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REVIEW ARTICLE

Biotechnological improvement of lignocellulosic feedstock for enhanced biofuel productivity and processing

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Abstract Secondary walls have recently drawn research interest as a primary source of sugars for liquid biofuel production. Secondary walls are composed of a complex mixture of the structural polymers cellulose, hemicellulose, and lignin. A matrix of hemicellulose and lignin surrounds the cellulose component of the plant's cell wall in order to protect the cell from enzymatic attacks. Such resistance, along with the variability seen in the proportions of the major components of the mixture, presents process design and operating challenges to the bioconversion of lignocellulosic biomass to fuel. Expanding bioenergy production to the commercial scale will require a significant improvement in the growth of feedstock as well as in its quality. Plant biotechnology offers an efficient means to create "targeted" changes in the chemical and physical properties of the resulting biomass through pathway-specific manipulation of metabolisms. The successful use of the genetic engineering approach largely depends on the development of two enabling tools: (1) the discovery of regulatory genes involved in key pathways that determine the quantity and quality of the biomass, and (2) utility promoters that can drive the expression of the introduced genes in a highly controlled manner spatially and/or temporally. In this review, we summarize the current understanding of the transcriptional regulatory network that controls secondary wall biosynthesis and discuss experimental approaches to developing-xylem-specific utility promoters.

Keywords Secondary wall · Biomass feedstock · Biofuel · Transcriptional regulator · Utility promoter

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Biofuel production from lignocellulosic feedstock

Secondary wall biosynthesis is one of the most important biological processes on Earth. Biologically, the development of a secondary wall is crucial to plant survival because it allows vascular plants to build strong mechanical tissues for water transport and support, which provide the foundation for their dominance on dry land. Economically, secondary walls are the dominant components of woody biomass, which is of primary importance to humans as timber for construction and pulp for paper manufacture, and as an environmentally cost-effective renewable source of energy.

First-generation biofuels are mainly produced from starch and sucrose of food crops, and this is associated with many societal problems, such as ensuring adequate food supplies, global crop price increases, and ethical issues (Somerville 2007). The development of next-generation

biofuels from lignocellulosic materials offers an alternative to food-based biofuels that could reduce our dependence on fossil fuels, encourage national economic growth, and mitigate the negative impacts of global climate change (Carroll and Somerville 2009; Pauly and Keegstra 2010). Lignocellulosic biomass from wood, agricultural residues and fast-growing grasses represents inexpensive, carbon-neutral, renewable and locally available feedstock for producing liquid transportation fuel (i.e., ethanol, diesel) (Gray et al. 2006; Han et al. 2007; Lin and Tanaka 2006; Simmons et al. 2010). For instance, it is estimated that 130 billion gallons of ethanol could be produced from 1.3 billion dry tons of plant biomass (Carroll and Somerville 2009).

While offering certain economic and environmental benefits, the production of biofuels from lignocellulosic feedstocks is currently not cost effective compared to fossil fuels or first-generation biofuels. The inherent recalcitrance, rigidity, complexity and diversity of plant cell walls drive up the cost of enzymatic release of fermentable sugars from the biomass. Variability in the proportions of the cell wall components (i.e., cellulose, hemicellulose and lignin) also presents design and operatonal challenges for the production of lignocellulosic biofuels. Breakthrough technologies are needed to overcome these barriers for cost-effective biomass conversion to fuels. Steps toward breaking-down such barriers include significant improvement in the development of biomass feedstock with desirable quality and quantity to ensure efficient 'low-cost' conversion (Mansfield 2009). A better understanding of the molecular mechanisms underlying the biosynthesis of secondary wall components will help us to identify candidate genes for genetically modifying key pathways that determine the quantity and property of the biomass.

