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Engineering of plant cell walls for enhanced biofuel production

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The biomass of plants consists predominately of cell walls, a sophisticated composite material composed of various polymer networks including numerous polysaccharides and the polyphenol lignin. In order to utilize this renewable, highly abundant resource for the production of commodity chemicals such as biofuels, major hurdles have to be surpassed to reach economical viability. Recently, major advances in the basic understanding of the synthesis of the various wall polymers and its regulation has enabled strategies to alter the qualitative composition of wall materials. Such emerging strategies include a reduction/alteration of the lignin network to enhance polysaccharide accessibility, reduction of polymer derived processing inhibitors, and increases in polysaccharides with a high hexose/pentose ratio.

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Introduction

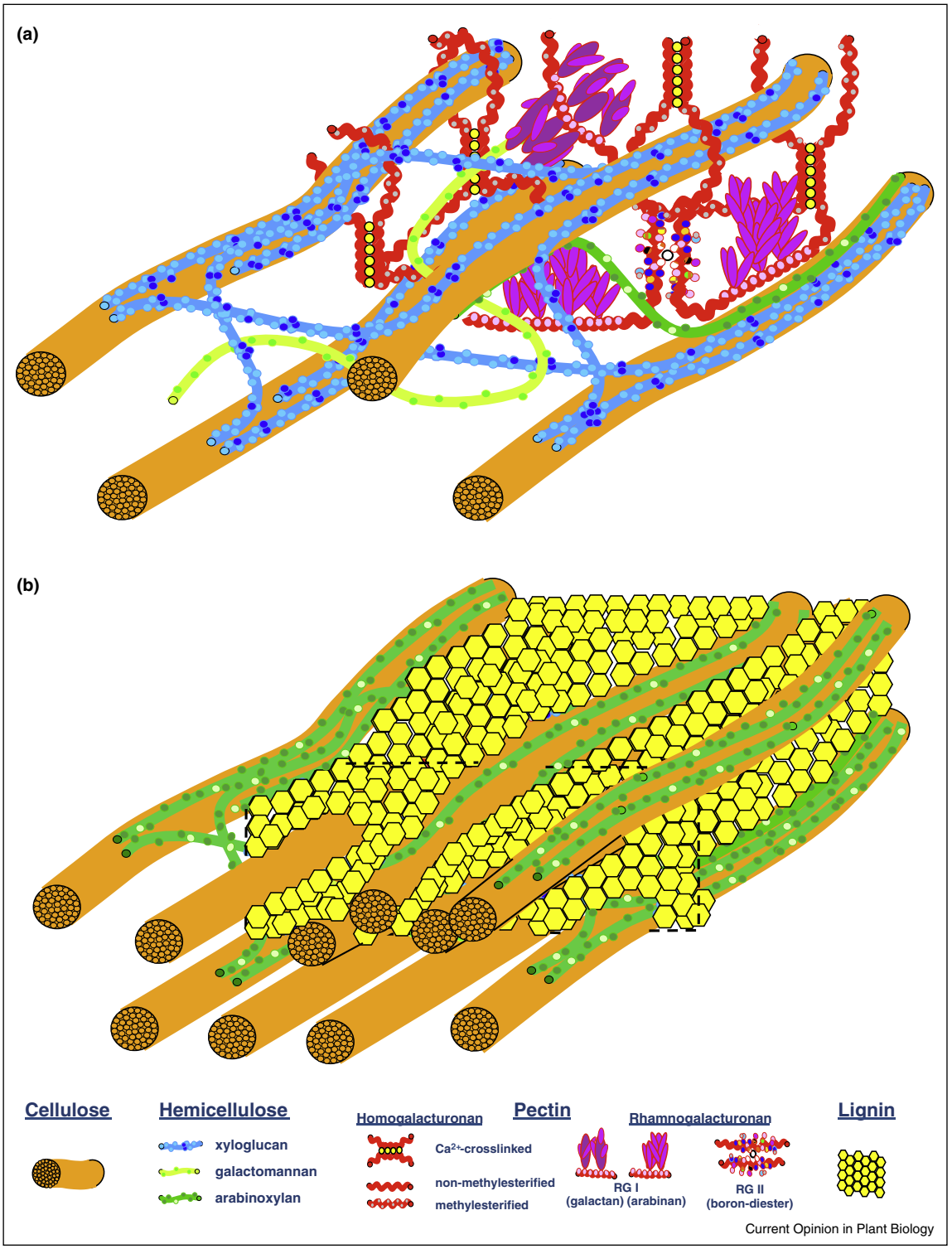
The annual production of cell walls of land plants alone has been estimated to be 150–170 billion tons [1]. To put this in perspective, the total annual output of the chemical industry worldwide (~1.2 billion tons/year; BASF report 2011) is produced by land plants in the form of cell walls in just two days. During this process massive amounts of atmospheric carbon dioxide are assimilated by plants through photosynthesis and deposited and stored in the wall, making the wall the dominant carbon-sequestration system on this planet. However, one should not consider the wall as a mere carbon storage device, but as a sophisticated material composite made of a number of polymer networks with numerous, essential functions in the life cycle of a plant.

