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IDENTIFICATION OF THE CELL WALL SYNTHESIS GENES IN *BETULA PENDULA*

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ABSTRACT

This study aims to provide information on *Betula pendula* cell wall synthesis genes regarding their potential physiological roles and the molecular mechanism associated. Here we identified 46 gene models in 7 gene families that encode cellulose synthase and related enzymes of *B. pendula*, and the transcript abundance of these genes in xylem, root, leaf, and flower tissues also be determined. Based on these RNA-seq data, we have identified 8 genes that most likely participate in cell wall synthesis, which include 3 cellulose synthase genes and 5 cellulose synthase-like genes. In parallel, a gene co-expression network was also constructed based on transcriptome sequencing. These analyses will help decipher the genetic information of *B. pendula* cell wall synthesis genes and alter its wood structure on the cellular level.

KEYWORDS: *Betula pendula*, cell wall, cellulose synthase, RNA-seq, WGCNA, transcription factors.

INTRODUCTION

Silver birch (*Betula pendula*) is a medium-sized deciduous tree that owes its common name to the white peeling bark on the trunk. This species is native to Europe and parts of Asia, and the range extends into Siberia, China, and southwest Asia in the mountains of northern Turkey, the Caucasus, and northern Iran (Hynynen et al. 2010). *B. pendula* is an ecologically and economically important plant species due to its strong tolerance to various climates. Flowering at an early age allows *B. pendula* has a faster succession of generations, which together with rapid juvenile development can shorten the breeding cycle. Large-sized logs are produced within relatively short periods with proper silvicultural treatment, and the wood characteristics allow versatile and valuable uses. In the context of societal evolutions and customer perceptions, *B.*

pendula will certainly play an increasing role in the building and furniture sectors, and among non-wood forest products (Dubois et al. 2020).

The cell periphery of higher plants is usually surrounded by the cell wall. Plant cell walls are complex networks of polymers that provide protection and structural properties to the cells (Buchanan et al. 2015). The cell wall mainly includes four major chemical polymers: cellulose, hemicellulose, lignin, and pectin (Pettersen 1984, Chen et al. 2020). Of these, cellulose is usually regarded as an outstanding commodity due to its abundance and distinctive structural properties. It is a linear homopolymer of β-1,4-linked glucose residues, and the coordinated synthesis of glucose chains is orchestrated by specific plasma membrane-bound cellulose synthase complexes. Annually, plants will produce about 180 billion tons of cellulose, making it the largest reservoir of organic carbon on earth (Festucci-Buselli et al. 2007). Pear et al. (1996) isolated and identified the *CESA* genes encoding cellulose synthase for the first time from cotton in 1996. Subsequent analysis of the *Arabidopsis thaliana* genome revealed that a total of 10 genes encode CESA proteins with 64% average sequence identity (Holland et al. 2000, Richmond 2000). In *Betula*-related studies, Liu et al. (2012) have isolated four full-length *CESA* cDNAs from *B. platyphylla* by using the RT-PCR method and calculated the phylogenetic relationship of them. Huang et al. (2014) have isolated eight full-length *CESA* cDNAs from *B. luminifera* based on transcriptome sequencing, and determined their positive influence in tension wood.

As an important tree species in papermaking, understanding the cellulose synthesis pathway of *B. pendula* will greatly contribute to its use in industrial production. Fortunately, the assembled sequences of *B. pendula* genome have become publicly available, which can help us understand this species at the genome expression level (Salojärvi et al. 2017, Chen et al. 2021). In this study, we identified the genes that likely encode cellulose synthase and related enzymes during cell wall synthesis in *B. pendula*, which will serve as a basis for further gene functional studies.

MATERIAL AND METHODS

Identification of *B. pendula* cell wall synthesis genes

The *B. pendula* genome (Salojärvi et al. 2017) and genomic structure information were downloaded from the CoGe (comparative genomics) platform. The putative cellulose synthase genes were first identified by BLASTP (Basic local alignment search tool - protein) v2.9.0 (Camacho et al. 2009) with the *A. thaliana* cellulose synthase genes as queries (e-value ≤ 1e-5). We then further manually examined these putative cell wall synthesis genes using the conserved domain database of NCBI (national center for biotechnology information) (Marchler-Bauer et al. 2015) to confirm if they were correctly annotated, and divided them into seven subgroups based on their functional type in *A. thaliana*. In addition, the chromosomal location of the *B. pendula* cell wall synthesis genes was visualized by using TBtools (toolbox for biologists) v0.67 (Chen et al. 2018).

