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*Review*

# Transcriptional regulatory network controlling secondary cell wall biosynthesis and biomass production in vascular plants

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**Secondary wall is an abundant component of plant biomass and has a potential to be a renewable resource of bioenergy and biomaterials. It is important to unravel the molecular mechanism underlying secondary wall formation and how it contributes to plant biomass production. In this review, we summarized the potential role of transcription factors (TFs) in secondary wall formation, and prospected to design the future bioenergy crops with high density biomass, low cellulose recalcitrance and lignin content.**

**Key words:** Transcription factors (TFs), secondary cell wall, plant biomass, NACs, mining yeast binding sites (MYBs).

## INTRODUCTION

Fears that increased energy demand, as well as the need for ecologically acceptable fuels that would replace highly polluting fossil fuels are the main reasons behind many different alternative energy researches. Burning fossil fuels results to emission of large amounts of carbon dioxide into the atmosphere which has been linked to global warming. One segment of the alternative fuel market that has been recently gaining plenty of popularity is the bioenergy. Bioenergy or biofuels are supposed to cause less pollution and they are also biodegradable. However, first generation biofuels make use of sugars derived from food crops such as sugar cane, corn, wheat, rice, and sugar beets, thus interfering with food security. Nowadays, the trend is towards the use of biomass consisting of the residual non-food parts of these crops, such as stems, leaves and husks that are left behind once the food crops have been extracted, as well as non-food crops such as switchgrass, grass, jathropa, whole crop maize, miscanthus and cereals that bear little grain, and also industrial wastes such as woodchips, skins and pulp from fruit pressing (Mansfield, 2009; Carriquiry et al., 2010). This is called second generation biofuels and does not interfere with food security and is thus the focus of this review.

Plant biomass has been considered as an important renewable source of bioenergy (Demura and Ye, 2010). The major types of biomass crop plants include grasses, angiosperms (hardwoods), and gymnosperms (soft-woods), and they have been considered as potential biomass sources for bioenergy production on the basis of their high biomass yield with low inputs.

Plant biomass is mainly found in the secondary cell wall. In general, plant biomass feedstock is made up of complex structures that are mainly comprised of cellulose, hemicellulose and lignin (Abramson et al., 2010. However, the presence of polymeric lignin prevents access of enzymes and chemicals to cellulose and hemicellulose, thus reducing degradability of the carbohydrate material. This natural resistance of plant cell walls to microbial and enzymatic deconstruction is collectively known as “biomass recalcitrance”. Thus, the

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plant biomass is more difficult to break down into sugars than starch and more exorbitant due to the high cost of lignocellulose conversion. One solution to this problem is to develop a plant with genetically-engineered secondary cell walls exhibiting low lignin content but high cellulose and hemicellulose content or crops that self-produce cellulose enzymes for cellulose degradation and ligninase enzymes for lignin degradation (Sticklen, 2006).

To effectively develop this kind of plant, it is therefore essential to understand the physiological, biochemical and gene regulatory networks. The biosynthesis of secondary walls is a highly coordinated developmental process that involves a coordinated expression of secondary wall biosynthetic genes regulated by a cascade of transcription factors (TFs) (Rubin, 2008). Therefore, it is important to understand the role of transcription factors in controlling the secondary wall formation. This review focuses on the recent understanding in the transcriptional network regulating secondary cell wall biosynthesis.

## THE SECONDARY CELL WALL

The principal components of secondary cell walls are cellulose, hemicelluloses and lignin (Zhong and Ye, 2009). The composition and the proportion of each component may vary between different plant species, tissues and cells, and may also change in different developmental and environmental conditions (Rubin, 2008). These chemical and structural complexities of plant cell walls that enable their diverse biological functions, including mechanical support, protection against pathogens, and regulation and transport of material, are also the root causes of recalcitrance to chemical and biological catalysts for bioenergy production (Wei et al., 2009). Secondary cell walls in the form of wood and fibers are the most abundant biomass produced by vascular plants, and are widely used for energy, pulping and paper-making, textiles and many other applications. Because of the abundance, it is also considered to be an important renewable source of bioenergy. Thus, there has been a tremendous interest in uncovering the mechanisms underlying the making of secondary cell walls in wood and fibers in the hope of modifying this rich renewable source to better suit this purpose (Rubin, 2008; Zhong et al., 2010).

## THE ROLE OF TRANSCRIPTION FACTORS IN REGULATING SECONDARY CELL WALL BIOSYNTHESIS

Transcription factors are proteins that have function in controlling the expression of target genes quantitatively, temporally, and spatially. To date, genetic analyses have revealed a number of transcription factors regulating vascular development (Yamaguchi and Demura, 2010a). Expression of transcription factors is one of several approaches to affecting the overall biomass yields. This can be done through manipulation of transcription factors that can lead to the expression of these desired characteristics (Abramson et al., 2010). So, an appreciation of the molecular mechanisms underlying the transcriptional regulation of secondary wall biosynthesis will be instrumental to design strategies for genetic improvement of plant biomass.