While plant cells are still elongating, they are surrounded by a primary wall, whose principle components are polysaccharides — glucans forming cellulose microfibrils, substituted polysaccharides representing various hemicelluloses, and a polyanionic polysaccharide network, the pectins — placed in water (water content: ~60–70% of the mass [2], Figure 1a). Once cell elongation ceases, a secondary wall is deposited in some types of cells. Secondary walls also consist of polysaccharides — cellulose microfibrils and various hemicelluloses — but the water content is much reduced (~5%) due to the presence of the hydrophobic polyphenol lignin (Figure 1b, [2]).

The specific composition of the wall and its components in a plant varies depending on plant species, tissue type, and developmental state of the tissue [3]. For example as shown in Figure 2, while the principal components of poplar wood and grass straw are similar, there are differences in the proportional abundance of cellulose, non-cellulosic polysaccharides and lignin and additional unique differences. Some of these unique structural properties include the abundance of mannose rich polymers and glucuronosylated xylans in hardwood vs arabinosylated xylans and the presence of *p*-hydroxy-cinnamic acids such as *p*-coumaric acid and ferulic acid in the lignin of grass straw.

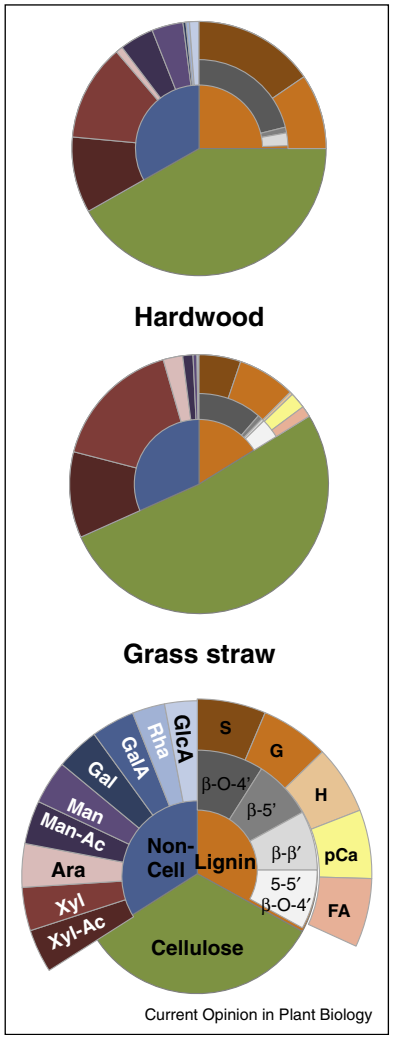
Due to their abundance plant cell walls, also termed lignocellulosics, are thought to represent a renewable resource for the production of biofuels and/or other commodity chemicals especially since lignocellulosic feedstocks do not represent a food source for humans [4]. In current commercial biorefinery processes lignocellulosic material is broken down using a chemical pretreatment at elevated temperature and the polysaccharides converted to monosaccharides via a cocktail of enzymes. The sugars can then be fermented by microbes or reformed by chemical catalysis to produce the desired chemical or fuel. For the biorefining processes to be economically viable a number of hurdles have to be overcome. One hurdle includes optimizing quantitative agronomic traits by increasing the yield of lignocellulosic biomass per ha, for example, through the selection of suitable feedstocks tailored to specific environmental weather/growth conditions [4]. Another hurdle includes qualitative traits of the lignocellulosic biomass such as reducing recalcitrance to degradation by making the carbohydrates more accessible for enzymes, or optimizing the composition for the fermentation step [5,6].

