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Emerging strategies of lignin engineering and degradation for cellulosic biofuel production

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Ethanol and other biofuels produced from lignocellulosic biomass represent a renewable, more carbon-balanced alternative to both fossil fuels and corn-derived or sugarcane-derived ethanol. Unfortunately, the presence of lignin in plant cell walls impedes the breakdown of cell wall polysaccharides to simple sugars and the subsequent conversion of these sugars to usable fuel. Recent advances in the understanding of lignin composition, polymerization, and regulation have revealed new opportunities for the rational manipulation of lignin in future bioenergy crops, augmenting the previous successful approach of manipulating lignin monomer biosynthesis. Furthermore, recent studies on lignin degradation in nature may provide novel resources for the delignification of dedicated bioenergy crops and other sources of lignocellulosic biomass.

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Introduction

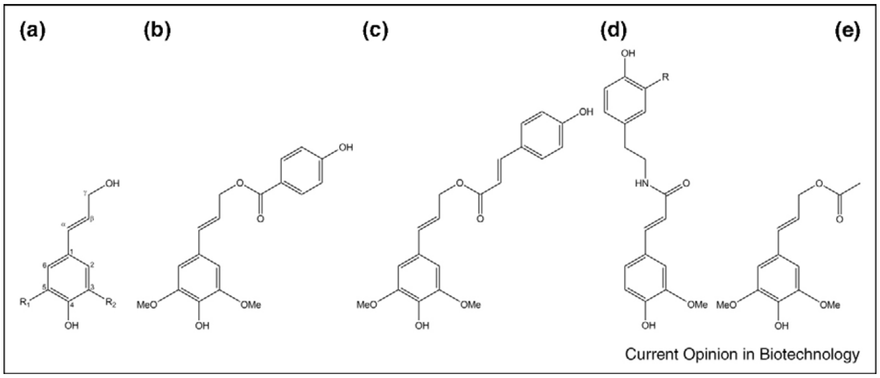
The cell walls of higher plants are strong, yet flexible composites of the biological polymers cellulose, hemicellulose, pectin, and lignin, which serve to maintain the structural integrity of plant cells. Cell wall polysaccharides can be used as a feedstock for biofuel production after being broken down into simple sugars (saccharification) [1••,2], but this process is strongly inhibited by the presence of lignin [3]. A highly degradation-resistant phenolic polymer, lignin is part of a complex matrix in which cellulose microfibrils are embedded. The inhibition of saccharification enzymes by lignin may result from the reduced accessibility of cellulose microfibrils, as well as the adsorbtion of hydrolytic enzymes to the lignin polymer. Furthermore, current chemical and physical strategies to remove lignin from biomass, such as treatment with steam or acid, result in the formation of compounds which can inhibit downstream processes of saccharification and fermentation [4•]. Taken together, these properties of lignin make its biosynthesis a key control point in determining the efficiency of biofuels production.

The ability of lignin to resist degradation can be attributed to its distinctive polymeric structure. Unlike cellulose, lignin is not a linear polymer of identical, repeating subunits. Instead, it is composed of a number of chemically distinct subunits, or monolignols, the abundance of which can vary among species, among individuals, and even among cell types within an organism [5]. The most common of these monolignols, the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monolignols (Figure 1a), are secreted to the extracellular millieu and subsequently radicalized by extracellular peroxidases and laccases [5,6]. These radicals are then able to form a variety of different ether or carbon–carbon bonds with the growing lignin polymer, forming a complex, branched network. The bonds formed among lignin monomers are less reactive than those of most other biological polymers and are of sufficient chemical diversity to preclude the ability of any single enzyme to recognize and degrade them all. Owing of the physical properties of lignin and its incorporation into the cell wall, current approaches used for its removal from biomass are sufficiently expensive and energy-intensive to make large-scale cellulosic biofuels production unfeasible [4•]. Previous attempts to modify lignin monomer biosynthesis have succeeded in down-regulating total lignin content and have been previously reviewed [3,7]; however, these approaches also frequently result in phenotypes undesirable for bioenergy crops, such as dwarfing, the collapse of vessel elements in the xylem, and increased susceptibility to fungal pathogens [8,9]. Thus, the challenge faced by researchers in this field is to simplify the removal of lignin from biomass or otherwise mitigate its effects on cellulose saccharification and downstream processes, while at the same time leaving the lignin polymer sufficiently strong to ensure the fitness of the plant itself. We describe several strategies for achieving such goals below.

