bamboo shoots, and maintained a high level in the metaphase of bamboo shoot development [29]. Therefore, although lignin biosynthesis in different tissues shares a same pathway, the selection of tissue-specific genes might be an important regulation mode to realize lignification at designated areas.

Endocarp is essential for the adaptations in seed protection and dispersal strategies in plants. Lignification in endocarp is a very different developmental program that requires unique sets of genes or enzymes [30]. The enzymatic activity of PAL, C4H, 4CL, CAD and POD increased rapidly in 30 days in the endocarps of peach (*Prunus persica*) [31]. Similar with our results, phenylpropanoid (*PAL* and *C4H*) and lignin (*CCoAOMT*, *peroxidase* and *laccase*) pathway genes were specifically induced in the endocarp layer over a 10 day time period. However, in walnut (*Juglans regia*), the enzymatic activity of both POD and PAL was decreased in endocarp during fruit development [32]. These evidences indicated that lignification in endocarps or fruits was under a very different control, which might be the reason for the lengthened pods we observed in transgenic Arabidopsis lines.

There was a positive correlation between expression levels of *DiCCoAOMT1* and

lignin content in both transgenic Arabidopsis and poplar lines (Fig. 2b and Fig. 4b), indicating the dosage effect of the function of *DiCCoAOMT1*. We observed that the cambium area was narrowed or replaced by the primary xylem cells in the stem cross section of transgenic poplar lines. However, this variation was not obvious in line P3 (Fig. 4c), in which the expression level of *DiCCoAOMT1* was lowest. Similarly, the results of lignin monomer composition also shown that lignin monomer content was not altered in line P3 (Fig. 5a). These findings showed that DiCCoAOMT1 functioned in lignin biosynthesis under high dosage.

Consistent with our results, overexpression of a *MlCCoAOMT1* gene from *Miscanthus lutarioriparius* also resulted in thickened secondary cell wall in transgenic *Arabidopsis* [33]. However, different results have been found in the study of an *LjCCoAOMT1* gene from *Lonicera japonica*. Overexpression of *LjCCoAOMT1* gene did not cause variations in plant height and leaves in transgenic Rice (*Oryza sativa* L.) lines, but there were significant differences in the number of tillers and the number of grains per ear [34]. Although the transgenic lines of Arabidopsis and poplar both exhibited elevated vigor, they have different morphological variations (Fig. 1a and Fig. 3a). Higher lignin content might provide stronger mechanical support to the transgenic lines, meanwhile promote the growth rate of stems and leaves.

It is unexpected that overexpression of *DiCCoAOMT1* increased the S/G rate in transgenic poplar lines. There are a number of reports proved that inhibiting CCoAOMT activity reduced the lignin content and G-unit content in transgenic plants, but the S-unit content was not affected, thus resulting in an increase of S/G ratio [17, 35, 36]. On the contrary, inhibition of COMT activity resulted in decrease of S-unit content, whereas G-unit content remains unchanged [37]. These evidences indicated that COMT is involved in the methylation of C5 position and specifically participates in the synthesis of S-unit, while CCoAOMT is mainly involved into the biosynthesis of G-unit. However, some studies found that *CCoAOMT* is not only involved in the synthesis of G-unit, but also in the synthesis of S-unit [38, 39]. DiCCoAOMT1 might promote S-unit biosynthesis through altering the conversion direction of conifer

aldehyde [40]. Conifer aldehyde is located at the shunt node of G- and S-unit. It comes from two sources, including caffeic acid CoA and ferulyl CoA. We speculate that the high expression level of *DiCCoAOMT1* caused massive accumulation of conifer aldehydes, and catalyzed the conversion from F5H to S-unit. In addition, overexpression of *DiCCoAOMT1* also caused a slight increase of H-unit content in some transgenic lines. Hydroxystilbenes, a new class of stilbenolignin polymers, were identified in the endocarp of some palm species, indicating that the lignin composition of endocarp is very different with those in other tissues. The endocarp of *Davidia* is much harder than that of its stems, indicating a different lignin composition in the endocarp. Whether the substrate of DiCCoAOMT1 is ferulyl CoA or other compounds remains to be further studied.

