Co-expression network of secondary cell wall biogenesis genes in *Eucalyptus tereticornis*

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Abstract

The composition of secondary cell wall determines the industrially relevant wood properties in tree species. Hence, its biogenesis is one of the most extensively studied developmental processes during wood formation. Presently, systems genetics approach is being applied to understand the biological networks and their interactions operational during secondary development. Genome-scale analyses of secondary cell wall formation were documented and gene regulatory networks were reported in *Arabidopsis*, poplar, pine, spruce, rice and sugarcane. In the present study, the expression patterns of 2651 transcripts representing different pathways governing secondary development was documented across four genotypes of *E. tereticornis*. A co-expression network was constructed with 330 nodes and 4512 edges and the degree ranged from 11 to 53. The network documented 75 (22 %) transcription factors with high degree of interaction. Secondary wall associated NAC domain transcription factor (*SND2*) was identified as the top hub transcript with 53 interactions. The present study revealed that functional homologs regulating secondary cell wall formation are conserved among angiosperms and gymnosperms.

Keywords: *: Cell wall, Eucalyptus, Expression, Network, Regulation, Transcript*

Introduction

Eucalyptus tereticornis Sm., commonly known as forest red gum has an extensive natural distribution from southern Papua New Guinea to southern Victoria of Australia. It ranks among the most extensively planted *Eucalyptus* species in the tropics and subtropics (Florence, 1996) and has been introduced as plantation crop in several countries due to its rapid growth and desirable wood properties when grown in a wide range of environmental conditions. Since Eucalypt species are preferred for paper and pulp production, intensive research has been conducted in the past two decades to understand the molecular regulation of wood formation in this genus (Kirst et al., 2004; Barros et al., 2009; Salazer et al., 2013; Thavamanikumar et al., 2014; Hefer et al., 2015; Shinya et al., 2016; Mizrachi et al., 2017).

Plant cell wall is composed of polysaccharides which determine its structural and functional properties. The secondary cell wall (SCW) biosynthesis occurs after cessation of cell growth and is chemically composed of cellulose, hemicelluloses and lignin and the proportion of each varies among different species (Zhong and Ye, 2014a). In tree species, the composition of SCW determines the industrially relevant wood quality traits and hence physiological, biochemical and molecular processes governing wood formation has been extensively reviewed in woody perennials (Andersson-Gunneras et al., 2003; Du and Groover 2010; Zhong and Ye, 2010; Wang and Dixon, 2012; Hussey et al., 2013; Zhong and Ye, 2014a, b; Hefer et al., 2015; Shinya et al., 2016; Mizrachi et al., 2017; Jokipii-Lukkari et al., 2018).

It is well documented that a large array of structural and regulatory genes are expressed during radial growth in woody stems. However, most of the studies have focused either on single gene or selected gene families from functionally characterized pathways, limiting understanding on the role of entire pathways or biological sub-networks which are essential, redundant, auxiliary or unique to wood formation (Mizrachi and Myburg 2016; Mizrachi et al., 2017). Hence, with the introduction of high throughput genomics technologies along with comprehensive computational pipelines, a holistic systems genetics perspective to comprehend the molecular architecture of complex trait like wood formation has emerged. Presently, genome-scale analyses of SCW biogenesis are reported and gene regulatory networks specific to SCW formation is documented in *Arabidopsis*, poplar, pine, spruce, rice and sugarcane (Yang et al., 2011; Palle et al., 2011; Ruprecht and

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Persson, 2012; Wang et al., 2012; Vanholme et al., 2012; Hirano et al., 2013; Cai et al., 2014; Taylor-Teeples et al., 2015; Lamara et al., 2016; Liu et al., 2015; Chandran et al., 2016; Davin et al., 2016; Ferreira et al., 2016; Zinkgraf et al., 2017; Shi et al., 2017; Jokipii-Lukkari et al., 2018).