Recently, several *Arabidopsis* transcription factors, including member of NAC, MYB (mining yeast binding sites) and other transcription factor families, have been discovered to be the regulators of the coordinated expression of secondary wall biosynthetic genes (Table 1 and Figure 1).

### The role of first- level master switches, NACs, in secondary cell wall biosynthesis in *Arabidopsis*

Secondary walls are typically composed of cellulose, xylan, and/or lignin. To make secondary walls, genes participating in the biosynthesis of cellulose, xylan, and lignin need to be coordinately expressed. One intriguing unknown is the identity of the switch that turns on the developmental program of secondary wall biosynthesis. Recent studies on the development of fibers, vessels, and endothecium have found that several members of the NAC and MYB transcription factors are key switches in regulating secondary wall biosynthesis.

The NAC (for NAM, ATAF1/2 and CUC2) domain transcription factors are plant-specific transcription factors characterized by a conserved NAC domain located at the N-terminal region and a divergent C-terminal activation domain (Olson et al., 2005). They have been extensively studied in vascular plants and non-vascular plants, particularly in *Arabidopsis thaliana* which genome contains at least 114 NACs (Zhong et al., 2010). Some of them have been shown to play diverse roles in plant growth development and plant defense (Olson et al., 2005; Ooka et al., 2003). Recent researchers have implicated a subgroup of phylogenetically, closely related NAC domain transcription factors, including SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1, also called NST3/ANAC012), NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1), NST2, VASCULAR-RELATED NAC DOMAIN6 (VND6), and VND7, function as the first level of master transcriptional switches that activates the secondary wall biosynthetic program in fibers, vessels, or endothecium of anthers (Zhong et al., 2010). The process stimulates the biosynthesis of cellulose, xylan and lignin, the main components in secondary cell wall (Ko et al., 2007; Kubo et al., 2005; Mitsuda et al., 2005; Yamaguchi et al., 2008; Zhong and Ye, 2007c; Zhong et al., 2006, 2007b, 2008). SND1 is specifically expressed in fibers, while VND6 and VND7 are specifically expressed in vessels. Simultaneous mutations of SND1 and NST1