Figure 1



Schematic models of plant cell walls. The emphasis is placed on wall composition rather than architecture. **(a)** Pectinaceous, aqueous primary cell walls found in dicots. **(b)** Lignified secondary walls with only minor water content.

Figure 2



Lignocellulosic composition of common feedstocks. Cellulose, lignin and non-cellulosic polysaccharide (non-cell) composition of Poplar wood and Miscanthus straw based on [87]. Bottom: legend. Monosaccharide composition: Xyl – xylose; Ara – arabinose; Man – mannose; Gal – galactose; GalA – galacturonic acid; Rha – rhamnose; GlcA – glucuronic acid; Xyl-Ac – acetylated xylose; Man-Ac – acetylated mannose. Lignin composition: S – syringyl-; G – guaiacyl-; H – p-hydroxyphenyl-; pCa – *p*-coumaryl-units; FA – ferulic acid. Lignin linkages: β-O-4′-β-arylether; β-5′-phenylcoumaran; β-β′-resinol; 5-5′-dibenzodioxocin; Note that the lignin linkage abundance is independent from the lignin composition.

Recently, several plant biotechnological approaches have been successful in addressing the recalcitrance without compromising plant growth and biomass accumulation [7,8]. Progress in these approaches are described here: firstly, overcoming recalcitrance by reducing/altering the lignin polymer network; secondly, reducing processing inhibitors that originate from the biomass; thirdly, enhancing fermentation yields by increasing the abundance of easily fermentable sugars in lignocellulosics.

Altering the lignin polymer network

The lignin biosynthesis pathway is already very well characterized and conserved across Tracheophyte kingdom. Lignin is mainly composed of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units derived from the random polymerization of *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, respectively. Significant progress on the modification of lignin structure, content or distribution has been achieved within the last years to reduce overall biomass recalcitrance and improve saccharification yield without compromising biomass yield (see recent reviews: [9–11]). The lignin-engineering field is rapidly evolving and multiple approaches are currently developed to reduce its recalcitrance, minimize lignin waste stream, and/or valorize it [7,12–14]. Rapid advances in genomics, transcriptomics and synthetic biology contributes significantly to the progress in lignin engineering. Omics studies enable the discovery of novel enzymes and metabolites from plants or microorganisms that can later be used to manipulate lignin content and composition. Furthermore, they facilitate the reconstruction of regulatory networks controlling biosynthesis of different secondary cell wall components and the identification of promoters with desired expression profiles that can subsequently be used to engineer plants and optimize tissue specific expression of transgenes. An example of highly successful engineering involved swapping the promoter driving the expression of the Arabidopsis cinnamate 4-hydroxylase (C4H) with a vessel-specific promoter to restrict C4H expression to vessel cells and disconnect it from the fiber regulatory network [15••]. This strategy allowed reducing lignin content by more than 20% without compromising plant growth. This approach highlighted the importance of vessel cell wall integrity for optimal plant development and the role of synthetic biology in lignin engineering. The approach depended on availability of a recessive lignin biosynthesis mutant and could therefore be difficult to translate to a range of crops. Developing dominant genetic traits would facilitate the translation of lignin technologies from model plants to crops. Ideally, approaches for engineering should efficiently target specific tissues and/or be turned on/off only during specific developmental stages of crops in order to reduce the risk of obtaining undesired phenotypes. Fine-tuning lignin engineering pathways would limit the negative impacts of lignin manipulations on the production of phenylpropanoid-derived secondary metabolites that are used for other purposes than lignification such as biotic stress resistance [16–18]. A tissue-specific dominant approach was developed by expressing a microRNA raised against CCR1 (cinnamoyl-CoA reductase 1) under the control of a secondary cell wall cellulose synthase promoter (pAtCesA7), and successfully reduced lignin accumulation in interfascular fibers while vessel integrity was retained [19•]. More recently, the first dominant and enzymatic-based strategy to reduce lignin content by half without compromising biomass yield was reported [20••].