In our earlier publication, we had reported that the expression variation of *EYE [EMBRYO YELLOW]* could presumably govern the phenotypic variation in wood properties across *Eucalyptus tereticornis*. Further, gene clusters discriminating the phenotypes were also reported (Dharanishanthi and Ghosh Dasgupta, 2016). However, the differentially expressed transcripts selected for the previous study did not include major transcripts regulating secondary cell-wall biogenesis, necessitating the present study, wherein a specific secondary cell wall related co-expression network was developed to identify major transcripts regulating secondary cell wall biogenesis in wood tissues of *E. tereticornis*.

Materials and Methods

Four genotypes of *Eucalyptus tereticornis* (SWMG-6, CW-8, KUP-14, NKR-49) were selected for expression profiling based on their holocellulose and klason lignin content which was determined by NIR spectroscopy. Percent klason lignin was 20.07 %, 21.57 %, 30.94 % and 25.27 % in SWMG-6, CW-8, KUP-14 and NKR-49 respectively, while their corresponding holocellulose content was 72.9 %, 73.15 %, 63.13 % and 69.36 % respectively. Wood core samples (in duplicate) of approximately 2.0 cm length were collected at a height of ~1.3m using increment borer (Haglof Inc., Sweden) from nineteen year-old standing trees available in the seed orchard established at Karunya Research Station, Coimbatore, India.

RNA isolation, Microarray Design and Hybridization

Total RNA was extracted from developing xylem tissues of all the four genotypes using Spectrum™ Plant Total RNA Kit (Sigma Aldrich, USA). The quality of RNA was checked NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, USA) and integrity was determined using 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). Total RNA from duplicate samples was pooled in equimolar concentration prior to labeling and hybridization. A 8x60K microarray chip was custom-designed in Agilent platform (AMADID: 059849) consisting of 44,817 probes representing 18,987 transcripts (Dharanishanthi and Ghosh Dasgupta, 2016). The size of the probes was sixty base pairs and a minimum of two probes per transcript was designed. RNA sample preparation, labeling and hybridization was done using one-color microarray-based gene expression analysis with Tecan HS Pro protocol (Agilent Technologies, CA, USA) as per manufacturer's protocol. Hybridization was conducted at 65°C for 16 hours and the slides were scanned using Agilent Microarray Scanner G2505C and the features were extracted with the Feature Extraction Software (Agilent Technologies, v12) (Dharanishanthi and Ghosh Dasgupta, 2016).

Selection of secondary cell wall related transcripts and functional annotation

A total of 2651 transcripts involved in cell wall biogenesis were manually mined from the expression datasets of 18,987 transcripts (Accession number GSE73030). Transcripts were functionally annotated and their position in chromosome, protein domains, biological pathways and gene ontology were defined based on the genome assembly of *E. grandis* using Phytozome v10. Further, *Eucalyptus* nucleotide sequences were used to search the complete protein sequences of *Arabidopsis* using BlastX with (e-value cutoff of 1^{e-5}) in the non-redundant database of NCBI and TAIR (v10) and the best hits (lowest e-value) was selected as *Arabidopsis* orthologs. Over-representation of gene ontology (GO) terms for the 2651 transcripts was conducted in AgriGO v2.0 (Tian et al., 2017).

Documentation of differentially expressed transcripts and hierarchical clustering

Feature extracted data was analyzed using GeneSpring GX Version 12 software (Agilent Technologies, CA, USA). After background correction, the data was log transformed and normalized. Global normalization of the data was done in GeneSpring GX using the 75th percentile shift and normalization across samples was done using median values. Transcripts expressed in all genotypes were used for analysis. The log2 fold expression data was filtered for significantly regulated (up and down regulated) transcripts across all genotypes and transcripts exhibiting ±2.0 fold difference in expression with a statistical significance of $p<0.05$ were considered as differentially regulated. The differential expression of transcripts across all possible pair-wise combination was performed. Hierarchical clustering was conducted with the CIMminer (http://discover.nci.nih.gov/cimminer/home.do) software using Euclidean distance method, average linkage cluster algorithm and distance represented as the average of all pairs from each cluster group (Dharanishanthi and Ghosh Dasgupta, 2016).