In a typical process of lignocellulosic feedstock biofuel production, a practice called 'pretreatment" is used to break-up the biomass and release polysaccharides, which are then saccharified by polysaccharide-degrading enzymes to generate monosaccharides. Subsequent fermentation of the monosaccharides by microbes produces ethanol. The remaining lignin residues are often burned to provide heating during the distillation process for ethanol separation. Deconstruction of the biomass during the "pretreatment" is often a bottleneck in the biofuel production process because plants have evolved to resist microbial attacks by developing strong secondary wall structures (Carroll and Somerville 2009). Currently, various chemical and physicochemical treatments such as hot steam, weak acid, or nonaqueous ammonia are used to deconstruct biomass (Sousa et al. 2009). However, those energy-intensive treatments are cost prohibitive for any commercial biofuel production. In order for these treatments to be economically feasible at the scale of commercial production, the properties of biomass feedstock need to be optimized for enhanced enzyme accessibility to release more fermentable glucose monomers from cellulose, more easily degradable and/or extractable lignin through the introduction of easily cleavable monolignols or the reduction of lignin–hemicellulose linkages, and improved fermentability of the monosaccharides released (Han et al. 2007; Mansfield 2009; Pauly and Keegstra 2008, 2010; Simmons et al. 2010; Vanholme et al. 2008; Van Vleet and Jeffries 2009). An absolute prerequisite for developing such "designer" feedstock is to secure the necessary knowledge base in the area of the molecular biology of the biosynthesis of the secondary wall and its components.

Genetic regulation of secondary wall biosynthesis is multifaceted and complex

A defining feature of vascular plants is the presence of xylem fibers and vessels that provide mechanical support for their growing bodies and serve as a conduit for long-distance transport of water and solutes. The secondary walls of these cells allow them to resist gravitational forces and/or the forces of tension associated with the transpirational pull on a column of water. Therefore, the proper formation of a secondary wall is crucial to the plant's survival. As such, land plants are equipped with "fool-proof" mechanisms to ensure the correct formation of the secondary wall in the cells of vascular tissues. The secondary wall is formed in a highly coordinated manner by successive encrustations and depositions of cellulose fibrils, hemicelluloses and lignin as soon as the cell has stopped growing (Lerouxel et al. 2006; Somerville 2006; Zhong and Ye 2007). Celluloses, the most abundant biopolymer on Earth, constitute the major polysaccharide of cell walls and are mostly present in the microfibril crystalline structure in the wall (Bhandari et al. 2006; Gardiner et al. 2003; Saxena and Brown 2005; Suzuki et al. 2006; Tanaka et al. 2003; Taylor et al. 2003). Hemicelluloses associate with the cellulose microfibrils, providing a crosslinked matrix. Recently, several genes involved in hemicellulose (e.g., glucomannan and xylan) biosynthesis have been identified (Brown et al. 2009; Dhugga et al. 2004; Lee et al. 2009; Liepman et al. 2005; Wu et al. 2009; Zhong et al. 2005). Xylans are the dominant component of the hemicelluloses in angiosperm species (York and O'Neill 2008) and are relatively difficult to ferment using commercial fermentation systems, which are mostly optimized for six-carbon sugars. Lignin protects and provides mechanical strength to the cell wall by filling the spaces between cellulose and hemicellulose. Many lignin biosynthesis genes have been characterized and used to genetically alter the content and composition of lignin in the cell walls (Boerjan et al. 2003; Vanholme et al. 2008).

