Biofuels have recently been the subject of intense debate with regard to ‘food versus fuel’. Consequently, attention has focused upon so-called ‘second-generation’ biofuels that use alternatives to food-based feedstocks. In the best-developed forms of second-generation biofuels, sugars from starch digestion could be replaced with sugars released from the plant cell walls. This biomass could come from either agricultural residue, such as part of the maize culm, or from purpose grown biofuel crops, such as Miscanthus or Switchgrass (Panicum virgatum), that generate huge yields even when grown on marginal land with minimal agricultural inputs. For these and other potential bioenergy crops such as trees, the majority of the plant biomass is composed of woody secondary cell walls. If all cell wall sugars were readily accessible to fermenting micro-organisms, a 5 kg log could theoretically produce up to 2.5 litres of ethanol. The secondary cell walls are frequently the first line of defence against pests and pathogens, as well as providing structure and support for upward plant growth (Figure 1). Consequently, by their very nature, secondary cell walls are designed for strength and to resist degradation. The compact organization of the wall makes its digestion, a process known as saccharification, very difficult so biomass is currently too costly to be a viable feedstock. Knowledge of how the walls are constructed, however, would allow us to efficiently deconstruct them. This article gives an overview of secondary walls and potential modifications expected to be beneficial to improved biofuel production.

Secondary walls are a complex matrix consisting chiefly of cellulose, hemicellulose and lignin. The most abundant constituent, cellulose, exists as a highly crystalline fibril consisting of individual glucan chains arranged in parallel and held firmly by both intra- and inter-molecular hydrogen bonds. This fibril, the microfibril, forms the foundation of the matrix. Coating the microfibril are hemicelluloses, and, in the case of secondary cell walls, this is most commonly xylan. Finally, lignin polymers fill the gaps within this network creating a strong water-resistant wall. Other minor constituents, such as cell wall proteins, make up the small remainder of the secondary wall. The most common analogy for secondary cell wall structure is reinforced concrete or fibre glass, where the cellulose microfibrils confer the tensile strength and lignin provides the matrix that confers rigidity. Although woody cell walls are not normally associated with small weeds, there is abundant evidence to suggest that the process of secondary wall formation in the model plant Arabidopsis is very similar to that of tree species such as poplar. The process of cell differentiation that leads to secondary cell wall deposition is associated with very large changes in transcription as the cell focuses almost exclusively on making cell wall polymers. This has been exploited in both Arabidopsis and poplar, where expression analysis has been used for identifying many candidate genes involved in secondary cell wall formation.

Much of what we know about the function of genes involved in building the secondary cell wall comes from Arabidopsis because it is amenable to live-cell imaging, forward and reverse genetics and biochemical analyses. Secondary walls are found in a number of different cell types. The xylem vessels, specialized for water transport, have been particularly useful for studying secondary cell wall deposition. At maturity, xylem vessels are essentially dead empty tubes. Therefore an essential part of xylem differentiation occurs just before programmed cell death when a patterned secondary wall is deposited. This mechanical strengthening is essential for resisting the large negative pressures that are generated as water is drawn up the plant. Any defects in this process lead to a characteristic phenotype in which the cell collapses inwards as it fails to resist this negative pressure (Figure 1). This phenotype has been useful in identifying ‘irregular xylem’ (rix) secondary cell wall mutants.

In this article, we focus on the biosynthesis of the three major secondary cell wall constituents: cellulose, xylan and lignin. The aim is not only to summarize recent progress in our understanding of their biosynthesis, but also to highlight their relevance to biofuel production and the technical problems that must be overcome before their efficient utilization for biofuels or other kinds of bioprocessing.

Cellulose

The individual glucan chains that make up the cellulose microfibril consist of a linear polymer of glucose residues connected by β1-4 glycosidic bonds. Although still debated, the elementary cellulose microfibril possesses a diameter of around 5 nm and most models suggest that it is composed of 36 glucan chains. Whereas other cell wall constituents are made inside the cell, cellulose is made at the plasma membrane by a large multiprotein complex known as the cellulose synthase complex. Freeze–fracture along the membrane bilayer and electron microscopy shows the cellulose synthase complex...
As a ‘rosette’, a six-lobed structure of 25–30 nm in diameter. One very unusual aspect of cellulose synthesis is the way in which the complex moves through the plane of the plasma membrane. Using cytoplasmic UDP-glucose as a substrate, the rosettes spin out the chains of cellulose resulting in microfibril deposition directly into the cell wall. Once formed, the cellulose microfibrils are believed to represent a rigid structure. The polymerization of glucose into the growing chains provides the energy that drives the movement of the cellulose synthase complex through the membrane as it is pushed against the rigid microfibril. Rosettes are particularly abundant at sites of secondary cell wall deposition, and localization to these sites is known to be dependent upon microtubules that form thick bundles at the cortex\(^3\). It is these microtubules that guide the complex as it moves beneath the secondary walls, but details on precisely how they achieve this are unclear. For example, it is still unclear whether the microtubules form direct attachments to the large cytoplasmic portion of the complex or whether they merely act as guide rails (see Figure 2).