Phylogenetic analyses of *B. pendula* cell wall synthesis genes

To investigate the phylogenetic relationships of the cellulose synthases (CESAs) and cellulose synthase-like proteins (CSLs), the phylogenetic tree was constructed for every subgroup. The multiple sequence comparison was performed by MUSCLE (multiple sequence alignment with high accuracy and high throughput) v3.8.1551 (Edgar 2004) with default parameters, and the constraint maximum likelihood phylogenetic trees of each subgroup were then be generated by RAxML (randomized accelerated maximum likelihood) v8.2.12 (Stamatakis 2014) with 1,000 bootstrap trials. The model was selected for the gamma model and visualized by iTOL (interactive tree of life) v5.0 (Letunic and Bork 2019).

RNA-seq expression analysis of *B. pendula* cell wall synthesis genes

Transcriptome sequencing data (PRJNA535361) from a previous study by us (Chen et al. 2019) were downloaded to investigate the expressional patterns of *B. pendula* cellulose synthase genes in different tissues. The clean reads of three replicates per tissue were aligned to the *B. pendula* transcriptome by using STAR (spliced trans alignment to a reference) v2.7.3a (Dobin et al. 2013), and the accurate transcript quantification was estimated by using RSEM (RNA-seq by expectation-maximization) v1.3.3 pipeline (Li and Dewey 2011) with paired-end sequencing mode. The normalized expression value was all selected as TMM (trimmed mean of M-values).

Transcription factor regulatory networks in *B. pendula* cell wall synthesis

The transcription factors of *B. pendula* were identified by PlantTFcat (Dai et al. 2013), and the conserved domain database of NCBI (Marchler-Bauer et al. 2015) was being used to determine whether they are correctly annotated. To perform the weighted correlation network analysis between cell wall synthesis genes and transcription factors, we used the WGCNA (weighted correlation network analysis) R package v1.69 (Langfelder and Horvath 2008) to construct the co-expression network. The TMM value from different tissues of *B. pendula* was as input expression data for this software, and only genes with TMM values larger than 10 for all samples were kept. The threshold power (β) value was determined to be 13 and the following settings were used: TOM-type, unsigned; mergeCutHeight, 0.15; deepSplit, 2; minModuleSize, 30; and eventually visualized by the Cytoscape v3.8.0 (http://cytoscape.org/).

RESULTS AND DISCUSSION

Identification of *Betula pendula* cellulose cell wall synthesis genes

A total of 28,153 coding genes in *B. pendula* genome (Salojärvi et al. 2017) were used to identify putative cell wall synthesis genes. In total, 46 gene models (Tab. 1) in 7 families were identified as putative cell wall synthesis genes in *B. pendula* genome. These 46 genes encode 10 cellulose synthase proteins (CESAs) and 36 cellulose synthase-like proteins (CSLAs, CSLBs, CSLCs, CSLDs, CSLEs, and CSLGs) in 7 families. Among these families, *CESA* was the predominant cellulose synthase gene family and contains seven members. The rest of the gene families all belong to the cellulose synthase-like family, *CSLG* was the largest cellulose synthase-like family containing eleven members, while *CSLA* was the smallest family with only

three members. We then applied quantitative criteria to assign the genes likely to be cell wall synthesis genes based on transcript abundance and specificity. The tissue-specific expressional data include xylem, roots, leaves, and flowers, and we calculated the expression of the 46 identified genes. A total of 8 genes showed that expression in the xylem was higher than the expression in both flower and leaf. These genes were identified as the cell wall synthesis genes *BpCESA4*, *BpCESA9*, *BpCESA10*, *BpCSLA2*, *BpCSLA3*, *BpCSLC1*, *BpCSLC4*, and *BpCSLD4*.

Tab. 1: Putative B. pendula cellulose synthase genes in 7 gene families.