Development of Co-expression network

A correlation matrix of differentially expressed transcripts was made by calculating pair-wise Pearson correlation coefficient using normalized expression value across all genotypes using Co-Express 1.5 software (http://www.bioinformatics.lu /CoExpress/) with default parameters (threshold >0.9). Edges were made based on results from Co-Express and network was constructed with 330 nodes and 4512 edges using Cytoscape software (http://www.cytoscape.org) using default parameters (Shannon et al., 2003). Duplicated edges and self loops were removed manually from the network. Assessment of over-representation of gene ontology (GO) was performed using ClueGO Cytoscape plugin (http://apps.cytoscape.org/apps/cluego). The statistical significance for all GOs for biological process, molecular function and cellular component was evaluated with default parameters (kappa score 0.4). GO annotation terms were considered significant if the corrected P-value (False discovery rate) was < 0.05 and if there were at least 4 transcripts associated with the same annotation (Bindea et.al., 2009).

Results

The customized array representing 2651 secondary cell wall related transcripts were categorized into different functional pathways including primary and secondary cell wall biosynthesis pathway represented by 481 transcripts; 383 transcripts belonging to cell wall related protein/ enzymes; 125 cell expansion related transcripts; programmed cell death/ senescence related pathways was represented by 214 transcripts and hormone signaling pathways consisted of 258 transcripts. A total of 1190 transcription factors related to cell wall biogenesis were included in the array for expression analysis. The functional annotation of the transcripts, their position in chromosome, protein domains, biological pathways and gene ontology is presented in supplementary table 1. The GO terms for biological process (Supplementary Figure 1), molecular function (Supplementary Figure 2) and cellular component (Supplementary Figure 3) revealed that the major GO terms represented in the analyzed transcript sets included metabolic process, organic substance metabolic process, primary metabolic process, cellular process, macromolecule metabolic process, nitrogen compound metabolic process biosynthetic process and cellular biosynthetic process (Figure 1).

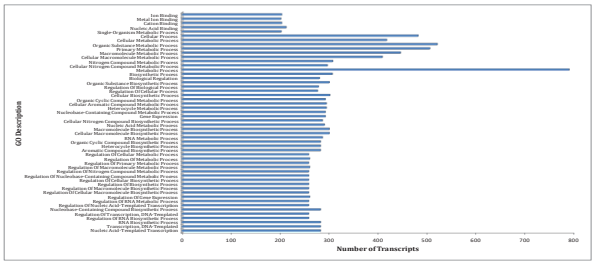
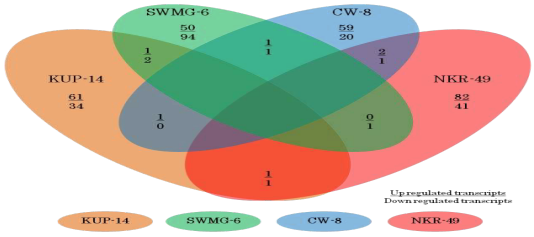


Figure 1
Major GO categories of transcripts involved in secondary cell wall biogenesis in *E. tereticornis*

The fold expression across all genotypes ranged from 10.42 to -8.9. In phenotypes with high holocellulose content (SWMG-6 and CW-8), the transcript expression ranged from 5.66 to -8.9, while in phenotypes with high lignin content (KUP-14 and NKR-49), the expression ranged from 10.42 to -8.39. All pair-wise comparison of differentially expressed transcripts is given in Figure 2 and the hierarchical clustering of differentially expressed transcripts is represented in Figure 3. The total number of transcripts differentially expressed across all genotypes (after removal of overlapping transcripts) was 394.

Co-expression network of major cell wall related transcripts

The number of significantly co-expressed transcripts (threshold >0.9) was 330 and the co-expression network was constructed with 330 nodes and 4512 edges and the degree ranged from 11 to 53 (Figure 4). Gene ontology enrichment confirmed that the network was significantly enriched with cell wall



KUP-14: Kupiano 14, SWMG-6: SW Mt.Garnet 6, CW-8: Cardwell 8, NKR-49: N Kenedy R 49.