The collapsed xylem of the \textit{irx1}, \textit{irx3} and \textit{irx5} mutants was found to result from severe cellulose deficiencies within the secondary wall. This also caused a large reduction in the mechanical strength of the stem, resulting in plants that have difficulty growing upright and instead readily topple over. \textit{IRX1}, \textit{IRX3} and \textit{IRX5} are all closely related members of the \textit{CesA} (cellulose synthase catalytic subunit) family that are believed to encode the catalytic subunits of the cellulose synthase complex as a ‘rosette’, a six-lobed structure of 25–30 nm in diameter.

Figure 1. Secondary cell wall in the giant grass \textit{Miscanthus} (top) showing the concentration of secondary cell walls around the vascular bundles and the edges of the stem where they form a ‘rind’ that represents the first and formidable line of defence against pests and pathogens. (Bottom) Vascular bundle of stem from \textit{Arabidopsis thaliana} wild-type (bottom left) and \textit{irx3} (bottom right) mutant showing misshapen and collapsed vessels.
The xylan backbone consists of β-1,4 linked xylopyranose residues. Further modifications found in dicotyledons such as Arabidopsis are highlighted in green and consists of a short oligosaccharide found at the non-reducing end of the backbone. An example of a side-chain modification found in some grasses is highlighted in lilac. Note that no reducing-end oligosaccharide has been found in arabinoxylan from grasses.
Despite its simple structure, a lack of basic knowledge about how cellulose is synthesized means few improvements have been made to facilitate its efficient industrial use. Rational engineering of cellulose synthesis to generate either a higher proportion of amorphous cellulose or less crystalline microfibrils is likely to require a much greater understanding of the function of the cellulose synthase complex. Manipulating cellulose biosynthesis in a way that would facilitate its breakdown therefore remains a very big challenge. One potential area for improvement is highlighted by the discovery of a novel enzyme that is able to work on crystalline chitin. In contrast with all cellulases described previously that work by hydrolysis, this chitinase acts as an oxidative enzyme and possesses a very flat active site. The absence of any cleft around the active site allows the enzyme to work on the flat crystalline surface of chitin and a similar enzyme that would work efficiently on cellulose would be a major advance.

**Xylan**

Whereas cellulose consists only of linear chains of glucose, hemicelluloses are used as a broad term to describe the shorter branched polysaccharides such as the pentoses xylose and arabinose. By far the most common hemicellulose in secondary walls of dicotyledons, such as *Arabidopsis*, is glucuronoxylan, often simply termed xylan. Glucuronoxylan consists of a backbone of β-1,4-linked xylopyranose residues that may be substituted with side chains of glucuronic acid or methyl glucuronic acid (Figure 3). Within the last 5 years, several glycosyltransferases involved in xylan biosynthesis have been identified through reverse genetics and screening for further *Arabidopsis* irx mutants. These include enzymes with proposed roles in the processive elongation of the backbone, addition of side chains or the formation of the short oligosaccharide chain that is found at the reducing end of the xylan backbone (Figure 3). While identifying the genes required for xylan biosynthesis represents an important breakthrough, it highlights a continued problem with understanding many aspects of plant cell wall biochemistry, namely the fact that, despite intensive effort, the precise substrates and products of only a handful of glycosyltransferases have been identified. Consequently, it is still largely unclear which enzymes catalyse which reactions and how they work together to synthesise long xylan chains. For example, it is unknown whether the oligosaccharide structure at the reducing end acts as a primer or to terminate the growing xylan chains. Details of how xylans are assembled and interact within the total wall matrix are poorly understood, but it is clear that the resulting hemicellulose network is a formidable barrier to saccharification. Recent studies, however, have highlighted how alterations in xylan biosynthesis can facilitate xylan breakdown and utilization. The identification of enzymes that add the glucuronic and methyl glucuronic side branches on to xylan has been recently identified. Surprisingly, mutating these enzymes results in xylan with a much simpler structure that contains no side branches, with little effect on the growth of the plant. Similarly, members of a family of genes containing a ‘domain of unknown function’ (DUF579) carry out an essential but unknown function in xylan biosynthesis. Mutating these genes results in *Arabidopsis* material with much higher yields during saccharification.