Gene family	Gene name	Gene ID (*B. pendula*)	Theoretical pI	Molecular weight
CESA	*BpCESA1*	*Bpev01.c0196.g0006*	6.36	122,491.46
	BpCESA2	*Bpev01.c0205.g0006*	6.36	127,415.18
	BpCESA3	*Bpev01.c0777.g0012*	6.70	121,270.01
	BpCESA4	***Bpev01.c0000.g0006***	**8.04**	**119,178.11**
	BpCESA5	*Bpev01.c0402.g0034*	6.81	122,759.64
	BpCESA6	*Bpev01.c0480.g0087*	7.43	122,927.82
	BpCESA7	*Bpev01.c0598.g0015*	6.46	124,289.77
	BpCESA8	*Bpev01.c0603.g0003*	6.05	96,459.34
	BpCESA9	***Bpev01.c0374.g0017***	**6.12**	**110,387.98**
	BpCESA10	***Bpev01.c0374.g0018***	**6.38**	**117,638.99**
CSLA	*BpCSLA1*	*Bpev01.c0902.g0015*	9.20	62,242.93
	BpCSLA2	***Bpev01.c0169.g0024***	**9.16**	**61,224.78**
	BpCSLA3	***Bpev01.c2286.g0004***	**9.31**	**74,447.19**
CSLB	*BpCSLB1*	*Bpev01.c1000.g0017*	8.61	23,261.32
	BpCSLB2	*Bpev01.c1000.g0013*	5.40	42,336.85
	BpCSLB3	*Bpev01.c1000.g0018*	7.94	86,260.11
	BpCSLB4	*Bpev01.c1193.g0003*	5.64	41,363.16
	BpCSLB5	*Bpev01.c1193.g0012*	6.07	42,637.45
	BpCSLB6	*Bpev01.c1193.g0006*	4.56	8,816.31
	BpCSLB7	*Bpev01.c1000.g0016*	5.96	17,352.82
CSLC	***BpCSLC1***	***Bpev01.c0094.g0029***	**8.73**	**76,234.32**
	BpCSLC2	*Bpev01.c0515.g0003*	8.84	79,284.87
	BpCSLC3	*Bpev01.c0058.g0002*	8.74	77,616.24
	BpCSLC4	***Bpev01.c0018.g0093***	**8.58**	**82,856.18**
CSLD	*BpCSLD1*	*Bpev01.c0016.g0057*	4.44	11,023.28
	BpCSLD2	*Bpev01.c0016.g0055*	7.34	118,350.37
	BpCSLD3	*Bpev01.c0423.g0009*	6.91	128,449.29
	BpCSLD4	***Bpev01.c0949.g0008***	**6.91**	**167,167.54**
	BpCSLD5	*Bpev01.c1082.g0006*	6.09	125,593.88
	BpCSLD6	*Bpev01.c1484.g0010*	6.16	121,322.33
	BpCSLD7	*Bpev01.c0364.g0008*	8.15	131,707.55
CSLE	*BpCSLE1*	*Bpev01.c1469.g0001*	6.41	98,907.76
	BpCSLE2	*Bpev01.c1782.g0020*	6.43	84,918.41
	BpCSLE3	*Bpev01.c1782.g0018*	7.54	63,693.94
	BpCSLE4	*Bpev01.c2470.g0006*	5.93	58,439.48
CSLG	*BpCSLG1*	*Bpev01.c1225.g0008*	8.49	82,497.93
	BpCSLG2	*Bpev01.c1739.g0002*	8.99	11,851.03

	BpCSLG3	*Bpev01.c1739.g0001*	7.85	69,509.20
	BpCSLG4	*Bpev01.c0188.g0037*	6.70	11,889.79
	BpCSLG5	*Bpev01.c2210.g0001*	5.74	20,329.79
	BpCSLG6	*Bpev01.c2469.g0001*	6.80	18,162.08
	BpCSLG7	*Bpev01.c0995.g0003*	7.82	83,335.89
	BpCSLG8	*Bpev01.c1270.g0001*	7.83	83,674.44
	BpCSLG9	*Bpev01.c0774.g0001*	6.83	84,300.74
	BpCSLG10	*Bpev01.c0774.g0003*	7.58	74,176.67
	BpCSLG11	*Bpev01.c0774.g0002*	7.53	84,509.94

Gene information in bold is for the genes most probably encode cell wall synthesis enzymes.

Chromosomal location and gene duplication

Cell wall synthetases mainly include cellulose synthases (CESAs) and cellulose synthase-like proteins (CSLs), so we investigated the formation of CESAs and CSLs based on the chromosomal location and intra-genome synthetic information. Similar to the *A. thaliana*, the multiple *BpCESAs* were scattered across the *B. pendula* genome and mapped in 13 of the 14 chromosomes (Fig. 1 and 2). The *BpCESAs* were concentrated on chromosome 6, 7, 8, 9, 10, and 11, with one or two genes per chromosome. The *BpCSLs* were scattered on 13 chromosomes except for chr5, and we found that some *BpCSLs* were organized into duplicated blocks, such as *BpCSLB1-7* on chr2, *BpCSLG2-7* on chr14, and *BpCSLG8-10* on chr1. This situation always originated from duplicative transposition.