**Table 1.** Transcription factors in regulating secondary cell wall biosynthesis in plants.

| Genes | Species | Accession number (NCBI) | Functions | References |
|---|---|---|---|---|
| NAC Family | | | | |
| AtNST1 | *Arabidopsis thaliana* | NM_130243 | Regulates formation of secondary walls. | Mitsuda et al. (2005, 2007) and Zhong et al. (2007b). |
| AtNST2 | *Arabidopsis thaliana* | NM_116056 | Regulates possibly the secondary wall thickening in various tissues. | Mitsuda et al. (2005) |
| AtNST3/ AtSND1/ ANAC012 | *Arabidopsis thaliana* | NM_103011 | Regulates formation of secondary walls. ANAC012 has been described as a negative regulator | Mitsuda et al. (2007), Zhong et al. (2006, 2007b) and Ko et al. (2007) |
| AtVND6 | *Arabidopsis thaliana* | NM_124617 | Regulates secondary wall formation. | Ohashi-Ito et al. (2010) |
| AtVND7 | *Arabidopsis thaliana* | NM_105851 | Regulates secondary wall formation. | Yamaguchi et al. (2011) |
| AtSND2 | *Arabidopsis thaliana* | NM_118992 | Regulates secondary cell wall development. | Hussey et al. (2011) |
| AtSND3 | *Arabidopsis thaliana* | NM_102615 | Regulates secondary cell wall development | Hussey et al. (2011) and Zhong et al. (2008) |
| AtXND1 | *Arabidopsis thaliana* | NM_125849 | Negatively regulates lignocellulose synthesis and programmed cell death in xylem. | Zhao et al. (2008) |
| MtNST1 | *Medicago truncatula* | GU144511 | Regulates the lignin biosynthesis. | Zhao et al. (2010) |
| MYB family | | | | |
| AtMYB26 | *Arabidopsis thaliana* | NM_112243 | Regulates secondary thickening in the endothecium | Yang et al. (2007) |
| AtMYB32 | *Arabidopsis thaliana* | NM_119665 | Negatively regulates SND1 expression and controls the lignin pathway | Wang et al. (2011) |
| AtMYB103 | *Arabidopsis thaliana* | NM_105065 | Regulates secondary cell wall development | Hussey et al. (2011) and Zhong et al. (2008) |
| AtMYB46 | *Arabidopsis thaliana* | NM_121290 | Regulates secondary wall biosynthesis. | Ko et al. (2009) and Zhong et al. (2007a) |
| AtMYB83 | *Arabidopsis thaliana* | NM_111685 | Regulates secondary wall biosynthesis. | McCarthy et al. (2009) |
| AtMYB58 | *Arabidopsis thaliana* | NM_101514 | Specifically regulate lignin biosynthesis | Zhou et al. (2009) |
| AtMYB63 | *Arabidopsis thaliana* | NM_106569 | Specifically regulate lignin biosynthesis. | Zhou et al. (2009) |
| AtMYB85 | *Arabidopsis thaliana* | NM_118394 | Specifically regulate lignin biosynthesis. | Zhong et al. (2008) |
| AtMYB75 | *Arabidopsis thaliana* | NM_104541 | Represses the lignin branch of the phenylpropanoid pathway. | Bhargava et al. (2010) |
| PtMYB1 | *Pinus taeda* | AY356372 | Putative actor in secondary cell wall biosynthesis. | Bomal et al. (2008) |
| PtMYB4 | *Pinus taeda* | AY356371 | Regulates lignin biosynthesis. | Patzlaff et al. (2003) |
| PtMYB8 | *Pinus taeda* | DQ399057 | Putative actor in secondary cell wall biosynthesis. | Bomal et al. (2008) |
| PtrMYB3 | *Populus trichocarpa* | XM_002299908 | Regulates secondary wall biosynthesis. | McCarthy et al. (2010) |
| PtrMYB20 | *Populus trichocarpa* | XM_002313267 | Regulates secondary wall biosynthesis. | McCarthy et al. (2010) |
| PttMYB21a | *Populus tremula* × *tremuloides* | AJ567345 | Represses regulators of lignin biosynthesis. | Karpinska et al. (2004) |
| EgMYB1 | *Eucalyptus gunnii* | AJ576024 | Represses secondary wall formation. | Legay et al. (2010) |
| EgMYB2 | *Eucalyptus gunnii* | AJ576023 | Regulates secondary cell wall formation and lignin biosynthesis. | Goicoechea et al. (2005) |
| OsMYB46 | *Oryza sativa* | JN634084 | Regulates secondary wall biosynthesis. | Zhong et al. (2011a) |
| ZmMYB46 | *Zea mays* | JN634085 | Regulates secondary wall biosynthesis. | Zhong et al. (2011a) |
| ZmMYB31 | *Zea mays* | NM_001112479 | Represses regulators of lignin biosynthesis. | Fornale´ et al. (2006) |
| ZmMYB42 | *Zea mays* | NM_001112539 | Represses regulators of lignin biosynthesis. | Fornale´ et al. (20060 and Sonbol et al. (2009) |
| TaMYB4 | *Triticum aestivum* | JF746995 | Negatively regulates the lignin biosynthesis. | Ma et al. (2011) |
| PvMYB4 | *Panicum virgatum* | JF299185 | Negatively regulates the lignin biosynthesis. | Shen et al. (2012) |
| AmMYB308 | *Antirrhinum majus* | Y15607 | Represses phenolic acid metabolism and lignin biosynthesis. | Tamagnone et al. (1998) |
| AmMYB330 | *Antirrhinum majus* | Y15607 | Represses phenolic acid metabolism and lignin biosynthesis. | Tamagnone et al. (1998) |
| VvMYB5a | *Vitis vinifera* | JQ308622 | Regulates lignin metabolism. | Deluc et al. (2006) |

**Table 1**. Contd.

| | | | | |
|---|---|---|---|---|
| Other families | | | | |
| AtWRKY12 | *Arabidopsis thaliana* | AF404857 | Negatively regulates secondary wall formation. | Wang et al. (2010) |
| AtSHN2 | *Arabidopsis thaliana* | NM_122448 | Downregulates lignin biosynthesis and upregulates cellulose synthesis | Ambavaram et al. (2011) |
| AtOFP4 | *Arabidopsis thaliana* | NM_100566 | Regulates secondary cell wall formation | Li et al. (2011) |
| AtKNAT7 | *Arabidopsis thaliana* | NM_104977 | Negatively regulates secondary wall formation. | Li et al. (2012) |
| MtSTP | *Medicago truncatula* | HM622067 | Negative regulator of secondary wall formation. | Wang et al. (2010) |
| Ntlim1 | *Nicotiana tabacum* | AB079513 | Regulates lignin biosynthesis. | Kawaoka et al. (2000) and Kawaoka and Ebinuma, (2001) |

cause a loss of secondary walls in fibers (Mitsuda et al., 2005, 2007; Zhong et al., 2008), whereas dominant repression of VND6 and 7 functions blocks secondary wall thickening in vessels (Kubo et al., 2005). Over-expression of any of these secondary wall NACs (collectively called SWNs) is able to activate the secondary wall biosynthetic program, leading to ectopic deposition of secondary walls in cells that are normally parenchymatous. These findings suggest that SND1 and NST1 are master regulators of secondary wall biosynthesis in fibers, while VND6 and 7 are master switches of secondary wall thickening in vessels. In addition NST1 together with its close homolog NST2 was also found to be the transcriptional switches regulating secondary wall thickening in the endothecium of anthers (Mitsuda et al., 2005).

Hence, all of the aforementioned findings suggest that NAC transcription factors are the primary activators of secondary wall biosynthesis in various secondary wall-containing cell types (Mitsuda et al., 2005).