DiCCoAOMT1 was specifically expressed in endocarp, and its expression level was elevated along with the lignification process of endocarp. The upstream regulation of *DiCCoAOMT1* is still a mystery. We cloned the promoter region of *DiCCoAOMT1*, and identified several cis-elements including a MBS (MYB binding site), a Sp1 element and a rbcS-CMA7a element involved in light response, and a TGACG-motif involved in the MeJA-responsiveness (unpublished data). This result indicated that the expression of *DiCCoAOMT1* might be controlled by light and endogenous hormones, and transcription factors, such as MYB, could play a critical role in regulating *CCoAOMT* gene and lignin biosynthesis. This finding was also reported in the study in hawthorn (*Crataegus* spp.), which performed the comparing transcriptome analysis between soft-endocarp and hard-endocarp hawthorn, and identified some MYB and NAC transcription factors that could potentially control lignin and flavonoid biosynthesis specifically in endocarp [41]. MYB and NAC transcription factors were also reported to be closely related to lignin accumulation in loquat fruit [42, 43].

Taken together, we identified an endocarp-specific *CCoAOMT* gene and verified its function in both herb and woody plants. The target gene significantly increased lignin content and altered lignin composition in transgenic lines, indicating its critical

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020 doi:10.20944/preprints202005.0454.v1

role in regulating the rapid lignification and determining the long seed dormancy in *Davidia*.

5. Conclusions

In summary, in this study, we verified the function of a gene called *DiCCoAOMT1* from the endocarp of *Davidia* in Arabidopsis and poplar. Overexpression of *DiCCoAOMT1* led to organ enlargement in transgenic plants, which also showed enhanced vigor. Moreover, overexpression of *DiCCoAOMT1* increased the lignin content by 45% and 20% in transgenic *A. thaliana* and poplar lines, respectively. Different with the function of *CCoAOMT* genes reported in other species, DiCCoAOMT1 contributed more on the biosynthesis of S-unit, thus increased the S/G rate and altered the lignin composition in transgenic poplar lines. The spatiotemporal expression of *DiCCoAOMT1* is important for the formation of the unique structure of *Davidia* endocarp, and the lignin composition of endocarp was supposed to be very different with that in xylem. Our results is not only laid a theoretical basis for elucidating the long seed dormancy of *Davidia*, but also important for understanding the molecular mechanism of lignification in specific organs.

Supplementary Materials:

Figure S1: The content of G-, S- and H-unit of lignin monomer detected by GC-MS. **a**, WT; **b**, P3; **c**, P8; **d**, P12. Table S1: Information of primers applied in the present study.

Author Contributions: Meng Li and Fuxiang Cao designed the experimental scheme; Xujie Dong provided the endocarp materials and transcriptome data of *D. involucrata*; Jian Li and Xiaomin Ji performed experiments and data analysis; Meng Li and Jian Li wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by grants from the Innovation Project of Forestry Science and Technology in Hunan Province (XLK201984) and the Key Research and Development Project of Hunan Province (2017NK2252).

Acknowledgements: The authority responsible for the *D. involucrata* resources is the Badagong Mountain Nature Reserve Management Division, who gave permission to collect the samples for our scientific research. We are grateful to the managers of Badagong Mountain Nature Reserve for their support of this study.

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

References

1. Whetten, R. Lignin Biosynthesis. *The Plant Cell* **1995**, *7*, 1001-1013.

2. Baucher, M.; Halpin, C.; Petit-Conil, M. Boerjan, W. Lignin: Genetic Engineering and Impact on Pulping. *Critical Reviews in Biochemistry and Molecular Biology* **2003**, *38*, 305-350.

3. Vanholme, R.; Demedts, B.; Morreel, K.; Ralph, J.; Boerjan, W. Lignin Biosynthesis and Structure. *Plant Physiology* **2010**, 153, 895-905.