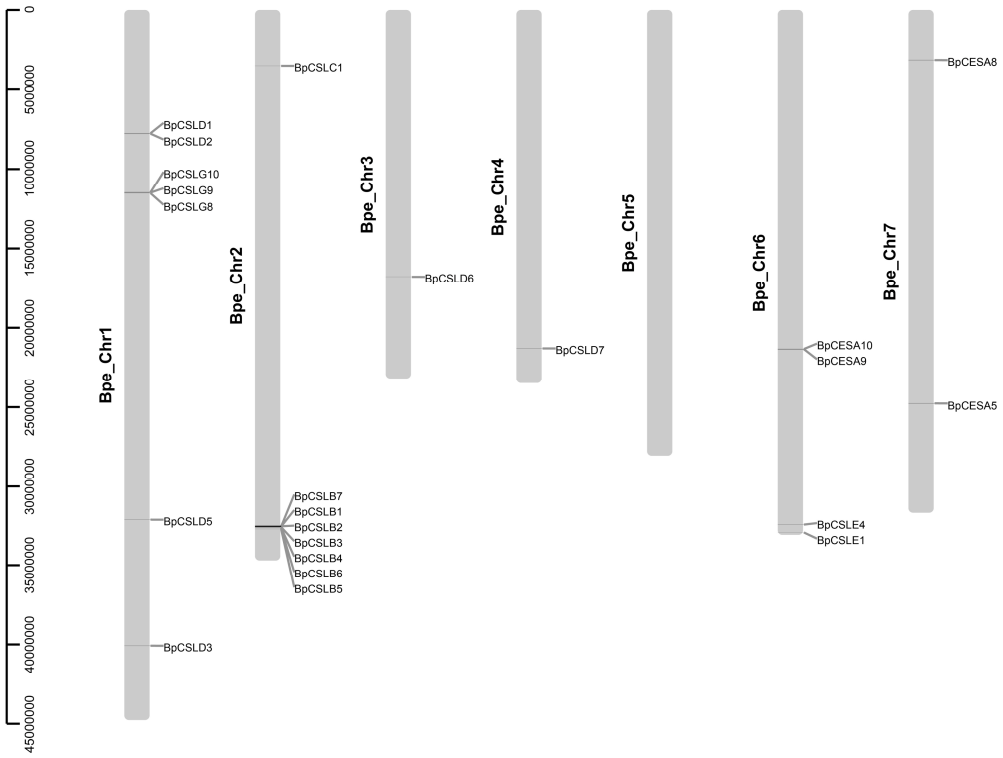

Fig. 1: The chromosomal location of B. pendula cell wall synthesis genes (Bpe_Chr1-7). The silver line represents the chromosome of B. pendula, and the black line represents the relative location of CESA and CSL genes on the chromosome.

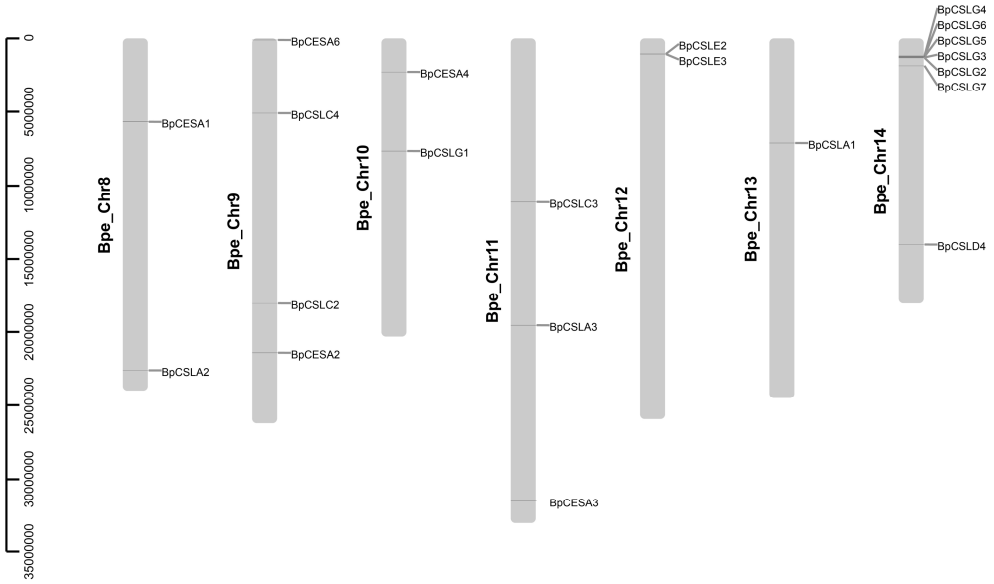

Fig. 2: The chromosomal location of B. pendula cell wall synthesis genes (Bpe_Chr8-14). The silver line represents the chromosome of B. pendula, and the black line represents the relative location of CESA and CSL genes on the chromosome.

Cellulose synthase (CESA) gene family

Cellulose is the principal ingredient of the cell walls in *B. pendula*, and the small microfibrils were crystallized by 36 tails of H-bonded-β-1,4-Glc chains catalyzed by cellulose synthases (Joshi 2003). Thus, cellulose synthase (CSEA) was one of the indispensable glycosyltransferases in plants, which plays a crucial role in regulating cell wall cellulose synthesis and plant cell morphogenesis.