Figure 2
Venn diagram showing differentially expressed cell wall related transcripts across *E. tereticornis* genotypes

biosynthesis related GOs including cell wall biogenesis (*GAUT12, XYL1, FLA12, PER64, LAC17, IRX3* and *IRX1*), glucosyl transferase activity (*UGT74F2, UGT87A2, UGT76E2, UGT84A1, CslG3* and *GolS2*), starch and sucrose metabolism (*BGLU17, BFRUCT1, XYL4* and *PME3*) and phenylpropanoid metabolic process (*COMT1, PAL2, HCT, 4CL1,4CL3, FAH* and *CYP42C4*) (Figure 5). The network documented 75 transcription factors with high degree of interaction including *SND2, WRKY23, SUVR2, SPT5L, AP2/B3 like, C3HC4 type* (ring finger), *NAC044* and *HB6*.

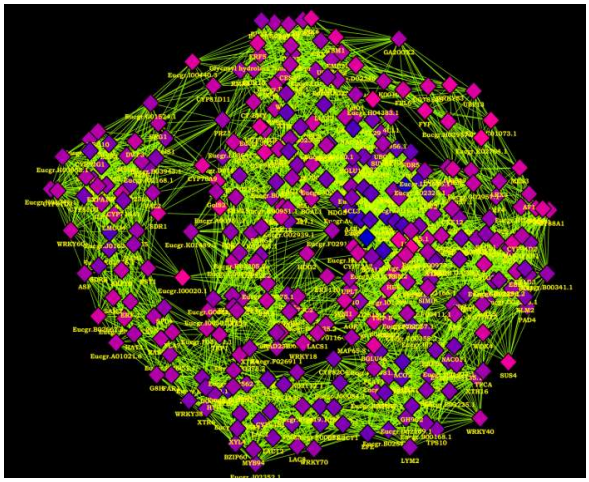
The list of top hub transcripts in the network is presented in table 1. Secondary wall associated NAC domain protein 2 (*SND2*) was found to be the major hub transcript in the network with 53 interactions. It co-expressed with functional genes like cellulose synthase (*CesA*), 4-coumarate: CoA ligase (*4CL*), fasciclin-like arabinogalactan (*FLA12*), beta-galactosidase (*BGAL8*), pectin methyl esterase (*PME*), ubiquitin (*UBQ9*), ascorbate peroxidise (*APX*) and eukaryotic aspartyl protease (*ASP*). Additionally, it interacted with ten transcription factors including homeodomain containing transcription factors *(HB6), WRKY (WRKY23),* AP2/B3-like TF (Eucgr.K02305) and C3HC4 type (RING finger) (Eucgr.I00623; Eucgr.D00969).

Two new TFs, *SUVR2* and *SPT5L* were also documented as top hub transcripts with 45 and 44 interactions. *SUVR2* co-expressed with 45 transcripts including *4CL1, 4CL3, IAA22, LEW2, NAC044, COMT1, PME, SND2, WRKY23* and *XTH9*, while *SPT5L* co-expressed with *SND2, CesA8, F3H, AAE14, ACO1, 4CL1, 4CL3, BGAL8* and *FLA12*.

Discussion

It is well documented that a large array of structural and regulatory genes are expressed during radial growth in woody stems, but a comprehensive understanding of how these genes interact to influence wood formation is currently limited (Liu et al., 2014). The reductionist genetics approaches have focused on either single gene or group of genes from functionally characterized pathways to ascertain their role during wood formation. The recent approach of systems genetics has enabled a deeper understanding on secondary development with an insight into the critically essential genes, pathways and