4. Boerjan, W.; Ralph, J.; Baucher. M. Lignin biosynthesis. *Annual Review of Plant Biology* **2003**, *54*, 519-546.

5. Simon, C.; Lion, C.; Huss, B.; Blervacq, A.S.; Spriet, C.; Guérardel, Y.; Biot, C.; Hawkins, S. BLISS: Shining a light on lignification in plants. *Plant Signaling & Behavior* **2017**, *12*, e1359366.

6. Manchester, S.R. Leaves and fruits of Davidia (Cornales) from the Paleocene of North America. *Systematic Botany* **2002**, *27*, 368-382.

7. Chen, K.R. A preliminary report on the research of dormancy of Davidia involucrata seeds. *Plant Physiology Communications* **1988,** *3*, 24-28.

8. Wan, C.K. Anatomical study on seed dormancy of Dove tree(Davidia involucrata). *Journal of Central South Forestry College* **1988**, *8*, 35-39.

9. Li, M.; Dong, X.J.; Peng, J.Q.; Xu, W.; Ren, R.; Liu J.; Cao, F.X.; Liu, Z.M. *De novo* transcriptome sequencing and gene expression analysis reveal potential mechanisms of seed abortion in dove tree (*Davidia involucrata* Baill.). *BMC Plant Biology* **2016**, *16*, 82.

10. Wu, X.B.; Yan, Z.W.; Dong X.J.; Cao, F.X.; Peng, J.Q.; Li, M. Cloning and characterization of a *CCoAOMT* gene involved in rapid lignification of endocarp in dove tree (*Davidia involucrata* Baill.). *Biotechnology & Biotechnological Equipment* **2019**, *32*, 1398-1406.

11. Wei, J.H.; Zhao, H.Y.; Zhang, J.Y.; Liu, H.R.; Song, Y.R. Cloning of cDNA Encoding *CCoAOMT* from *Populus tomentosa* and Down-regulation of Lignin Content in Transgenic Plant Expressing Antisense Gene. *Acta Botanica Sinica* **2001**, *43*,

1179-1183.

12. Rakoczy, M.; Femiak, I.; Alejska, M.; Figlerowicz, M.; Podkowinski, J. Sorghum *CCoAOMT* and CCoAOMT-like gene evolution, structure, expression and the role of conserved amino acids in protein activity. *Molecular Genetics and Genomics* **2018**, *293*, 1-13.

13. Zhao, H.Y.; Sheng, Q.X.; Lü, S.Y. Characterization of three rice CCoAOMT genes. *Chinese Science Bulletin* **2004**, *49*, 1602-1606.

14. Do, C.T.; Pollet, B.; Thevenin, J.; Sibout, R.; Denoue, D.; Barriere, Y.; Lapierre, C.; Jouanin, L. Both caffeoyl Coenzyme A 3-O-methyltransferase 1 and caffeic acid O-methyltransferase 1 are involved in redundant functions for lignin, flavonoids and sinapoyl malate biosynthesis in Arabidopsis. *Planta* **2007**, *226*, 1117-1129.

15. Pang, S.L.; Ong, S.S.; Lee, H.H.; Zamri, Z.; Kandasamy, K.I.; Choong, C.Y.; Wickneswari, R. Isolation and characterization of CCoAOMT in interspecific hybrid of Acacia auriculiformis x Acacia mangium --a key gene in lignin biosynthesis. *Genetics and Molecular Research:GMR* **2014**, *13*, 7217–7238.

16. Wagner, A.; Tobimatsu, Y.; Phillips, L.; Flint, H.; Torr, K.; Donaldson, L.; Pears, L.; Ralph, J. CCoAOMT suppression modifies lignin composition in Pinus radiata. *The Plant Journal* **2011**, *67*, 119-129.

17. Eom, I.Y.; Kim, K.H.; Lee, S.M.; Yi, Y.S.; Choi, J.W. Characterization of Chemical Composition in Poplar wood (Populus deltoides) by Suppression of CCoAOMT Gene Expression. *Journal of the Korean Wood Science and Technology* **2010**, *38*, 213-222.