Although *Arabidopsis* represents a good model for xylan biosynthesis in dicotyledons, including trees such as poplar, other species possess a more complex xylan structure. In particular, monocotyledons including potential biofuel crops such as *Miscanthus* and switchgrass, as well as cereals that generate large amounts of straw waste, have xylan that also contains arabinose side chains. Lignin in grass cell walls is rich in hydroxycinnamic acid, particularly ferulates, which forms strong cross-links with xylan via these arabinose side chains (Figure 3). Identification of the enzymes that add these arabinose units to xylan and form the links between ferulates and arabinose is a high priority. Down-regulation of these activities will substantially improve cell wall digestibility.

One of the challenges of viable ethanol production is how to use the large number of pentoses that arise from xylan digestion. Although micro-organisms, including yeasts, are being developed that can efficiently ferment pentoses, one undesirable property of xylan is that it is acetylated, and the acetate released during breakdown is detrimental to the physiology of the fermenting micro-organisms. One area rich for biotechnology is therefore to limit the amount of xylan in the cell wall and to control the degree of acetylation during secondary wall formation.

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**Figure 4.** Monolignol biosynthetic pathway. Lignin is formed mainly from three different monomers called H-, G- and S-units and share a common pathway from phenylalanine to coumaroyl-CoA. Enzyme abbreviations: PAL (phenylalanine–ammonia lyase), 4CL (trans-cinnamate 4-hydroxylase), 4CL (4-coumarate-CoA ligase), HCT (hydroxycinnamyl-CoA:shikimate), C3H (p-coumaryl shikimate), CCoAOMT (caffeyl-CoA-O-methyltransferase), COMT (caffeic acid O-methyltransferase), CAD (cinnamyl alcohol dehydrogenase). Red arrows indicate those enzyme-encoding genes that have been down-regulated and for which saccharification data have been presented. The degree of saccharification is represented by the thickness of the red arrows; for example, HCT down-regulation results in the highest degree of saccharification compared with wild-type control experiments and so has the thickest line.
Secondary walls are impregnated with lignin, an extremely hydrophobic polymer consisting of a range of phenolic subunits derived from monolignols. The biosynthesis of the monolignols p-coumaryl alcohol (yielding H-lignin subunits), coniferyl alcohol (G-lignin subunits) and sinapyl alcohol (S-lignin subunits) is outlined in Figure 4. All three monolignols are ultimately derived from phenylalanine and share a common biosynthetic pathway to p-coumaryl CoA. It is the unpolymerized monolignols that are deposited into the wall. Subsequent polymerization is thought to involve the oxidation of the subunits, possibly by wall-associated peroxidases and/or laccases, creating free radical intermediates that permit coupling. Once lignin is formed, it is very difficult to extract from the structural polysaccharides cellulose and xylan. Lignin therefore represents a formidable barrier to improvements in saccharification. One of the most comprehensive studies on the effects of alteration in lignin biosynthesis on the release of cell wall sugars has been carried out in alfalfa\(^1\). Down-regulation of several of the steps involved in monolignol biosynthesis leads to improved saccharification (Figure 4). This study also highlights one of the major problems with cell wall alterations. Down-regulation of the enzymes that gave the best improvements in saccharification also resulted in plants with lower biomass. The reason for this decrease in biomass is unclear, but it highlights the need to improve digestibility without affecting growth. Manipulating monolignol biosynthesis in plants could also involve increased incorporation of unusual monolignols that are only present normally in very low quantities and some of which are readily removed. Consequently, an alternative strategy to simple alterations in monolignol biosynthesis is to increase the production of these unusual monolignols or to engineer the biosynthesis of entirely novel monolignols. When polymerized into lignin, these novel molecules will generate more labile bonds that could be more easily cleaved to generate a more open structure that would facilitate further breakdown.

### Concluding remarks

Increased focus on using secondary cell walls for biofuels has contributed to an improved understanding of many of the aspects of secondary cell wall biosynthesis. Successfully combining improvements in cellulose, hemicellulose and lignin is likely to result in plants with dramatically improved digestibility. An alternative to making incremental changes to different cell wall polymers is to exploit the way in which some plants are able to make dramatic alterations to their cell wall composition. One example is a type of wood called tension wood that occurs on the upper side of trees that are bent over. The tension wood is composed almost exclusively of polymers is to exploit the way in which some plants are able to make dramatic alterations to

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