Figure 3
Hierarchical clustering of differentially expressed transcripts



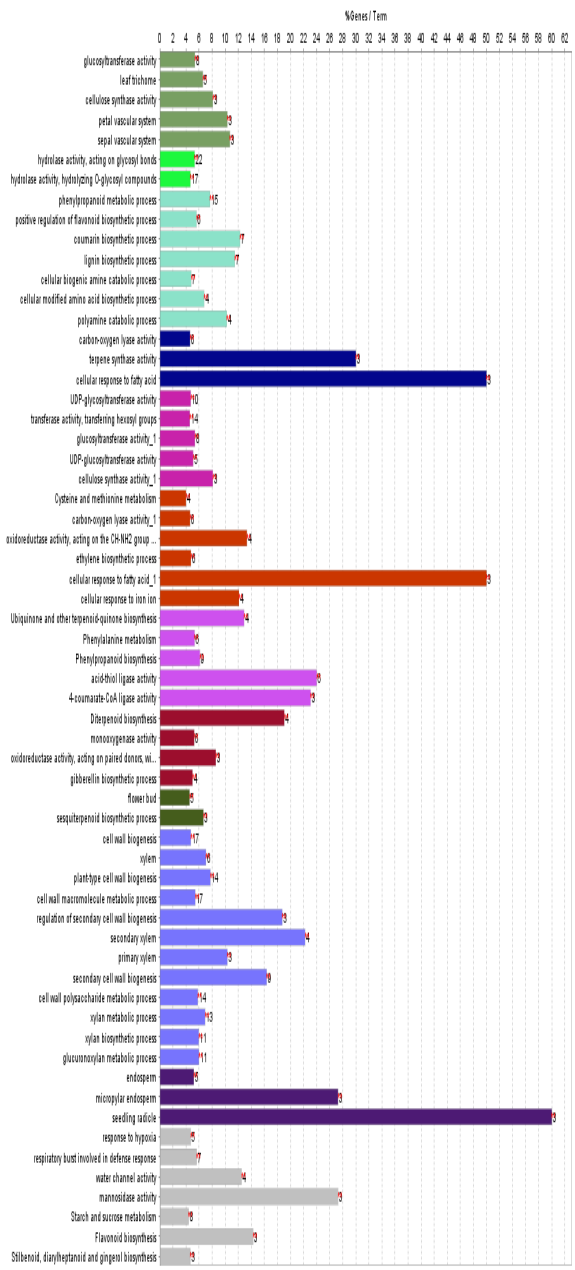
Nodes: Represented in pink-purple to color (based on degree)Edges: Depicted in green color

Figure 4
Gene co-expression network of secondary cell wall related transcripts in E. tereticornis

networks which are unique to wood formation in tree species (Mizrachi and Myburg, 2016; Mizrachi et al., 2017).

Gene networks for secondary cell wall formation are reported in several species like *Arabidopsis* (Yang et al., 2011; Ruprecht and Persson, 2012; Taylor-Teeples et al., 2015; Davin et al., 2016), poplars (Yang et al., 2011; Cai et al., 2014; Liu et al., 2015; Lamara et al., 2016; Zinkgraf et al., 2017; Shi et al., 2017), *Pinus taeda* (Palle et al, 2011), sugarcane (Ferreira et al., 2016), rice (Guo et al., 2014; Chandran et al., 2016). Recently, in *E. grandis* × *E. urophylla* hybrid population, a network based eQTL analysis tagging biomass and bio-energy related traits was reported. Molecular networks associated with wood density, DBH, glucose released, and lignin content were generated to understand the complex trait (Mizrachi et al., 2017). However, a comprehensive SCW related network is not reported in *Eucalyptus* species and hence the present investigation was undertaken to document the expression profiles of cell wall related transcripts and develop the co-expression network to identify major regulators of SCW in *E. tereticornis*.

Co-expression networks for cell wall biogenesis have been reported in *Arabidopsis* by several research groups. Yang et al. (2011) reported a network encompassing 694 cell wall related genes and the major gene families represented in the network were cellulose synthases, glycoside hydrolases, glycosyl transferases, exostosin, kinase/LRR superfamily, plastocyanin-like and TF from *MYB* family. Subsequently, Wang et al., (2012) documented cellulose synthases, glycosyl transferases, xyloglucan endotransglucosylase/hydrolases, expansin and COBRA- families in the cell wall related network. They also reported the presence of 45 TF families including *MYB, NAC, HB*, and *WRKY*. Transcriptional regulatory networks controlling secondary wall biosynthesis was also reported in *Arabidopsis* (Cassan-Wang et al., 2013; Hussey et al., 2013; Taylor-Teeples et al., 2015; Davin et al., 2016) and these networks documented the major cell wall associated gene families mentioned earlier. *Populus* genome wide co-expression network and