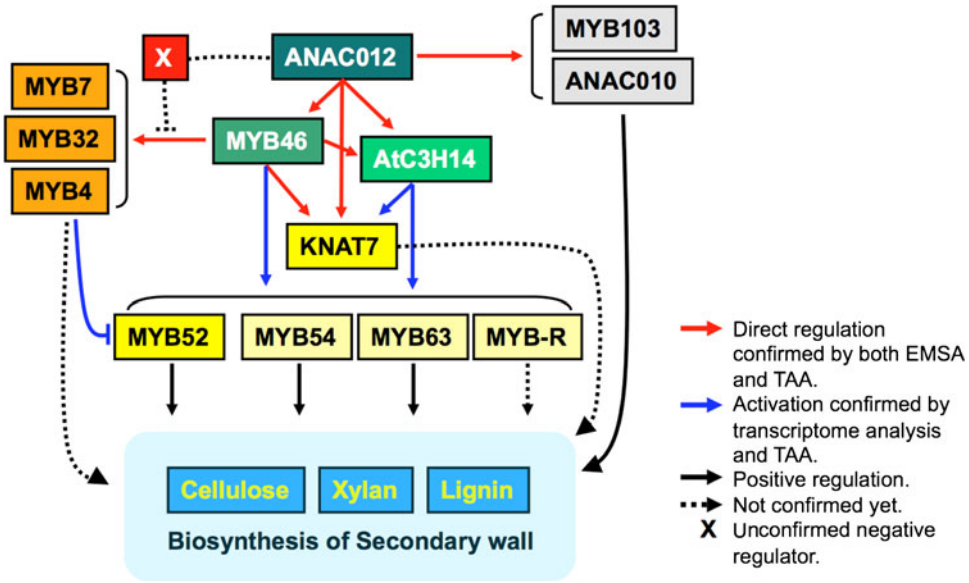

Fig. 1 Tentative model of the transcriptional regulatory network of secondary wall biosynthesis. The hierarchy of the transcription factors involved in secondary wall biosynthesis is displayed. The *red arrows* indicate the direct regulation of the target gene, as confirmed by both transient transactivation assay (TAA) and electrophoretic mobility shift assay. *Blue arrows* indicate direct or indirect regulation, as confirmed by TAA. *Black arrows* indicate positive regulation reported by Zhong et al. (2008). *Dashed arrows* not yet confirmed

While the transcriptional control of the coordinated expression of the regulatory genes involved in the biosynthesis of these components during secondary wall formation is still poorly understood, available observations suggest that the coordination is multifaceted and multilayered (Fig. 1) (Kim WC, Ko JH, Han KH; unplublished observation; Ko et al. 2009; Zhong et al. 2010). The exact nature of this multifaceted regulatory network remains to be elucidated.

Pathway-specific transcriptional regulators of secondary wall biosynthesis

Coordinated transcriptional activation of biosynthetic pathways of secondary wall components (i.e., cellulose, hemicellulose and lignin) leads to secondary wall formation. Recent studies on NAC and MYB transcription factors have provided insight into the complex process of the transcriptional regulation of secondary wall biosynthesis (Demura and Ye 2010; Zhong and Ye 2007). However, only fragmented information is available about pathway-specific regulation of the biosynthesis of secondary wall components. Uncoupling the transcriptional regulation of each component of the secondary wall will have a direct impact on fundamental plant biology as well as the genetic manipulation of feedstock properties.

Several reports have described that a group of closely related NAC transcription factors, including ANAC043/NST1, ANAC066/NST2, ANAC012/NST3/SND1, VND6 and VND7, function as key transcriptional regulators of secondary wall biosynthesis in various sclerenchyma cell types in *Arabidopsis thaliana* (Ko et al. 2007; Kubo et al. 2005; Mitsuda et al. 2005, 2007; Zhong et al. 2006, 2007). Overexpression of these NAC transcription factor genes resulted in ectopic deposition of secondary walls in the cells that are normally parenchymatous, while suppression of their function resulted in a significant reduction of secondary wall thickening (Kubo et al. 2005; Mitsuda et al. 2005, 2007; Zhong et al. 2006, 2007). These results suggest that these secondary wall NAC genes act as transcriptional switches that turn on the entire biosynthetic pathways of the wall components (i.e., cellulose, hemicellulose, and lignin), which leads to the deposition of secondary walls.

In addition, several MYB transcription factors were identified as important regulators of secondary wall biosynthesis in *Arabidopsis*, pine and eucalyptus (Goicoechea et al. 2005; Patzlaff et al. 2003a; Steiner-Lange et al. 2003; Yang et al. 2007). For instance, mutation of MYB26 led to a loss of secondary wall deposition (Steiner-Lange et al. 2003), while its overexpression caused ectopic secondary wall thickening in parenchyma cells (Yang et al. 2007). Also, *Pinus taeda* MYB1 and MYB4 and *Eucalyptus grandis* MYB2 have been suggested to be regulators of lignin biosynthesis (Goicoechea et al. 2005; Patzlaff et al. 2003a, b). Recently, Zhong et al. (2008) reported that a battery of SND1-regulated transcription factors such as SND2, SND3, MYB103, MYB85, MYB20, and KNAT7 are part of the transcriptional network regulating secondary wall biosynthesis.