## The role of second-level master regulators, MYBs, in secondary cell wall biosynthesis in *Arabidopsis*

The MYB proteins are a large family of transcription factor which regulate diverse facets of plant metabolism and development (Rogers and Campbell, 2004). MYB proteins are characterized by a highly conserved deoxyribonucleic acid (DNA)-binding domain: the MYB domain. This domain generally consists of up to four imperfect amino acid sequence repeats (R) of about 52 amino acids, each forming three α–helices. It has been divided into different classes depending on the number of adjacent repeats (that is, one, two, three or four). R2R3-MYB class is one of the largest families of transcription factors in plants, with over 120 members encoded by genes in the *Arabidopsis* genome. Numerous R2R3-MYB proteins have been characterized by genetic approaches and found to be involved in the control of plant-specific processes including (i) primary and secondary metabolism, (ii) cell fate and identity, (iii) developmental processes and (iv) responses to biotic and abiotic stresses (Dubos et al., 2010; Riechmann et al., 2000).

In *Arabidopsis*, the MYB proteins have been identified as the direct target of secondary wall NACs master switches regulating secondary wall biosynthesis. Studies have identified MYB46 and its close homolog MYB83 as SND1 direct targets. These MYB proteins are equally expressed in both fibers and vessels, suggesting that they are also direct targets of the fiber-specific SND1 and NST1, in addition to the vessel-specific VND6 and 7. Furthermore, promoter activity of MYB46 and 83 were detected both in proxylem and metaxylem vessels (Nakano et al., 2010).

It is shown that simultaneous mutations of MYB46 and 83 results in lack of secondary wall thickening in vessels and a subsequent growth arrest at the seedling stage (Zhong and Ye, 2012; Zhong et al., 2010). Over-expression of MYB46 or 83 in *Arabidopsis* induces activation of secondary wall biosynthetic genes for cellulose, xylan and lignin and concomitantly results in ectopic deposition of secondary walls in cells that are generally parenchymatous (McCarthy et al., 2009; Zhong and Ye, 2012). These findings indicate that MYB46 and 83 act as second-level master regulators controlling the downstream genes in secondary wall formation (Wang and Dixon, 2012) and that this is a common mechanism in diverse groups of vascular plants (Goicoechea et al., 2005; McCarthy et al., 2010; Patzlaff et al., 2003; Zhong et al., 2010).

## Transcriptional complex network regulating the secondary wall biosynthetic program in *Arabidopsis*

It has been reported that a transcriptional regulatory network consisting of a cascade of transcription factors is involved in regulating secondary wall biosynthesis in *Arabidopsis* (Zhong et al., 2010). Among of these, the secondary wall NACs (SWNs) master switches