We identified 10 *BpCESAs* in the *B. pendula* genome, of which *BpCESA4*, *BpCESA9*, and *BpCESA10* were abundant in xylem (Fig. 3). *BpCESA4* was the highest expressed gene in the root and xylem of the CESA family. The most similar protein to BpCESA4 was AtCESA4 in *A. thaliana*, which confers plant resistance to bacterial and fungal pathogens while encoding a cellulose synthase. Handakumbura et al. (2013) reported that AtCESA4 loss-of-function mutants of *A. thaliana* and *Oryza sativa* have weak stems and thin or irregular cell walls. The protein most similar to BpCESA9 and BpCESA10 was AtCESA8 in *A. thaliana*, Glass et al. (2015) reported that endo-β-1,4-glucanases AtGH9B5 and AtGH9C2 can impact cellulose crystallization and plant cell wall development by influencing cellulose synthase AtCESA8. In addition, Kim et al. (2014a) reported that transcription factor AtMYB46 can directly regulate the secondary wall-associated cellulose synthase AtCESA4 and AtCESA8 in *A. thaliana*.

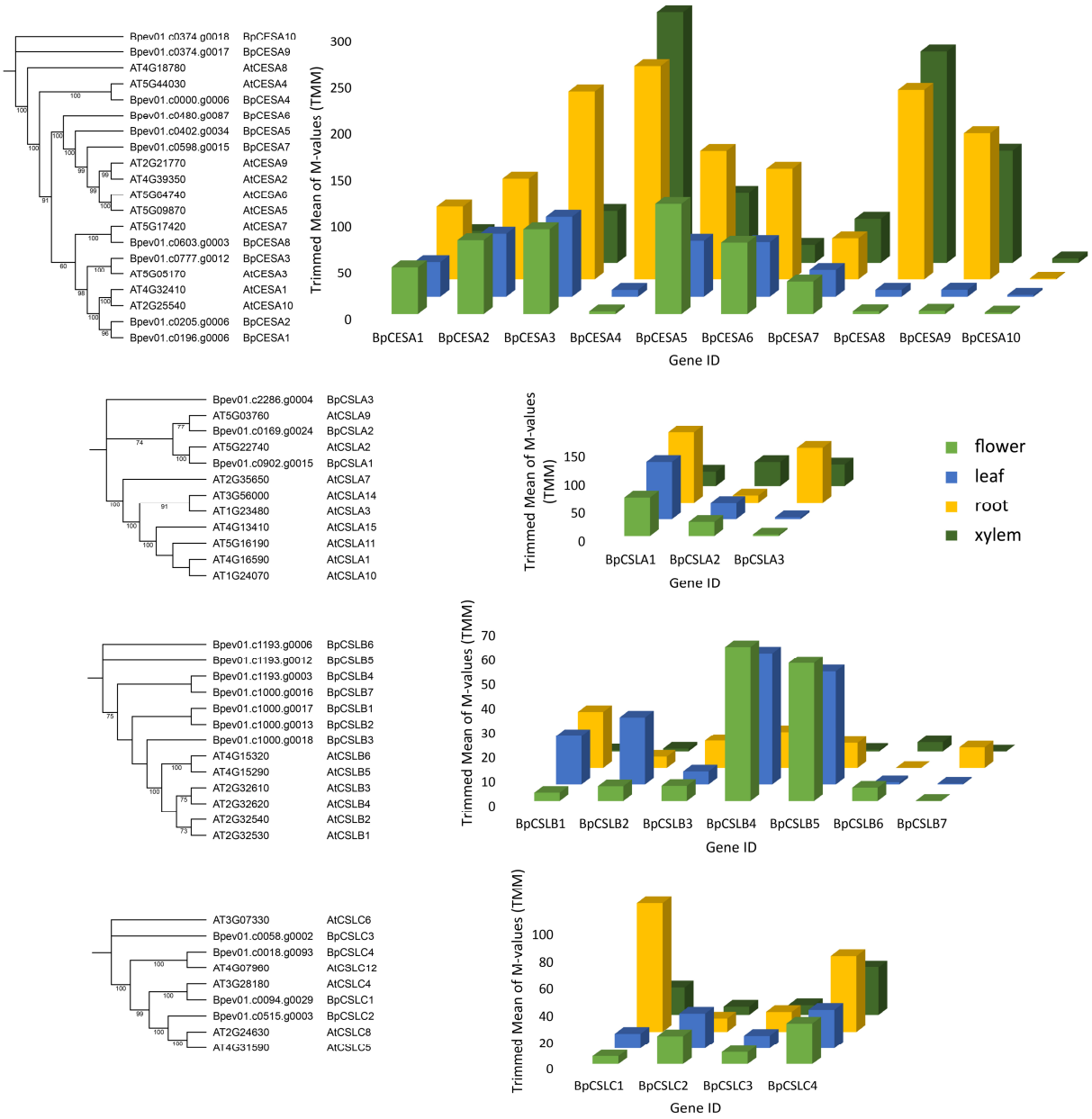

Fig. 3: Tissue-specific expression profiles and phylogenetic analysis of BpCESA, BpCSLA, BpCSLB, and BpCSLC families in B. pendula. The expression was analyzed in three independent biological replicates of each tissue, and the phylogenetic tree (1000 bootstraps) was constructed by RAxML using the maximum likelihood algorithm.