Number indicates the percent transcript in each GO term

Figure 5
GO enrichment categories of secondary cell wall related network in *E. tereticornis*

transcriptional network related to cell wall biosynthesis was reported by Yang et al. (2011), Cai et al. (2014) and Liu et al. (2015). These networks also comprised of members from cellulose synthases, glycoside hydrolases, glycosyl transferases, exostosin, kinase/LRR superfamily, plastocyanin-like family and xyloglucan endotransglucosylase/hydrolases (*XTH*) and TF families like *MYB, NAC* and *HB*, as reported in *Arabidopsis*.

The cell wall specific networks from gymnosperms like *Pinus taeda* (Palle et al., 2011), *Picea glauca* (Lamara et al., 2016) and from monocots like *Oryza sativa* (Guo et al., 2014; Chandran et al., 2016) and *Saccharum* Spp., (Ferreira et al., 2016)

Table 1
List of top hub transcripts represented in the secondary cell wall related co-expression network of *E. tereticornis*

Transcript ID	*E.grandis* ID (Phytozome)	Gene description	Degree	Major co-expressed transcripts
SND2	Eucgr.E03226	NAC domain containing protein 73	53	*CesA1, 4CL, FLA12, BGAL8, PME, UBQ9, APX, ASP, HB6, WRKY23*
Eucgr.A02817	Eucgr.A02817	Integrase-type DNA-binding superfamily protein	51	*XTH5, 4CL, FLA12, CesA8, IAA17, UBQ9,* UGT84A1, HB6, *WRKY*
PME	Eucgr.E01468	pectin methylesterase	49	*XTH9, 4CL1, 4CL3, BGLU17, IAA22, CesA8, NAC044,* COMT1, F3H
Eucgr.J02205	Eucgr.J02205	HD domain-containing metal-dependent phosphohydrolase family protein	48	*XTH9, 4CL1, 4CL3, BGLU17, IAA22, CesA8, NAC044, COMT1, F3H*
AAE14	Eucgr.D00173	Acyl-activating enzyme 14	47	*FLA12, HB6, IAA17, CesA8, PME, SND2, UGT84A1, WRKY23, F3H, XTH5*
WRKY23	Eucgr.H00996	WRKY DNA-binding protein 23	47	*4CL1, 4CL3, BGLU17, IAA22, CesA8, NAC044, COMT1, F3H*
CYP72A8	Eucgr.F01386	cytochrome P450, family 72, subfamily A, polypeptide 8	47	*4CL, FLA12, CesA8, IAA17, UBQ9, UGT84A1, HB6, WRKY*
F3H	Eucgr.J02430	Flavanone 3-hydroxylase	45	*4CL1, 4CL3, FLA12, CesA8, NAC044, UBQ3, UBQ9, UGT84A1, WRKY23*
SUVR2	Eucgr.K01433	SET-domain containing protein lysine methyltransferase family protein	45	*4CL1, 4CL3, IAA22, LEW2, NAC044, COMT1, PME, SND2, WRKY23, XTH9*
SPT5L	Eucgr.J01043	kow domain-containing transcription factor 1	44	*SND2, CesA8, F3H, AAE14, ACO1, 4CL1, 4CL3, BGAL8, FLA12.*

documented the presence of gene families like *CesA, GH, FLA, EXPA* and TFs like *MYB, NAC, HB, WRKY* and *Znf* in the network. Comparison of cell wall co-expression networks across *Arabidopsis*, poplar, rice, barley, soybean, *Medicago* and wheat (Ruprecht et al., 2011); *Arabidopsis* and rice (Hirano et al., 2013; Hansen et al., 2014) and *Arabidopsis* and poplar (Yang et al., 2011) were also reported. These studies revealed that the genes regulating cell wall biogenesis pathways are highly similar.