The approach focused on the creation of a competitive metabolic pathway in cells producing secondary walls to reduce the availability of shikimate, which is necessary for the activity of hydroxycinnamoyl-CoA shikimate hydroxycinnamoyltransferase (HCT), a key enzyme of the lignin pathway.

Alternative approaches focusing on altering lignin composition have also been developed. For example, monolignol ferulate esters could be produced in lignifying tissues and incorporated in lignin polymers by expressing a feruloyl-CoA monolignol transferase in poplar using a secondary cell wall cellulose synthase promoter (pPtCesA8) [21••]. The incorporation of these novel monolignols into lignin polymers reduced their recalcitrance to a mild alkaline pretreatment and improved saccharification yield. More recently, a Cα-dehydrogenase (LigD) enzyme from the bacterium *Sphingobium* sp. SYK-6 was expressed to oxidize the α-hydroxy functionalities in β-O-4-linked monolignols resulting in an enriched level of G-type α-keto-β-O-4-linkages in lignin [22]. The enrichment is still very limited but there are many opportunities for improvement such as the replacement of the p35S promoter used in their study by one that would restrict and allow stronger expression of LigD in secondary cell wall. In addition, manipulating monomeric composition of lignin could be used to reduce lignin degree of polymerization (DP) and overall biomass recalcitrance. For example, lignin enrichment with H units is known to facilitate lignin extractability and lower its degree of polymerization [23,24]. Extreme H enrichment was recently achieved by stacking multiple recessive approaches. Repressing the activity the *p*-coumaroyl-shikimate 3′-hydroxylase (C3′H) in an Arabidopsis mutant defective in two mediator-encoding genes resulted in lignin composed of 95% of H units with limited negative impact on plant growth [25]. Although this approach is very effective to generate H-rich lignin and reduce biomass recalcitrance in model plants, it will be difficult to translate it to crops due to the number of genes that need to be repressed in specific tissues. Alternatively, H unit enrichment and lignin DP reduction can be achieved by the inhibition of HCT activity with a dominant approach via either the reduction of shikimate availability in lignifying tissues [20] or coexpression of NahG (salicylate reduction approach) and an HCT silencing construct [26].

Importantly, several crop mutants affected in various lignin biosynthetic steps have been isolated from various collections (e.g. sorghum) or generated with silencing strategies (e.g. alfalfa) and most of them exhibit reduced biomass recalcitrance [27,28]. Unfortunately, compromises between altering the degree of biosynthesis and plant biomass accumulation and fitness need to be made because most mutations will not only affect lignin structure, and wall integrity in important tissues (e.g. vessels) but also impact negatively the biosynthesis of the phenylpropanoid derived metabolites involved in stress resistances. Interestingly, the mutants affected in cinnamoyl aldehyde dehydrogenase (CAD) activity, which is the last lignin enzymatic step and a lignin specific enzyme reaction, show no growth phenotypes [27,29,30].

Reducing endogenous process inhibitors

The polysaccharides in lignocellulosic biomass are not composed exclusively of carbohydrates, but additionally contain acetyl esters, methyl esters and ethers. These modifications to the polysaccharide increase recalcitrance because they hinder access of enzymes to the polysaccharides. Furthermore, the esters are eventually released and can inhibit subsequent fermentation. In particular acetic acid that is released from the biomass is highly inhibitory to yeast fermentation [31].