The transcription factor MYB46, a direct target of ANAC012/SND1, was identified as a transcriptional master switch that turns on the biosynthetic pathways of secondary wall components (Ko et al. 2009; Zhong et al. 2007). Constitutive overexpression of MYB46 led to ectopic deposition of secondary walls. In order to gain further

insights into the MYB46-mediated transcriptional network regulating secondary wall biosynthesis, Ko et al. (2009) developed an inducible secondary wall thickening system in transgenic *Arabidopsis* plants by ectopically over-expressing the *MYB46* gene under the control of dexamethasone (DEX)-inducible promoter. The induction of ectopic secondary wall thickening was confirmed in the rosette leaves of T_3-homozygous seedlings, where no apparent secondary wall thickening was observed in wild-type plants. High resolution time-course transcriptome profiling, using Affymetrix GeneChip Arrays and Illumina Digital Gene Expression analysis, uncovered that MYB46 induced a battery of genes involved in secondary wall biosynthesis within 6 h of induction treatment, including *CesA4*, *CesA7*, *CesA8*, *FRA8/IRX7*, *IRX8*, *IRX9*, *CCR1/IRX4* and *LAC4/IRX12* (Ko et al. 2009). The whole-transcriptome analyses also identified a number of novel transcriptional regulators, such as AtC3H14, MYB63 and MYB7, the expression of which coincided or preceded with the induction of secondary wall biosynthesis genes (Table 1; Ko et al. 2009). MYB63 was recently identified as a direct regulator of the lignin biosynthetic pathway (Zhou et al. 2009). Further analysis identified a complex and multifaceted transcriptional network leading to the biosynthesis of secondary wall polymers (Fig. 1). Functional characterization of the candidate regulators in the network will facilitate the development of a transcriptional roadmap that can serve as a strategic platform for the genetic manipulation of biomass feedstock for tailored "quality" and quantity.

Development of utility promoters for biomass engineering

Various target traits for the domestication of biomass crops include growth rate, stress tolerance, fermentable sugar content, lignin content and composition, lowering biomass recalcitrance to fermentation, and biomass density. Genetic engineering offers a means to effectively change the quality and quantity of the biomass to attain the desired traits. However, it is necessary to control temporally and/or spatially the expression of the introduced gene to avoid unintended consequences of the transgene for the growth of the resulting transgenic plants. Such control of transgene expression can be achieved by using appropriate utility promoters that can drive gene expression in a highly development- or tissue-specific manner. For instance, the use of developing-xylem-specific promoters will allow direct manipulation of the chemical and physical properties of the wood (i.e., secondary xylem) without any negative impact of such manipulation on other aspects of the growth and development of the resulting plants.

Several xylem-specific promoters have been isolated, characterized and utilized successfully to drive transgene expression in xylem tissue (Coleman et al. 2006, 2009; Hu et al. 1999; Li et al. 2003; Osakabe et al. 2009; Rahantamalala et al. 2010; Shi et al. 2010; Winzell et al. 2010). However, the reported observations were made with different promoters driving different transgenes in different hosts, so it is difficult to quantitatively interpret the effectiveness of any given utility promoters. In order to identify strong developing-xylem-specific utility promoters, a series of tissue type-specific transcriptomes were obtained from the young stems of poplar using Affymetrix Poplar Genome Array (Kim HT, Ko JH, Han KH; unpublished data). Subsequent bioinformatics analyses identified several genes that were specifically expressed when developing the xylem of poplar. The tissue-specific activity of their promoters was tested and confirmed in transgenic poplars (Fig. 2). These promoters have the potential to provide a means for developing the xylem-specific expression of pathway-specific transcriptional regulators of secondary wall biosynthesis. For example, the modification of each biosynthetic pathway for secondary wall polymers in a developing-xylem-specific manner will enable us to produce "tailored" biomass optimized for biofuel production.