18. Li, X.Y.; Chen, W.J.; Zhao, Y.; Xiang, Y.; Jiang, H.Y.; Zhu, S.W.; Cheng, B.J. Downregulation of caffeoyl-CoA O-methyltransferase (CCoAOMT) by RNA interference leads to reduced lignin production in maize straw. *Genetics and Molecular Biology* **2013**, *36*, 540-546.

19. Zhang, X.S.; Ni, R.; Wang, P.Y.; Zhu, T.T.; Sun, C.J.; Lou, H.X.; Cheng, A.X. Isolation and functional characterization of two Caffeoyl Coenzyme A 3-O-methyltransferases from the fern species Polypodiodes amoena. *Plant Physiology and Biochemistry* **2019**, *136*, 169-177.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020 doi:10.20944/preprints202005.0454.v1

20. Zhang, G.Y.; Qi, J.M.; Xu, J.T.; Niu, X.P.; Zhang, Y.J.; Tao, A.F.; Zhang, L.W.; Fang, P.P.; Lin, L.H. The CCoAOMT1 gene from jute (Corchorus capsularis L.) is involved in lignin biosynthesis in Arabidopsis thaliana. *Gene* **2014**, *546*, 398-402.

20. Zhang, X.R.; Henriques, R.; Lin, S.S.; Niu, Q.W.; Chua, N.H. Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. *Nature Protocol* **2006**, *2*, 1-6.

21. Murashige, T.; Skoog, F. Arevised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum* **1962**, *15*, 473-497.

22. Jia, Z.C.; Gou, J.Q.; Sun, Y.M.; Yuan, L.; Tian, Q.; Yang, X.Y.; Pei, Y.; Luo, K.M. Enhanced resistance to fungal pathogens in transgenic Populus tomentosa Carr. by overexpression of an nsLTP-like antimicrobial protein gene from motherwort (Leonurus japonicus). *Tree Physiol* **2010**, *30*, 1599–1605.

23. McCown, B.H.; Lloyd, G. Woody Plant Medium (WPM)-a mineral nutrient formulation for microculture for woody plant species. *Horticultural Science* **1981**, 16, 453.

24. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402-408.

25. Fukushima, R.S.; Hatfield, R.D. Comparison of the Acetyl Bromide Spectrophotometric Method with Other Analytical Lignin Methods for Determining Lignin Concentration in Forage Samples. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 3713-3720.

26. Lapierre, C.; Pollet, B.; Rolando, C. New Insights Into the Molecular Architecture of Hardwood Lignins by Chemical Degradative Methods. *Research on Chemical Intermediates* **1995**, *21*, 397-412.

27. He, B.; Li, Z.G.; Hao, X.J.; He Y.C. A New Technique of Fast Paraffin Sectioning in Plant Tissues. *Chinese Bulletin of Botany* **2014**, *49*, 203-208.

28. Raes, J.; Rohde, A.; Christensen, J.; Van de Peer, Y.; Boerjan, W. Genome-Wide Characterization of the Lignification Toolbox in. *Plant physiology* **2003**, *133*, 1051-1071.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020 doi:10.20944/preprints202005.0454.v1

29. Chen, L.N.; Guo, X.J.; Yang, H.Q. Cloning and Expression Analysis of CCoAOMT，a Key Gene in Lignin Biosynthesis of Dendrocalamus sinicus. *Journal of Plant Genetic Resources* **2019**, *20*, 476-484.

30. Dardick, C.; Callahan, A. Evolution of the fruit endocarp: Molecular mechanisms underlying adaptations in seed protection and dispersal strategies. *Frontiers in plant science* **2014**, *5*, 284.

31. Cao, A.J.; Wang, Y.N.; Ge, S.L.; Yang, A.Z.; Xin, Y.J.; Hua, B.G. Changes of enzyme activity during the depositive process of lignin in peach endocarp. *Journal of Beijing Agricultural College* **2009**, *24*, 5-8.