Cellulose synthase-like (CSL) gene family

The cellulose synthase-like (CSL) gene family was divided into six families, which were CSLA, CSLB, CSLC, CSLD, CSLE, and CSLG. The functions of the CSL family are still being explored, but a substantial number of studies were published in recent years. Jensen et al. (2012) reported that the CSL genes is associated with hemicellulose synthesis, Schreiber et al. (2014) and Doblin et al. (2009) reported that cellulose synthase-like protein CSLFs and CSLHs mediate

the synthesis of the cell wall (1,3)(1,4)-β-D-Glucans, but the vast majority of CSL genes functions require further study.

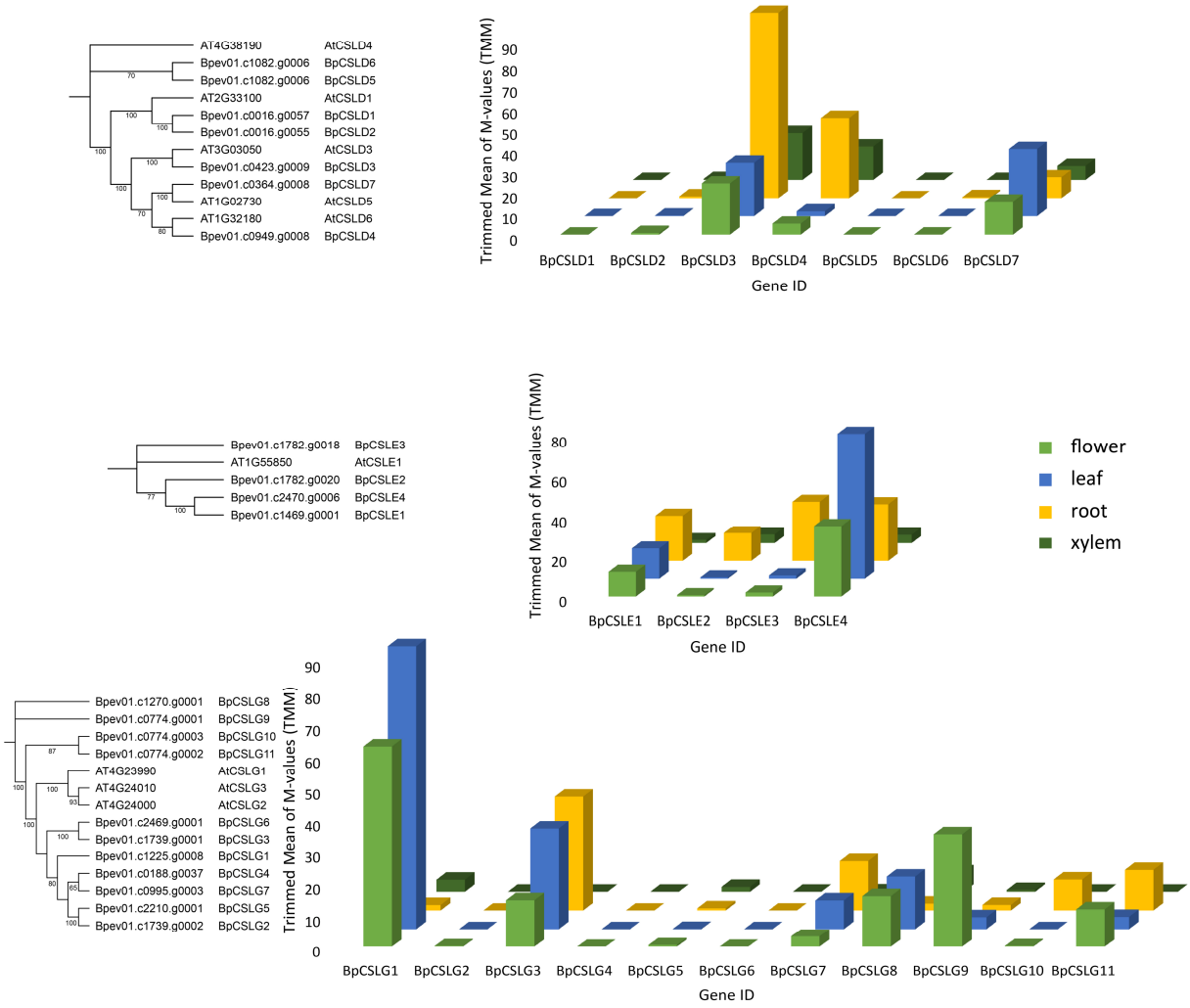

Fig. 4: Tissue-specific expression profiles and phylogenetic analysis of BpCSLD, BpCSLE, and BpCSLG families in B. pendula. The expression was analyzed in three independent biological replicates of each tissue, and the phylogenetic tree (1,000 bootstraps) was constructed by RAxML using the maximum likelihood algorithm.