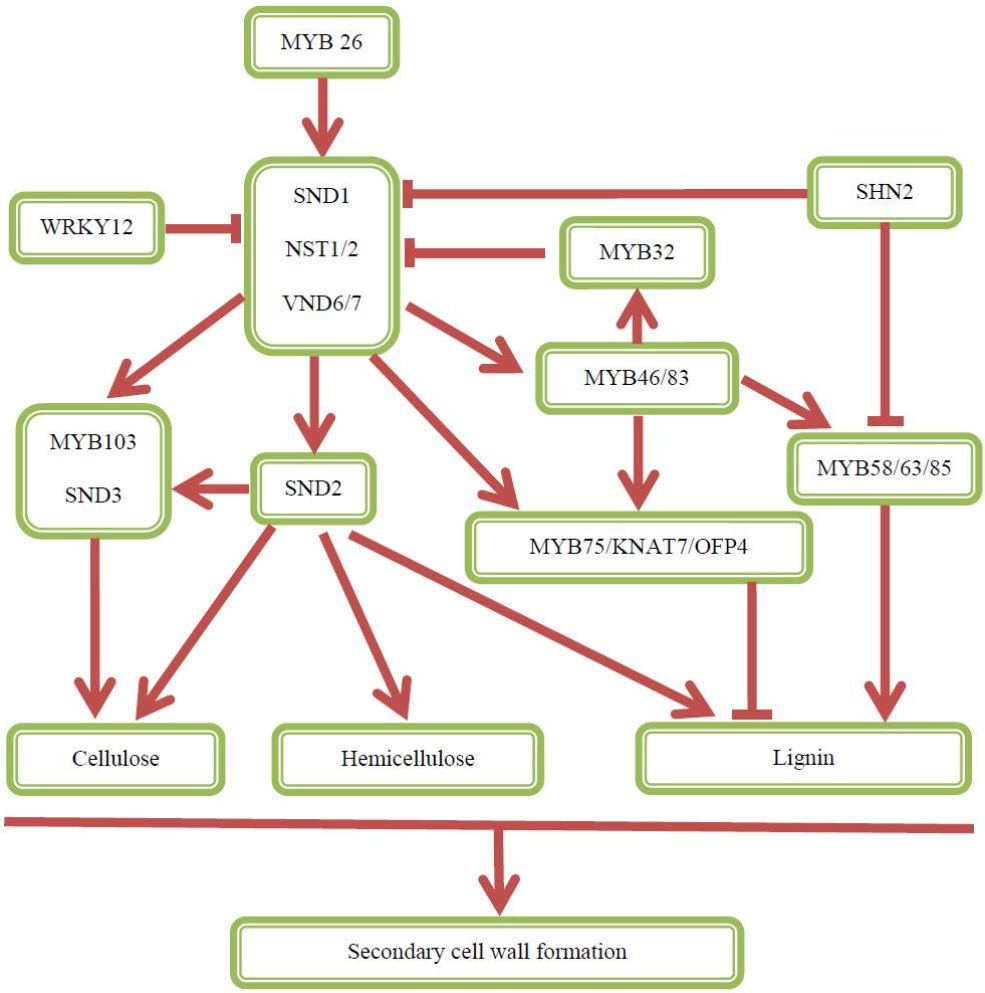


**Figure 1.** The model of the transcriptional network of secondary cell wall formation in *Arabidopsis*.

including first and second-level master switches regulate a hierarchy of downstream transcription factors in secondary wall biosynthetic pathways. As an example of downstream transcription factors, SND3 and MYB103 are proposed to be direct targets of SND1 and also of its close homologs, NST1, 2, and vessel-specific VND6 and 7 (Abramson et al., 2010). Dominant repression of these TFs resulted to reduced secondary wall thickening in fiber cells. However, over-expression of SND3 and MYB103 increased secondary wall thickening in fibers and were able to induce the expression of a cellulose synthase (CesA8) gene (Zhong et al., 2008). This result indicates that SND3 and MYB103 have the ability to specifically induce the expression of cellulose biosynthetic gene. Additional downstream transcription factors include SND2, which is indirectly, but strongly activated by SND1 (Zhong et al., 2008). SND2 also acts downstream of MYB103. It has been shown to regulate cellulose and hemicelluloses biosynthetic genes in addition to its involvement in lignin polymerization and signaling (Hussey et al., 2011).

Three other MYB transcription factors, including MYB58, 63, and B85, are specifically involved in the regulation of lignin biosynthesis (Demura and Ye, 2010). The expression of MYB58 and 63 was shown to be regulated by the SND1 close homologs NST1, 2, VND6, and 7 and their downstream target MYB46 (Zhou et al., 2009). MYB85 activates lignin biosynthetic genes and cause ectopic lignin deposition when over-expressed (Zhong and Ye, 2009).

Comparably, the expression of two members of ASYMMETRIC LEAVES2 (AS2)/LATERAL ORGAN OUNDARIES DOMAIN (LBD) family proteins, ASL19/LBD30 and ASL20/LBD18 have been shown also to be regulated by VND6 and 7. ASL19/LBD30 and ASL20/LBD18 are expressed in immature tracheary elements and were shown to be direct targets of VND7. Over-expression of ASL19/LBD30 and ASL20/LBD18

induced the formation of tracheary element-like cells, and ectopic expression of VND7 was detected in ASL20/LBD18 over-expressing plants, which indicates that ASL20/LBD18 is involved in a positive feedback loop for VND7 expression (Soyano et al., 2008; Yamaguchi et al., 2011).

It is worth mentioning that some cell wall synthesis genes may be directly regulated by the transcription master switches, which gives the transcriptional regulation network more flexibility but also more complexity.

Recently, there have been mounting evidences that SWNs may themselves be under both positive and negative regulation (Wang and Dixon, 2012). Some researchers have noted examples of TFs in control of negative feedback regulation of SWNs such as MYB32, WRKY12 and SHN2 which is summarized as follows.

MYB32 is a known target for regulation by SND1 and can be activated by MYB46 (Ko et al., 2009). In addition, the mutation of MYB32 caused down-regulation in the nst1nst3 double mutant of *Arabidopsis* (Wang and Dixon, 2012). These results indicate that MYB32 is a downstream component of SWNs. However, the protein sequence of MYB32 has been shown to be a transcriptional repressor. This was confirmed by transactivation assays and *in vivo* transgenic studies, which also showed that expression of SND1 is negatively regulated by MYB32 (Wang et al., 2011).

The second TF, WRKY12, may also act as a negative regulator of SWNs. Loss of function of AtWRKY12 in *Arabidopsis* or its ortholog in *Medicago* results in secondary cell wall thickening in pith cells associated with ectopic deposition of secondary cell wall formation. The mutation of AtWRKY caused up-regulation of NST2 together with other secondary cell wall-related TFs and biosynthetic genes. Using electrophoretic mobility shift assay (EMSA) and in planta transgenic experiments, it was confirmed that AtWRKY12 directly binds to the NST2 gene promoter and expression of WRKY12, leads to repression of NST2. The result showed that when the mutant *wrky12-1* allele was crossed with the nst2 mutant, the pith cells of the double mutant were restored to wild-type (Wang et al., 2010). Thus, these results indicate that WRKY12 controls the cell fate in pith cells by acting as a negative regulator of SWNs.