Conclusion

Plant biotechnology plays a critical role in the improvement of crops in both agriculture and forestry. The successful application of biotechnology requires two enabling techniques: gene discovery and utility promoters for controlled gene expression. There has been an increasing body of literature on the discovery of regulatory genes of secondary wall biosynthesis. The regulatory network identified so far appears to be multifaceted and multilayered. Furthermore, additional pathway-specific regulators for the components of secondary walls need to be discovered. Recent advances in the molecular biology of secondary wall biosynthesis provide good prospects for gene discovery aimed at the biotechnological improvement of biomass feedstock in the near future. However, commercial applications of biotechnology to crop improvement are often limited by a lack of suitable utility promoters. Developing-xylem-specific promoters can be used to drive transgenes to modify the quantity and quality of the biomass in a developing-xylem-specific manner (Fig. 2). The combination of pathway-specific transcriptional regulators

Table 1 Transcriptional regulators upregulated within 6 h by the induction of secondary wall formation

Transcription factor	AGI[a]	TF family name[b]	Co-expression with MYB46[c]	Transcriptional activation by MYB46[d]	Secondary wall related[e]
ATERF9 (ERF domain protein 9)	At5g44210	AP2-EREBP		ND	ND
ERF/AP2 transcription factor family	At3g14230	AP2-EREBP		ND	ND
AtbHL044/BEE1 (BR ENHANCED EXPRESSION 1)	At1g18400	bHLH		ND	ND
bHLH family protein	At2g40200	bHLH	129.8	ND	ND
bHLH family protein	At5g50915	bHLH		ND	ND
G-BOX BINDING FACTOR 6, GBF6/bzip11	At4g34590	Bzip		ND	ND
GATA13 (GATA type) family protein	At5g25830	C2C2-Gata	40	ND	ND
GATA9 (GATA type) family protein	At3g51080	C2C2-Gata		ND	ND
Zinc finger (C2H2 type) family protein	At1g14580	C2H2		ND	ND
AtC3H14, C3H-type zinc finger protein	At1g66810	C3H	8.3	Yes	Yes
Zinc finger family protein	At3g10810	C3H		ND	ND
Zinc finger family protein	At5g15790	C3H		ND	ND
Zinc finger family protein	At1g72220	C3H	155.6	ND	ND
GARP-G2-like family	At2g40970	G2-like		ND	ND
BELI-LIKE HOMEODOMAIN 10 (BEL10)	At1g19700	Homeobox		ND	ND
BLH6 (BELLI-LIKE HOMEODOMAIN protein 6)	At4g34610	Homeobox		ND	ND
HOMEODOMAIN GLABROUS11 (HDG11)	At1g73360	Homeobox		ND	ND
KNAT7 (KNOTTED-LIKE HOMEOBOX 7)	At1g62990	Homeobox	312.9	Yes	Yes
AGL82	At5g58890	MADS		ND	ND
AtMYB1	At3g09230	MYB		ND	ND
AtMYB109	At3g55730	MYB		ND	ND
AtMYB32	At4g34990	MYB		Yes	ND
AtMYB4	At4g38620	MYB		Yes	ND
AtMYB46	At5g12870	MYB	0	ND	Yes
AtMYB52	At1g17950	MYB	149	Yes	Yes
AtMYB54	At1g73410	MYB		Yes	Yes
AtMYB58	At1g16490	MYB	246.2	ND	Yes
AtMYB63	At1g79180	MYB	147.1	Yes	Yes
AtMYB7	At2g16720	MYB		Yes	ND
CAPRICE (CPC), MYB transcription factor	At2g46410	MYB		ND	ND
MYB75/PAP1	At1g56650	MYB		ND	Yes
MYB-R, myb-related transcription factor	At2g38090	MYB		Yes	ND
ANAC002/ATAF1	At1g01720	NAC		ND	ND
ANAC003	At1g02220	NAC		ND	ND
ANAC040	At2g27300	NAC		ND	ND
NAC/ANAC075	At4g29230	NAC		Yes	ND
NAC1	At1g56010	NAC		ND	ND
Argonaute, AGO10 PNH/ZLL	At5g43810	Not classified		ND	ND
Scarecrow family protein	At4g08250	Not classified		ND	ND
Zinc finger family protein	At1g72200	Not classified		ND	ND
TCP transcription factor family	At3g15030	TCP		ND	ND
ATHB27 (HOMEOBOX PROTEIN 27)	At5g42780	ZF-HD		ND	ND