We identified 36 *BpCSLs* in the *B. pendula* genome, of which 5 genes were abundant in the xylem (Fig. 3 and Fig. 4). They were *BpCSLA2*, *BpCSLA3*, *BpCSLC1*, *BpCSLC4*, and *BpCSLD4*, respectively. BpCSLA2 and BpCSLA3 were most similar to AtCSLA9 in *A. thaliana*. Expression of *CSLs* in *A. thaliana* cells revealed that AtCSLA glycosyltransferases can encode cell wall glucomannan and intervene in the progression of embryogenesis (Goubet et al. 2009, Liepman et al. 2005). In addition, Kim et al. (2014b) reported that transcription factors AtNAC41, AtbZIP1, and AtMYB46 can directly regulate the expression of *AtCSLA9* in *A. thaliana*. The most similar protein to BpCSLC1 was AtCSLC4 in *A. thaliana*, which encodes a protein similar to cellulose synthase and its mRNA can move in cell-to-cell. The 1,4-beta-glucan synthase AtCSLC4 can form the xylosylated glucan backbone with three xylosyltransferases

AtXXT1, AtXXT2, and AtXXT5 in *A. thaliana* (Chou et al. 2012). Intriguingly, glucan synthase AtCSLC4 has opposite orientations in the Golgi membrane (Davis et al. 2010) with mannan synthase AtCSLA9, which may cause the functional differences between them. The most similar protein to BpCSLD4 was AtCSLD3 in *A. thaliana*, which part in the cell-wall synthesis of tip-growing root-hair cells (Park et al. 2011). Galway et al. (2011) reported that root hair-specific disruption of cellulose and xyloglucan in AtCSLD3 mutants.

Involvement of transcription factors in cell wall synthesis

Based on transcriptome sequencing data, we performed an extensive analysis between putative cell wall synthesis proteins and 2,816 transcription factors of *B. pendula* (Supplementary table S1 on https://doi.org/10.6084/m9.figshare.17056994). The results showed that a total of 51 transcription factors were co-expressed with 6 cell wall synthesis proteins, which were BpCESA4, BpCESA9, BpCSLA2, BpCSLC1, BpCSLC4, and BpCSLD4 (Fig. 5).