One way to reduce lignocellulosic acetyl content is by genetically interfering with the synthesis of the acetylated polysaccharides. Three groups of enzymes have been identified that are required for wall polysaccharide *O*-acetylation, the Reduced Wall Acetylation (RWA) proteins, the Altered XYloglucan 9 protein, and the Trichome Birefringence/Trichome Birefringence-Like (TBR/TBL) proteins [32–34]. Downregulating the activity of RWA proteins results in plants with less wall acetylation and based on studies with Arabidopsis this is a viable strategy for reducing acetylation levels by about 25% [32,35,36]. If wall acetylation is further reduced by inactivating or downregulating the transcription of multiple *RWA* genes plant growth is adversely affected [36]. A similar severe dwarfed phenotype has been observed, when AXY9 was knocked-out in Arabidopsis [34]. The *O*-acetylation level in knock-out allels of AXY9 is reduced by 70% in stem and 30% in leaf tissue. The precise role of AXY9, a plant specific, unique gene in Arabidopsis, in the polysaccharide *O*-acetylation process remains enigmatic. The RWA proteins and AXY9 appear to work upstream to the TBR-TBL proteins as they affect acetylation of multiple polysaccharides. In contrast, TBL proteins have been shown to be specific for a particular type of polysaccharide [33,37,38]. For example, the TBL29 protein is an acetyltransferase that specifically acetylates xylan, which is the most abundant source of acetate in biomass [38,39•]. However, inactivation of TBL29 results in severe plant growth retardation and reduced biomass accumulation, but it seems conceivable that this could be amended, for example, by restricting the inactivation to fiber cells similarly to what was done with xylan and lignin in recent studies [15,40]. Surprisingly, increased expression of a xylan glucuronosyltransferase in a *tbl29* mutant background resulted in plants with virtually normal growth while maintaining the low acetylation level of the original mutant [41•]. Saccharification was substantially improved. Unfortunately, mutants and engineered plants with reduced cell wall acetylation have so far only been studied in Arabidopsis and it remains to be seen to

what extent the findings can be translated to bioenergy crops.

Grass xylans contain less acetylesterification than hardwood xylan, but instead the grass xylans are esterified with ferulic acid and to a lesser extent *p*-coumaric acid. Ferulate esters are often dimerized in the wall, thereby generating intra-molecular and inter-molecular crosslinks of xylans that contribute to the recalcitrance of grass biomass. The pathway for feruloyl esterification is not fully understood but appears to involve acyltransferases in the BAHD family [42]. However, since these enzymes are generally cytoplasmic while xylan feruloylation takes place in the Golgi lumen there must be additional steps involved [43]. Overexpression of a rice gene in the BAHD family, *OsAT10*, resulted in a significant increase in *p*-coumaric acid esterification and a decrease in feruloyl esterification without any substantial impact on plant growth [44]. The mechanism for the decrease in feruloylation is unclear, but a possible explanation is that OsAT10 is a *p*-hydroxycinnamoyltransferase and lower feruloyl incorporation into xylan by saturating the xylan backbone with *p*-coumaric acid esters or by depleting the availability of feruloyl-CoA. Grasses have many *BAHD* genes and an extensive analysis of the role of the grass-specific genes has not been reported. Using an RNAi directed against several *BAHD* genes in rice resulted in walls with about 20% less ferulic acid [45]. Unfortunately, it is not completely clear which *BAHD* gene(s) were responsible for ferulic acid incorporation in this study. Another strategy for reducing ferulic acid in the wall is to express ferulic acid esterase heterologously in plants. Such an approach has been used in a number of species with mixed success. In *Festuca* and *Lolium* the plants were reported to grow normally and the biomass to be more easily saccharified [46–48]. However, in wheat the same approach resulted in a severe negative impact on plant growth and development [49].

Increasing wall sugars

Carbohydrates are the principal components of the wall currently used for biorefining. These carbohydrates are mainly utilized by fermenting microbes in form of monosaccharides in particular hexoses such as glucose. However, the wall contains also a significant amount of pentoses such as xylose derived from the hemicellulose xylan. Pentoses cannot be fermented as efficiently as hexoses [50] and thus current strategies aim at reducing xylan thereby increasing the hexose/pentose ratio in wall materials.

Adjusting carbon partitioning from cell primary metabolism to wall glucan biosynthesis has been successful [51] by for example overexpression of a sucrose synthase in poplar [52]. Sucrose synthase produces UDP-glucose for cellulose synthesis and the result was an absolute increase in cellulose (2–6%). Cellulose abundance was higher in secondary walls due to the use of specific promoters. This increase was achieved without any apparent detrimental effect on plant growth or biomass yield. Interestingly, the opposite experiment, reducing the transcription of sucrose synthase in poplar, did lead to a reduction in wall density, but all wall polymers including the hemicelluloses and lignin were affected, not only cellulose [53]. Another target for influencing carbon allocation is UDP-glucose pyrophosphorylase, which produces UDP-glucose. A corresponding UDP-glucose pyrophosphorylase double mutant in Arabidopsis displayed a significant reduction in cellulose synthase expression [54], while overexpression of this enzyme in Jute lead to an increase in cellulose content [55]. These approaches clearly indicate that it is possible to alter carbon flux towards wall polymers even without affecting overall plant biomass accumulation, but our knowledge of this area is too rudimental to predict which polymers might be affected.