Data was adopted from Ko et al. (2009)

ND not determined yet

[a] *Arabidopsis* gene index

[b] Transcription factor family classified from AtTFDB (http://arabidopsis.med.ohio-state.edu/AtTFDB)

[C] Mutual rank of genes co-expressed with MYB46 (ATTED-II; http://atted.jp/)

[d] Results of transient transcriptional activation assay (Ko et al. 2009)

[e] Genes reported to be involved in the secondary wall biosynthesis (Ko and Han, unpublished results; Bhargava et al. 2010; Zhong et al. 2008; Zhou et al. 2009)

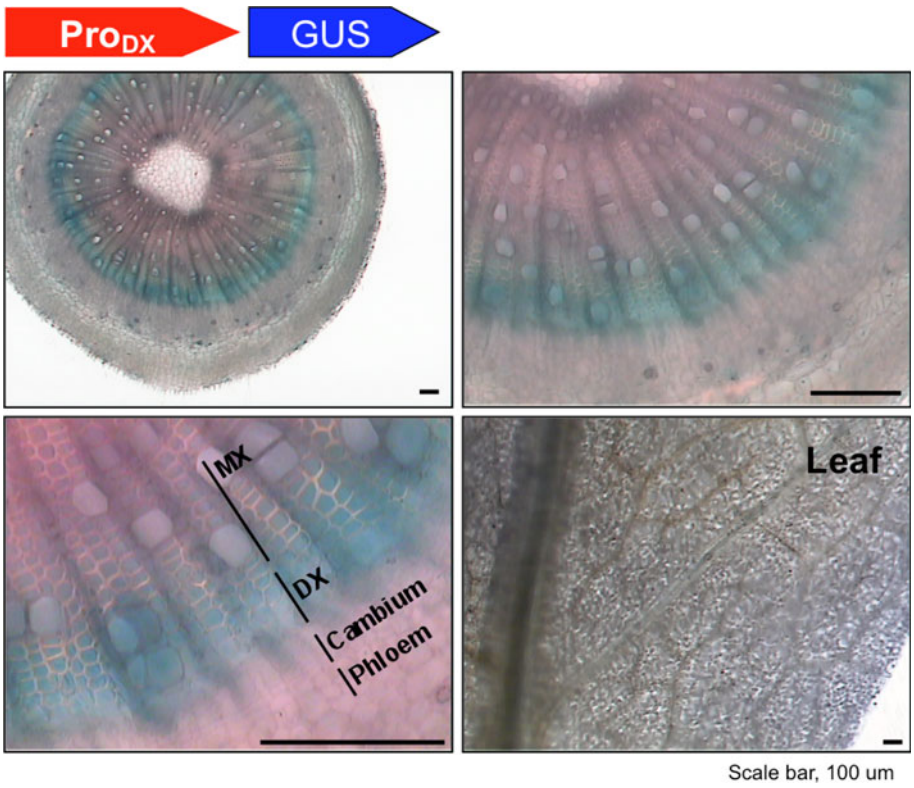

Fig. 2 The developing-xylem (DX)-specific promoter provides a means for "targeted" changes in the chemical and physical properties of woody biomass. Stem cross-sections of a transgenic poplar expressing a DX::GUS construct show exclusive GUS staining in the developing xylem region, while no GUS staining is observed in the leaf. *Scale bar* indicate 100 μm

and their tissue-specific expression can provide a novel means of biomass engineering for improved productivity and processing.

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