On the other hand, the transcription factor, SHN2, acts as a negative and positive regulator of SWNs. SHN2 is a member of SHINE/WAX INDUCER (SHN/WIN) transcription factors. It has been reported that over-expression of SHN2 in rice (*Oryza sativa*) has resulted to an increase in the rice's cellulose content while its lignin content was reduced by 45%. Furthermore, expression of SHN2 increases wood digestibly (that is, elevated S:G ratio) with no compromise in plant strength and performance (Ambavaram et al., 2011). SHN2 has also been shown to negatively regulate NAC TFs, SND1/NST1/2 and VND6, which are first level master witches of secondary cell wall synthesis and controls MYB expression. In addition, SHN2 also affects lignin formation indirectly through negative regulation of the MYBs TFs, MYB58/63, and cellulose synthesis through direct activation of MYB20/43 and other related TFs (Ambavaram et al., 2011; Marques et al., 2011).

Further studies also showed that the KNAT7, MYB75 and OFP4 could be involved in the regulation of secondary cell wall biosynthesis in *Arabidopsis*. These TFs also act as negative regulators of secondary wall formation (Bhargava et al., 2010). MYB75, also called PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1), is a known regulator of the anthocyanin branch of the phenylpropanoid pathway in *Arabidopsis*. A loss-of-function mutation in MYB75 (*myb75-1*) results in increased cell wall thickness in xylary and interfascicular fibers within the inflorescence stem. Moreover, the total lignin content and S/G ratio of the lignin monomers were also affected. In addition, transcript profiles from the *myb75-1* inflorescence stem revealed marked up-regulation in the expression of a suite of genes associated with lignin biosynthesis**.** These results suggested that MYB75 acts as a negative regulator of the lignin branch of the phenylpropanoid pathway. Since MYB75 physically interacts with another secondary cell wall regulator, the KNOX transcription factor KNAT7, these regulatory proteins may also form functional complexes that contribute to the regulation of secondary cell wall deposition in the *Arabidopsis* inflorescence stem (Li et al., 2011).

The KNAT7, one of seven *Arabidopsis* KNOTTED ARABIDOPSIS THALIANA (KNAT) genes, has been reported to be one of the direct targets of both master switches in secondary cell wall biosynthesis – the SND1 and MYB46, a first- level and second-level master switch, respectively (Ko et al., 2009; Legay et al., 2010; Zhong et al., 2008). Previous report has shown that KNAT7 loss-of-function mutants have an increased cell wall thickness of interfascicular fibers, while KNAT7 over-expression mutants have the opposite phenotype. These result suggested that KNAT7 acts as a transcriptional repressor rather than an activator in regulating secondary cell wall biosynthesis (Legay et al., 2010). In addition, KNAT7 has been reported to interact with OFP4, a member of the Ovate Family Protein (OFP) transcription co-regulators. Both OFP4 and KNAT7 act as transcriptional repressors. In planta interactions between KNAT7 and OFP4 enhance KNAT7's transcriptional repression activity. An *ofp4* mutant exhibited similar fiber cell wall phenotypes as *knat7*, and the phenotype of a double *ofp4* knat7 mutant was similar to those of the single mutants. These result indicated that that OFP4 plays a role in regulating secondary cell wall formation through its interaction with KNAT7. Thus, MYB75, KNAT7, and OFP4 act as a negative regulator of the lignin biosynthesis in secondary cell wall biosynthesis.

Reports show that the TF MYB26 can switch NAC TFs on or off in anther endothecium (Wang and Dixon, 2012).

Expression of MYB26 in anthers is critical for the development of secondary thickening in the anther endothecium (Legay et al., 2010). Double knockout of NST1 and NST2 showed a similar phenotype to that of myb26 (Mitsuda et al., 2005). MYB26 affects the expression of both NST1 and NST2 and its over-expression results in ectopic secondary thickening in both *Arabidopsis* and *tobacco* (Legay et al., 2010). These results suggest that MYB26 is an activator of secondary wall formation and is an upstream regulator of SWNs in the anther endothecium (Wang and Dixon, 2012).

In another study, the NAC domain protein, VND-INTERACTING2 (VNI2) is expressed in xylem vessel formation. Transient reporter assays showed that VNI2 is a transcriptional repressor and can repress the expression of vessel-specific genes regulated by VND7. Expression of C-terminally truncated VNI2 under the control of the VND7 promoter inhibited the normal development of xylem vessels in roots and aerial organs. These data suggest that VNI2 negatively regulates xylem vessel formation that interacts with VND proteins. (Yamaguchi et al., 2010b).

The first-level master switches (SND1, NST1/2, VND6/7) in the network are key transcriptional switches regulating a cascade of downstream transcription factors, leading to the activation of the secondary wall biosynthetic program. Although, MYB26 and WRKY12 are shown to be specific regulators of NST2 but not the other first-level switches. Arrows indicate positive and the straight-line-end bars indicate negative regulation.