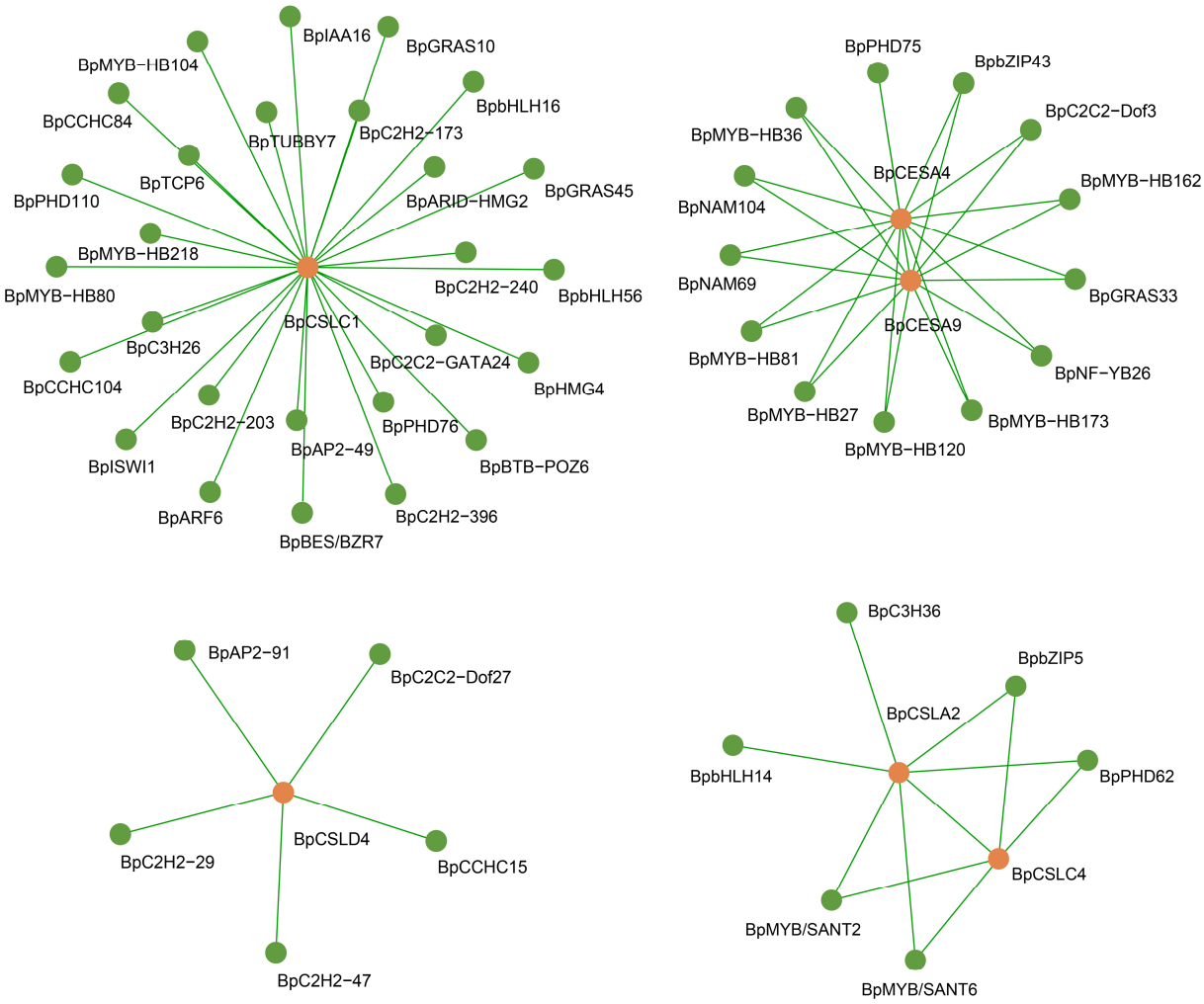

Fig. 5: The transcription factor regulatory network calculated by WGCNA. The green dots are transcription factors, and the orange dots are cell wall synthesis genes.

The highest number of transcription factors were co-expressed with BpCSLC1, up to 27, including ARF, IAA, and several other auxin-related transcription factors. BpARF6 was most similar to AtARF17 in *A. thaliana*, Yang et al. (2013) reported that AtARF17 is essential for the primexine formation and pollen wall development. BpIAA16 was most similar to AtIAA16, which has transcriptional wiring with cell wall-related genes in *A. thaliana* (Mutwil et al. 2009), too. In addition to BpCSLC1, there was a co-expression relationship between BpCESA4 and BpCESA9, with 13 transcription factors regulating these two cellulose synthase genes. BpNAM69 was most similar to AtNAC43 (NST1) in *A. thaliana*, which is known to be involved in cellulose synthesis. Zhong et al. (2007) reported that inhibition of the expression of both *AtSND1* and *AtNST1* by RNA interference (RNAi) results in loss of secondary wall formation in stem fibers, and several fiber-associated transcription factor genes will be down-regulation in *A. thaliana*. BpMYB-HB162 was most similar to AtMYB83 in *A. thaliana*, Ko et al. (2014) reported that the AtMYB46/AtMYB83-mediated transcriptional regulatory program is a gatekeeper of secondary wall synthesis.

CONCLUSIONS

Cellulose synthesis requires the plant hormones, nitric oxide, cellulose synthase, and a complex transcriptional regulation network. In this study, we identified a total of 10 *BpCESA* genes and 36 *BpCSL* genes in *B. pendula*, which include 8 genes that are most likely involved in cell wall synthesis. These genes showed striking consistency compared to the cell wall synthesis genes in *P. trichocarpa*, demonstrating that the cellulose synthesis family is conserved during species evolution.

Given the importance of cellulose synthase importance to cellulose synthesis, maybe we can limit the rate of cellulose synthesis by directly or indirectly inhibiting the expression of related genes, thereby reducing the cellulose content of *B. pendula*. Oomen et al. (2004) reported that reducing of the cellulose content of *Solanum tuberosum* tuber by antisense expression of *StCESA3* clones. Zhong et al. (2003) reported that the *AtCESA7* mutant of *A. thaliana* has lower fiber cell wall thickness and cellulose content. However, the process of increasing cellulose content is not as simple as reducing it. Tan et al. (2015) reported that overexpressing *HvCESA* showed no increase in cellulose content or stem strength in *Hordeum vulgare*, despite the use of a powerful constitutive promoter. Previous studies (Doblin et al. 2002) have shown that individual CESA and CSL proteins play different roles in the synthase complex and require tightly regulation, so we need more complex strategies in the plant engineering of increasing cellulose content.

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