Another target for altering sugar content in the wall is by manipulating the nucleotide sugar conversion pathway, which produces specific activated sugars for specific polymers and the corresponding nucleotide sugar transporters, which are responsible for relocating the nucleotide sugars to their corresponding transferases. For example, one target for increasing the hexose/pentose ratio of biomass is β-1,4-galactan. β-1,4 galactans are sidechains of rhamnogalacturonan I domains of pectin and normally not abundant in secondary walls, with the exception of tension wood and gelatinous fibers. Overexpression of a recently characterized UDP-galactose transporter, URGT1, in Arabidopsis resulted in increased β-1,4 galactan deposition in leaves [56]. However, to what extent URGT1 can improve β-1,4 galactan accumulation in woody tissues has yet to be reported.

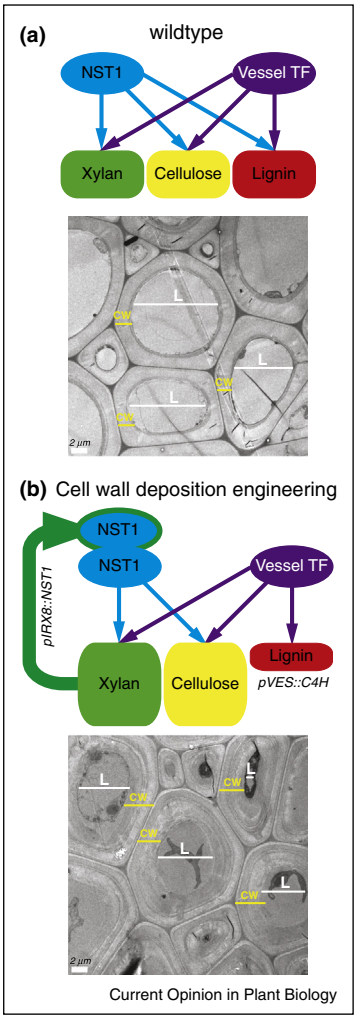
A promising strategy for enhancing specific wall sugars is the engineering of the glycan synthases and glycosyltransferases, which are directly involved in wall polymer synthesis. Cellulose is synthesized by a multiple transmembrane spanning protein, CesA, that forms complexes in the plasma membrane [57,58]. Only recently has the protein structure of a cellulose synthase from *Rhodobacter* been elucidated giving valuable insights into the working of this enzyme [59••]. De novo and homology modeling of the catalytic domain of a cotton cellulose synthase shows good agreement with the bacterial one [60•] indicating that the cellulose synthesis is processive and that cellulose synthesis does not require a primer unlike previously thought [61]. Overexpression of CesAs in poplar did not lead to the desired accumulation of cellulose, but instead resulted in a reduction in wall cellulose due to silencing of endogenous CesAs [62]. The plants showed severe, aberrant, pleiotropic phenotypes implying that manipulating the expression levels of CesAs might not be a fruitful approach. Moreover, two Arabidopsis mutants containing CesAs with point mutations in specific regions displayed

dwarfed phenotypes or a reduction in cellulose content, respectively, thus compromising biomass accumulation [63,64]. Both mutations cause a decrease in cellulose crystallinity. Expression of a defective cellulose synthase CesA3 (*eli1*) is correlated with a lignin enrichment in H units [65] suggesting that over-expression of a defective cellulose synthase could be used to enhance the ratio between crystalline and amorphous cellulose as shown in tobacco [66•] and boost the accumulation of H units to reduce lignin DP and recalcitrance.

With the recent advance of establishing gene regulatory networks responsible for secondary wall formation [67••], overexpressing certain transcription factors leads to ectopic production of secondary walls [68]. Hence, the utilization of emerging transcription factors specific for the cellulose synthase could represent a viable strategy to increase cellulose production. The successful engineering of increased cell wall density was achieved by overexpressing the NST1 master transcription factor controlling secondary cell wall biosynthesis in fiber cells in Arabidopsis [15]. The *NST1* gene was driven by a downstream promoter of a secondary cell wall glycosyltransferase (IRX8), thereby effectively generating a positive feedback loop that ensured a high level of NST1 expression but only in cells already expressing NST1 to begin with (Figure 3). By introducing this construct into plants that had already been engineered to have low lignin content, the cell walls did not only have higher density but also a markedly higher carbohydrate content and improved saccharification [15].