## Transcription factors regulating the secondary cell wall biosynthetic program in other plants

The role of transcription factors in regulating secondary cell wall biosynthesis has also been studied in other plants (Table 1). In the case of *Eucalyptus globulus*, its TF EgMYB2 have been suggested to have a role in the coordinated control of genes belonging to the monolignol-specific pathway, and therefore in the biosynthesis of lignin and the regulation of secondary cell wall formation (Goicoechea et al., 2005). Another of its TF, EgMYB1, has been found to be a repressor of secondary wall formation (Legay et al., 2010).

In *maize* (*Zea mays*), few members of the MYB family have been characterized and it was shown that two of them, ZmMYB31 and ZmMYB42, act as repressors of lignin biosynthesis and produces a decrease in lignin content of the transgenic plants (Fornale et al., 2006). In addition, *ZmMYB46* has been found to have role function similar to OsMYB46. ZmMYB46 and OsMYB46 are orthologs of Arabidopsis MYB46/MYB83 and, when over-expressed in *Arabidopsis*, they were able to activate the entire secondary wall biosynthetic program. Furthermore, the promoters of OsMYB46 and ZmMYB46 contain secondary wall NAC-binding elements (Zhong et al., 2011a).

The potential of MYB proteins to bind AC elements and regulate lignification was first demonstrated with a family member from *Antirrhinum majus*, *Am*MYB308 and *Am*MYB330 (McCarthy et al., 2010; Zhong and Ye, 2009). Over-expression of the *Antirrhinum* MYB proteins in transgenic tobacco plants caused a reduction in the expression of several lignin biosynthetic genes and a decrease in lignin content, suggesting that the *Antirrhinum* MYBs are able to regulate the expression of lignin biosynthetic genes and thereby affect lignin biosynthesis (Zhong and Ye, 2009).

In poplar (*P. trichocarpa*), the PtrMYB3 and PtrMYB20 are functional orthologs of *Arabidopsis* MYB46 and 83. Therefore, in a similar fashion, these TFs are direct targets of the poplar secondary wall NAC master regulators poplar wood-associated NAC domain proteins (PtrWNDs) which activate the biosynthetic pathways of cellulose, xylan and lignin when over-expressed in *Arabidopsis* (McCarthy et al., 2010; Zhong et al., 2010, 2011a). Additionally, the WND-regulated transcription factors PtrNAC150, PtrNAC156, PtrNAC157, PtrMYB18, PtrMYB74, PtrMYB75, PtrMYB121, PtrMYB128, PtrZF1, and PtrGATA8 are able to activate the promoter activities of poplar wood biosynthetic genes for all three major wood components (Zhong et al., 2011b). Furthermore, transient expression assays showed the variation in PtrWND transactivation activity toward downstream genes, even between duplicate gene pairs (Ohtani et al., 2011). On the other hand, PttMYB21 a (Populus tremula × tremuloides) is a transcriptional repressor of regulating lignin biosynthesis (Karpinska et al., 2004).

*Medicago truncatula* has also been used as a model in studying the regulation of secondary cell wall biosynthetic program. Its TFs, *MtNST1* and *MtSTP* are suggested to have roles in regulating the lignin biosynthesis and negative regulation of secondary wall formation, respectively (Zhao et al., 2010). Thus, AtWRKY-12 and MtSTP are true homologs that function in controlling pith cell wall formation.

The widely popular and important bioenergy crops, wheat (*Triticum aestivum*) and switchgrass (*Panicum virgatum*) have also been investigated for the roles of their respective MYB TFs, TaMYB4 and PvMYB4. Both TFs can bind with AC elements that have been considered as the MYB-binding sites in lignin biosynthetic genes. It has been reported that over-expression of TaMYB4 in transgenic tobacco led to transcriptional reduction of both cinnamyl alcohol dehydrogenase (CAD) and cinnamoyl-CoA reductase (CCR) genes involved in the lignin biosynthesis that substantially decreased the levels of total lignin. Similarly, ectopic over-expression of PvMYB4 in transgenic switchgrass also resulted in reduced lignin content. Thus, these findings suggest that TaMYB4 and PvMYB4 are negative regulators of lignin biosynthesis (Ma et al., 2011; Shen et al., 2012).

NtLIM1, a transcription factor from tobacco, has also

been shown to have the capacity to regulate the expression of some lignin biosynthetic genes. NtLIM1 shows sequence similarity to members of the LIM protein family and is able to activate the AC element- driven GUS reporter gene expression in tobacco protoplasts. Antisense inhibition of NtLIM1 expression in transgenic tobacco plants caused a reduction in lignin content in stems up to 27%, indicating that it is required for normal lignin biosynthesis (Kawaoka et al., 2000). However, it remains to be determined whether NtLIM1 directly regulates the expression of lignin biosynthetic genes.