New insights into the regulation of lignin biosynthesis

Lignification is associated with secondary cell wall thickening, which occurs only in certain types of plant cells, such as xylem and fibers. This developmental program requires the expression of lignin biosynthetic

Figure 1



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Conventional monolignols and related molecules that can be incorporated into lignin polymer. **(a)** *p*-Hydroxycinnamyl alcohols — R_1=H, R_2=H: *p*-coumaryl alcohol; R_1=H, R_2=OCH_3: coniferyl alcohol; R_1=OCH_3, R_2=OCH_3: sinapyl alcohol. **(b)** Sinapyl benzoate. **(c)** Sinapyl *p*-coumarate. **(d)** Hydroxycinnamic acid amides — R=H: feruloyltyramine; R= OCH_3: feruloyl-3′-methoxytyramine. **(e)** Sinapyl acetate.

genes to be coordinately regulated with each other and with genes controlling other aspects of plant growth and development [10]. A detailed understanding of how these processes are regulated and coordinated would enable us to modify lignification in a more efficient and precise way, possibly through altered expression of relevant transcription factors (Figure 2). Several R2R3-MYB transcription factors, such as *Antirrhinum majus* MYB308 (AmMYB308) [11], *Pinus taeda* MYB4 (PtMYB4) [12], *Arabidopsis thaliana* MYB61 (AtMYB61) [13], and *Eucalyptus gunnii* MYB2 (EgMYB2) [14], have been found to regulate the expression of multiple monolignol biosynthetic genes by interacting with *cis*-regulatory sequence motifs that are rich in adenosine and cytosine residues, known as AC elements, present in their promoters. These transcription factors have bioengineering potential for reducing carbon flux into lignin biosynthesis through coordinated downregulation of multiple monolignol biosynthetic genes. This would be advantageous because in some cases, deleterious phenotypes associated with the downregulation of lignin biosynthetic enzymes have been attributed to the build-up of pathway intermediates [15].

Given the intimate relationship between secondary wall thickening and lignification, it is not surprising that some transcription factors controlling secondary wall formation also affect lignin biosynthesis. For example, the *Arabidopsis* MYB transcription factor AtMYB46 was recently reported to regulate the formation of secondary cell wall in inflorescence stems [16•]. Overexpression of AtMYB46 in *Arabidopsis* increases the expression of biosynthetic genes for all three major components of the secondary cell wall, including lignin. Interestingly, in these plants the thickness of the secondary wall of tracheary elements in the inflorescence stem is increased but that of the interfascicular fibers is reduced, compared to wild-type *Arabidopsis* plants [16•]. The reason for this observation is unknown, but from the viewpoint of biotechnology, this suggests the possibility of changing both the secondary cell wall and lignin in different cell types. The expression of AtMYB46 is in turn regulated by a NAC transcription factor SND1/NST3 [16•], which, together with NST1, was found to redundantly regulate tissue-specific secondary wall thickening in *Arabidopsis* inflorescence stems [17••]. The secondary wall thickening of interfascicular fibers is completely lost in the *Arabidopsis NST1*, *SND1/NST3* double knockout mutant (*nst1 nst3*), but that of vascular vessels is not affected. Although the stem of the *nst1 nst3* double mutant has reduced strength and cannot hold the inflorescence upright, both the growth rate and overall size of the *nst1 nst3* mutant are similar to that of wild-type plants, consistent with the mutant's normal vascular development. Dominant suppression of either NST1 or NST3 results in similar phenotypes to that of the double knockout mutant [17••,18]. This suggests a possible means of downregulating lignification in a tissue-specific manner, while avoiding vascular collapse [8,9].

Controlling lignin polymerization through monolignol-specific oxidases

Monolignol polymerization is another important step during lignification which remains poorly understood and needs to be explored further in the context of lignin engineering. The dehydrogenative polymerization of monolignols is thought to be catalyzed by peroxidases and laccases [3]; however, because most of the genes encoding these enzymes show high redundancy in plant genomes, the exact roles for individual isozymes have not been clearly defined. As a result, relatively few studies have utilized these enzymes to manipulate lignification [5]. Most peroxidases characterized till date can efficiently oxidize coniferyl alcohol, showing less activity toward sinapyl alcohol [5]; however, several recent studies have suggested the presence of sinapyl alcohol-specific peroxidases, potential targets for lignin engineering (Figure 2) [19]. For example, Sasaki *et al.* reported the