The glycan backbones of the hemicelluloses contain β-1,4 glycosidic linkages similarly to cellulose and are often synthesized by enzymes homologous to the CesAs, belonging to glycosyltransferase family 2 [69]. Consequently, the backbone of matrix polysaccharides such as mannan, xyloglucan, and mixed-linkage glucans are synthesized by CslA, CslC, and CslF/H proteins, respectively [70]. Overexpression of a guar CslA (ManS) in soybean embryos led to a 3% increase in mannan in soybean seeds [71]. CslF and CslH were identified as playing a key role in the synthesis of mixed-linkage glucan, a non-cellulosic polysaccharide occurring in the walls of grasses, by heterologous expression of their rice and barley orthologs, respectively, in Arabidopsis [72,73]. Arabidopsis does not contain endogenous mixed-linkage glucans in its walls, but expression of these genes resulted in the presence of β-glucans as detected by immunolabelling techniques using a specific antibody. However, the level of mixed-linkage glucan level in the leaf tissue was estimated to be only 0.1% and 0.4% (wt/wt) of the walls for the expression of *OsCslF* and *HvCslH*, respectively. A more promising result was obtained when a barley CslF (HvClsF6) was overexpressed in barley resulting in a 3–4-fold increase in mixed-linkage glucans in the leaf tissue leading to a plant biomass with a higher hexose content [74]. However, this

Figure 3



Engineering of increased cell wall density and decreased lignin in Arabidopsis. Schematic of simplified regulatory network controlling secondary cell wall biosynthesis in vessel and fiber cells in plants and images from wildtype **(a)** and engineered **(b)** Arabidopsis plants depicting interfascicular tissues composed of fiber cells. Engineered plants were generated from a *c4h* defective mutant (mutant affected in the second lignin biosynthesis step) that was transformed with the wild-type version of the mutated *C4H* gene driven by a vessel-specific promoter (pVES) which rescued the negative effect of low lignin content. Generated plants were further transformed with a construct (e.g. pIRX8::NST1) that led to higher expression of master transcription factor controlling secondary cell wall biosynthesis in fiber cells (e.g. NST1). This approach has been described in Ref [15]. Scale is 2 μm; CW: cell wall; L: Cell lumen.

overexpression utilizing the constitutive 35S promoter often resulted in yellow, wilted leaves and plant lethality compromising plant biomass accumulation. Similar plant growth defects were observed when using the 35S promoter for transient expression of *OsCslF6* in tobacco or stable transformation in Arabidopsis resulting in accumulation of mixed-linkage glucan at levels up to 13% and 6%, respectively [75]. Since the presence of β-glucan was

enhanced in the vasculature it is possible that the additional β-glucan lead to clogging of the water-transporting elements resulting in wilting. When placed under the control of an endosperm specific promoter a near doubling of the mixed-linkage glucan content in the transgenic barley grain was recorded without detriment to plant growth [74]. Also, the use of a senescence associated promoter to drive *OsCslF6* allowed accumulation of mixed-linkage glucan up to 2.5% of the wall while negative effects on growth were avoided [76]. Again, the choice of promoter and hence in which tissues to overexpress the genes is vital to retain a healthy plant performance.