### Utilization of transcription factors involved secondary cell wall biosynthesis to potentially enhance biomass sources for bioenergy production

Studies on the transcriptional regulation of secondary wall biosynthesis have revealed several important points. First, designing future bioenergy crops through genetic modification is made possible, chiefly, by the immense discoveries on the various regulation mechanisms of secondary cell wall formation. The transcription factors particularly reviewed in this paper are good candidates for improving biomass properties of crops since most of them control the biosynthesis of multiple cell wall components. Genetic modification of secondary cell wall composition through manipulation of these transcription factors can help reduce the cost of pretreatment of bioenergy crops and therefore facilitate subsequent fermentation (Wang and Dixon, 2012).

In this endeavor, primary focus should be in developing methods that will lower lignin and improve availability and levels of cellulose (Ambavaram et al., 2011). It should be noted that decrease in lignin caused by downregulation of the genes responsible for its biosynthesis is accompanied by an increase in cellulose content. Therefore, this suggests that a metabolic network is involved in regulating secondary cell wall biosynthesis (Demura and Ye, 2010).

In curbing the lignin content of the secondary cell wall of bioenergy crops, it is important to note two significant aspects for lignin modification. First, both lignin content and composition are important. Although it is interdependent on efficient processes to fractionate lignin, a more uniform lignin structure might facilitate more efficient cell-wall degradation for fuel production. Second, the pre-treatment of biomass might even be rendered unnecessary if lignin content falls below a critical threshold, which would enhance downstream enzymatic saccharification (that is, the release of products such as cellulobiose and glucose from cellulose via chemical hydrolysis or enzymatic reactions) and fermentation steps for improved efficiency (Abramson et al., 2010; Yuan et al., 2008).

In order to modify the lignin to generate feedstocks with diminished recalcitrance (that is, resistance of plant cell walls to hydrolysis for the release of fermentable sugars), several TFs that can downregulate lignin biosynthesis can be potentially used to meet this objective (Table 1). The MYB TFs *Am*MYB308/330 triggers 17% reduction of lignin content. Other MYBs, such as *Eg*MYB1, *Zm*MYB31/42, *Zm*MYB31, *Zm*MYB42, *Ta*MYB4 and *Pv*MYB4 also reduce lignin content. Additionally, reduction of lignin content in stems by up to 27% can also be instigated by LIM TF and *Nt*LIM1 (Fornale´ et al., 2006; Kawaoka et al., 2000; Ma et al., 2011; Rogers and Campbell, 2004; Shen et al., 2012; Tamagnone et al., 1998; Zhong and Ye, 2009).

In terms of modifying cellulose for biomass production to improve bioenergy, the TF WRKY is a good candidate. Some evidence from the mutation of WRKY TF suggests that it also represses secondary cell wall thickening in pith cells associated with ectopic deposition of lignin, xylan, and cellulose, leading to an ~50% increase in biomass density (Wang et al., 2010).

## CONCLUDING REMARKS

We have witnessed unprecedented progress in the past few years in the characterization of cell wall biosynthetic genes and in studies of transcriptional regulation of secondary cell wall biosynthesis (Zhong and Ye, 2007c). The recent discovery of transcription factors that globally regulate secondary cell wall synthesis in *Arabidopsis* suggests that these may be of use in biomass crops to increase secondary cell wall formation (Mitsuda et al., 2005; Zhong et al., 2006). Secondary cell wall biosynthesis is highly controlled by first- and second-master switches which regulate a cascade of downstream transcription factors that activate the secondary cell wall biosynthetic program. Several of these transcription factors have been identified in this review together with their respective effects upon expression. Most important of these transcription factors are those that negatively regulate lignin biosynthesis (for example, SHN2, MYB75, KNAT7 and OFP4) and those that positively regulate cellulose biosynthesis (for example, SND2, MYB103 and SND3) during secondary cell wall formation. It is also important to know that some of these transcription factors positively regulate both cellulose and lignin (for example, SND2) so that a compromise may be necessary.

Following these findings, it is imperative to further investigate ways on how to exploit these important transcription factors as to how they can be genetically manipulated to improve biomass properties (that is, cellulose and hemicellulose) in bioenergy crops and reduce cellulose recalcitrance and lignin content of secondary cell walls. The deposition of cellulose, xylan and lignin is normally coordinated during wall biosynthesis. If it is possible to control the deposition of each separately, then it should be possible to modify secondary walls in ways that will allow the production of

designer walls in biomass crops (Pauly and Keegstra, 2010). Such modifications can lead to improved biomass density and can potentially reduce land use and transportation costs. High density biomass can further be improved by re-engineering for liquid bioenergy production via fermentation using existing “low lignin” technologies (Wang and Dixon, 2012). Exploration of methods in harnessing the identified TFs for bioenergy crops engineering may eventually help in overcoming existing obstacles leading to the realization of renewable, sustainable and efficient liquid bioenergy. It is, however, important to note that when such bio-engineering practices are carried in food crops with the end view of using their non-food parts, the food quality must not be compromised nor its safety.

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