In the present study, the co-expression network was constructed for major cell wall related transcripts with 330 significantly co-expressed transcripts in *E. tereticornis*. Several gene families present in the cell wall biosynthesis network of *Arabidopsis*, poplar, white spruce, pine, rice and sugarcane were present in *E. tereticornis* including *CesAs, GT, GH, COBRA-like, GATL, EXPA, TUB, CCR, OMT*, O-fucosyl transferaser family and TF families like *MYB, NAC, WRKY, bHLH* and *Znf-C2H2*. Genes reported in cell wall biosynthesis of other plant species were also found in Eucalyptus network including *CesA7, IRX6 (COBL4), IRX15, XTH9, CCR, SND, MYB20* and *VND7*. The studies from both annual and perennial species including *E. tereticornis* indicate that biological pathways functionally relevant to secondary cell wall development are conserved across species (Hansen et al., 2014).

Molecular studies in vascular plants have indicated that the expression of several families of TFs is associated with the secondary cell wall biosynthesis. The transcriptional network was described as a complex multi-leveled feed-forward loop regulatory system (Zhong and Ye, 2014 b). The secondary wall related NAC TFs (*SND1, NST1/2* and *VND6/7*) act as the top level master regulators which activated the second-level master switches like *SND3, XND* and *MYBs* and they synergistically induced the expression of downstream TFs like *BES1, SND2, C3H14, KNAT7* and lignin specific *MYB* and cell wall related structural genes involved in biosynthesis of cellulose,

hemicelluloses, lignin and signaling (Cassan-Wang et al., 2013; Hussey et al., 2013; Zhong and Ye, 2014 a, b; Ye and Zhong, 2015).

In *Arabidopsis*, it was reported that *SND1* is a master switch that regulated the secondary wall thickening in fibers (Zhong et al., 2006), while in poplar it was identified as a critical transcriptional switch of secondary wall biosynthesis (Cai et al., 2014). *SND1* is reported to regulate the expression of several other TFs in *Arabidopsis* and *P. trichocarpa* (Zhong et al., 2006, 2007; Hussey et al., 2013; Zhong and Ye, 2014a, b; Ye and Zhong, 2015). In the gymnosperm *P. taeda*, *SND1* was reported as the master regulator in cell wall related networks and interacted with *NST1, KNAT7, MOR1, PtMYB8, MYB85, XET2* and lignin biosynthetic genes (Palle et al., 2011), suggesting that *SND1* acted as master regulator in both gymnosperm and angiosperm.

Recently, Zinkgraf et al (2017) reported the conservation of gene families in co-expression modules in poplars and documented *NST1, VND1* as first-layer master regulators of *ANAC075, GATA12, SND2, WRKY12* which in turn regulated second-layer switches like *MYB46, MYB83* and several downstream TFs involved in cell wall formation. This module also included several major structural genes involved in lignin, cellulose and hemicellulose biosynthesis. In another study in *P. trichocarpa, PtrSND2/3-A2, PtrSND2/3-B1*, and *PtrSND2/3-B2* was identified as major regulators of wood formation and co-expressed with cell wall component genes (Shi et al., 2017). Further, they had also reported that cell wall biogenesis related transcripts were redundantly controlled by TFs during wood formation (Shi et al., 2017). The results in the present study is in consensus with the earlier reports, wherein SND2 was identified as a master regulator of cell wall biogenesis regulating the expression of 53 transcripts including *CesA1, 4Cl, FLA12, BGAL8, PME, UBQ9, APX* and *ASP* and other TFs like *HB6, WRKY23* and *C3HC4 type* (RING finger) in *E. tereticornis*.

Evolutionary studies have indicated that the ability to produce secondary xylem has been independently lost and gained several times in the angiosperm lineage, supporting the hypothesis that the key genes required for secondary growth are conserved among angiosperms (Kirst et al., 2004; Groover, 2005; Dejardin et al., 2010; Spicer and Groover, 2010; Lens et al., 2012) and between angiosperms and gymnosperms (Pavy et al., 2008). Additionally, reports suggest that the conservation of gene families involved in cell wall biogenesis and secondary development preceded the divergence of gymnosperms and angiosperms (Nairn et al., 2008; Del Bem and Vincentz, 2010).

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