Overexpression of CslC, a xyloglucan glucan synthase, to augment the abundance of this easily degradable hemicellulose in plant biomass has not been reported to date. Reduing xylan biosynthesis is an attractive strategy to increase the hexose/pentose sugar ratio in biomass. However, mutants in xylan biosynthesis show a dwarf phenotype and have collapsed xylem vessels. Interestingly, xylan, which also contains a glycan backbone of β-1,4 linkages, can be formed in vitro by an enzyme with a single transmembrane domain that is not related to CesA in GT family 2, but instead is a member of glycosyltransferase family 47 [39,77]. Many other proteins including numerous other glycosyltransferases disturb xylan biosynthesis in the corresponding Arabidopsis mutants [78], highlighting that our knowledge of the synthesis of these polymers in vivo is still in its infancy. Silencing of glycosyltransferases involved in xylan biosynthesis such as Irregular Xylem 8 (*IRX8*) in Arabidopsis leads to a reduction in wall xylan, but also collapsed xylem vessels and thus plant dwarfism [79]. A similar *IRX8* silencing approach in poplar also lead to a significant reduction in xylan and in increased glucose yields through saccharification [80]. However, in contrast to Arabidopsis the Poplar transformants displayed an increase in plant growth indicating that results obtained with Arabidopsis do not necessarily translate directly to crop plants and will have to be tested in each case. Alternatively, to overcome the negative impact of reduced xylan Arabidopsis mutants in xylan biosynthesis were transformed with constructs that restored xylan biosynthesis in xylem vessels but not in interfascicular fibers [40]. This strategy using vessel-specific promoters resulted in plants with reduced xylan content but no negative effect on growth or mechanical properties. Translating this engineering strategy to crops will be challenging since it depends on transformation of a severely dwarfed mutant. Therefore, dominant approaches that can be readily introduced in elite cultivars would be better, but at present none are available for xylan reduction.

Another polymer that is easily digestible in the walls is callose, a β-1,3 linked glucan, that is mainly deposited in the wall during stress situations such as pathogen infection or wounding. Overexpression of a callose synthase, a member of the GT48 family, in Arabidopsis results indeed in an ectopic deposition of callose [81]. The total biomass of the plant was not affected and as an additional benefit pathogen penetration into the leaf is inhibited rendering the plant more resistant to powdery mildew. While the increase in callose content has not been quantified in these transgenic plants the chosen approach could be a promising strategy to enhance the conversion properties of plant biomass. However, application of this approach to crops will need to be assessed on a case by case basis, as not only wall properties are affected but also pathogenicity and the plant's response to pathogens.

The pectic sidechain β-1,4 galactan is synthesized by a galactan synthase, GalS, present in GT family 92 [82], but overexpression of GALS1 in Arabidopsis did not result in increased β-1,4 galactan content in secondary cell walls [83•]. However, when UDP-galactose epimerase was overexpressed together with GALS1 either with constitutive or secondary cell wall specific promoters, the secondary cell walls contained significant amounts of β-1,4 galactan, and the galactose content in stems was doubled [83]. As mentioned above, overexpression of a UDP-galactose transporter also enabled increased β-1,4 galactan accumulation [56] and hence it would be interesting to determine the effect of overexpression of all three proteins at the same time.

Some wall polysaccharides are turned over and degraded by plant glycosyl hydrolases. Hence, one strategy is to stop polymer degradation allowing the polymer to remain in the wall thus contributing to wall biomass. One example is mixed-linkage glucan, which is degraded in young grass tissues presumably to provide ample carbon for the elongating coleoptile [84]. Mixed-linkage glucan is degraded specifically by plant licheninases. A maize mutant called candy-leaf 1 (*Cal-1*) harboring a mutation that leads to the inactivation of a maize licheninase resulted in a more than doubling of β-glucan not only increasing the hexose content of the plant biomass, but also a 35% increase in saccharification yield of its leaf and stalk material was observed [85]. Field trials indicated that the mutant's biomass accumulation was not affected.

Concluding remarks

As reviewed above, multiple strategies have been devised for engineering plants to make the biomass more suitable for biofuel production. Techno-economical modeling indicates that the most important target for engineering is lignin, but other properties can also be improved with significant benefit [86]. According to the model based on dilute acid pretreatment of corn stover, biofuels costs can readily be reduced by 10–25% by affecting inhibitor content, monosaccharide composition and/or lignin. Other types of biomass or pretreatment processes would

require modification to the model, but the main plant engineering goals would remain similar. An important question that remains to be answered is how well the model studies can be translated to crops. Although no negative impact was observed on plant growth in many of the engineering studies reviewed here, it is unclear if there would be negative impact on growth and development under field conditions. Another question is how to combine different engineering targets in a single plant. Stacking of multiple traits is technically challenging and the extent to which improvements in biomass composition are compatible and additive needs to be explored. In a broader context, the successful engineering of plants demonstrates the potential for devising modifications to plant structures and biosynthetic pathways. Adverse effects on plant growth and development can often be avoided by using promoters with the right spatio-temporal expression profile.

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