32. Zhao, S.G.; Wen, J.; Wang, H.X.; Zhang, Z.H.; Li, X.B. Changes in Lignin Content and Activity of Related Enzymes in the Endocarp During the Walnut Shell Development Period. *Horticultural Plant Journal* **2016**, *3*, 141-146.

33. Wang, H.M. Cloning and Functional Characterization of MICCoAOMT1 in Miscanthus. Lutarioriparius. *Lanzhou University* **2014**, Thesis.

34. Jiang, X.H.; She, C.W.; Zhu, Y.H.; Liu, X.M. Cloning and expression analysis of the Lonicera japonica Thunb. chlorogenic acid synthetase gene (LjCCoAOMT1) in rice. *Genetics and Molecular Research:GMR* **2014**, *13*, 2166-2176.

35. Guo, D.J.; Chen, F.; Inoue, K.; Blount, J.W.; Dixon, R. Downregulation of Caffeic Acid 3-O-Methyltransferase and Caffeoyl CoA 3-O-Methyltransferase in Transgenic Alfalfa: Impacts on Lignin Structure and Implications for the Biosynthesis of G and S Lignin. *The Plant cell* **2001**, *13*, 73-88.

36. Meyermans, H.; Morreel, K.; Catherine, L.; Pollet, B.; Bruyn, A.; Busson, R.; Herdewijn, P.; Devreese, B.; Beeumen, J.; Marita, J.; Ralph, J.; Chen, C.; Burggraeve, B.; Van Montagu, M.; Messens, E.; Boerjan, Wout. Modifications in Lignin and Accumulation of Phenolic Glucosides in Poplar Xylem upon Down-regulation of Caffeoyl-Coenzyme A O-Methyltransferase, an Enzyme Involved in Lignin Biosynthesis. *Journal of Biological Chemistry* **2000**, *275*, 36899-36909.

37. Rossitza, A.; Noëlle, F.; Françoise, M.; Brigitte, C.; Marie-Thérèse, T.; Bernard, M.; Bernard, F.; Michel, L. Altered lignin composition in transgenic tobacco expressing O-methyltransferase sequences in sense and antisense orientation.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2020 doi:10.20944/preprints202005.0454.v1

The Plant Journal **2003**, 8, 465-477.

38.Ye, Z.H.; Zhong, R.; Morrison, W.H.; Caffeoyl coenzyme A O-methyltransferase and lignin biosynthesis. Phytochemistry **2001**, *57*, 1177-1185.

39.Schmitt, D.; Pakusch, A.E.; Matern, U. Molecular cloning, induction, and taxonomic distribution of Caffeoyl-CoA 3-O-methyltransferase, an enzyme involved in disease resistance. Journal of Biological Chemistry **1991**, *266*, 17416-17423.

40. Chapple, C. Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. *Annual Review of Plant Physiology and Plant Molecular Biology* **1998**, *49*, 311-343.

41. Dai, H.Y.; Han, G.F.; Yan, Y.J.; Zhang, F.; Liu, Z.C.; Li, X.M.; Li, W.R.; Ma, Y.; Li, H.; Liu, Y.X.; Zhang, Z.H.; Transcript Assembly and Quantification by RNA-Seq Reveals Differentially Expressed Genes between Soft-Endocarp and Hard-Endocarp Hawthorns. *PLOS ONE* **2013**, *8*, e72910.

42. Ge, H.; Zhang, J.; Zhang, Y.J.; Li, X.; Yin, X.R.; Grierson, D.; Chen, K.S. EjNAC3 transcriptionally regulates chilling-induced lignification of loquat fruit via physical interaction with an atypical CAD-like gene. *Journal of Experimental Botany* **2017**, *68*, 5129-5136.

43. Wang, W.Q.; Zhang, J.; Ge, H.; Li, S.J.; Li, X.; Yin, X.R.; Grierson, D.; Chen, K.S. EjMYB8 Transcriptionally Regulates Flesh Lignification in Loquat Fruit. *PLOS ONE* **2016**, *11*, e0154399.