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Xylans

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Introduction

The biosynthesis of the plant cell wall is an important area for study as it is the basic resource for all areas that involve dietary fibre and structural and commercial use of wood fibre. The wall is a composite structure and the relative proportions of individual constituents and their associations determine the properties of the particular resource. Unfortunately, there are some major gaps in our knowledge of the biosynthesis and assembly of the plant cell wall but the extent of our knowledge of the role of hemicelluloses, xyloglucan in the primary and xylan in the secondary cell wall and their biosynthesis is improving. The properties of the cell wall have many commercial consequences, including the determinants of fibre quality in paper making. Modern printing technology requires speciality papers with a smooth finish and appropriate tear strength. The non-cellulosic polymers are key components that affect the adherence of the cellulose microfibrils to each other and the compactness and porosity of the paper. They also affect the ability of the pulp to bind additives important in the finish of the paper. The xylan and xyloglucan components of the cell wall are important constituents of these non-cellulosic materials. Other non-cellulosic components such as lignin have been targetted for manipulation with some considerable success (Halpin *et al.*, 1994; Van Doorsselaere *et al.*, 1995, Boudet *et al.*, 1995, 1996) and consequences for the pulping and paper industry are beginning to be evaluated. Attention is now beginning to be directed towards hemicelluloses as a second target for such manipulation in order to improve the resource. To date, it has been necessary to modify the process of paper-making to achieve higher quality papers but this is expensive in terms of waste of unsuitable material, use of energy and reagents and inefficiency in general. Approximately 1.2 mha of forest is grown for pulp in the EU. Taking the UK as an example, pulp imports for use in paper making are in excess of 400 000 tonnes (Hummel, 1988). While considering the fluctuating nature of the price market, even very modest improvements in efficiency would have clear benefit for UK industry, reducing import costs and improving UK competitiveness. The feasibility and possible consequences of the manipulation of xylans to the pulp and paper industry therefore need to be addressed.

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The structure of xylans and association with other cell wall components

Xylans are heterogeneous polymers of the secondary wall, and are the main component of hemicelluloses deposited during differentiation of the xylem (Northcote 1972; Bolwell, 1993). They broadly consist of 1,4-linked β-D-xylp residues, substituted with 4-O-methyl-D-glucuronic acid, D-glucuronic acid, arabinose and acetate. There are at least six types of linkages involved in their biosynthesis. In dicots, the 4-O-methylglucuronic acid side chain is the main substituent attached to C-2 positions and hardwood xylans are acetylated. The acetyl content is variable and is about 10% for beechwood and birchwood, for example, (Biely *et al.*, 1985), but may be as high as 70%. L-araf residues are less abundant and attached to some C-3 positions. Gymnosperm xylans contain less of these arabinosyl units and are not acetylated. In graminaceous xylans, arabinosyl units predominate. The other main secondary wall hemicelluloses, glucomannans are also a feature of gymnosperm walls, while they are of minor occurrence in angiosperms. These polysaccharides consist of 1,4 -linked β-D mannose residues in which a significant proportion (20–50%) are replaced by D-glucosyl units. Only mannose is found contiguously though. The distribution of the sugars is also not regular. Small amounts of D-galactose have been detected and some residues are acetylated.

Studies using immunogold localization showed that xylan, as distinct from xyloglucan, could be detected in primary walls but not cell plates, but was present in largest amounts in secondary thickenings of vascular cells (Northcote *et al.*, 1989). Xylan was also immunodetected in membrane vesicles in differentiating xylem cells in transit to the wall. Ingold *et al.* (1988) also reported that the hemicelluoses that accumulate in the secondary cell walls of cultured mesophyll cells of Zinnia during the differentiation of tracheary elements are xylans. In addition to the chemical content and total levels of xylan present, the associations of xylans with other components of the cell wall also have commerical significance and have been studied in secondary wall. There is evidence that, during the assembly, the synthesis and depositon of xylan is intimately linked with that of cellulose (Taylor and Haigler, 1993). Xylans have also been shown to be linked with secondary wall proteins. The only examples of specific secondary wall proteins localized directly other than inferred from cDNA sequence, have been shown in Loblolly pine (Bao *et al.*, 1992), hypocotyl of French bean (Wojtaszek and Bolwell, 1995) and in differentiating Zinnia cells (Stacey *et al.*, 1996). The French bean protein proved to be hydroxyproline poor, glycosylated, being recognized by wheatgerm agglutinin and localized to tracheary elements, xylary and phloem fibres and can be localiszed in secondary walls induced in bean cultures. Alkaline extracts of hemicellulose showed that this protein could still be detected immunologically indicating a strong association with xylan. The epitope conferring recognition by wheat germ agglutinin is a particular feature of secondary thickenings (Benhamou and Asselin, 1989). The chitotriose motif recognized is highly conserved and found in all vascular plants examined (Bonham V., Burrell M.M. and Bolwell G.P., unpublished data). Many of these secondary wall-specific proteins are lysine rich. A number of these lysine rich proteins have also been recognized from their cognate cDNAs Another epitope found in secondary thickenings of Zinnia cells was recognized by a monoclonal antibody (JIM 13), which is specific for an arabinogalactan protein. There are probably AGPs specifically expressed in xylem (Loopstra and

Sederoff, 1995). As yet, there is no defined function for these proteins, although they may also form structural associations with secondary wall xylan. The deposition of lignin in these walls involves generation of mesomeric phenoxy-radicals from the hydroxy-cinnamyl- alcohol precursors. These will rapidly form linkages with the polysaccharides of the wall and these linkages may take place randomly. Other factors govern whether the lignin encrusting some of the xylan will be of the *p*-hydroxyphenylpropane, guaiacyl- or syringyl-type. However the extent of lignin–xylan associations, however, is of fundamental importance to the pulping and paper making properties of the source material.

The biosynthesis of xylans

The precursors for xylan biosynthesis are UDP-xylose to form the backbone and UDP-glucuronic acid and UDP-arabinose for the side chains (*Figure 1*). The source for these is probably either sucrose or starch. Recently, an intimate association has been demonstrated between sucrose synthase, thought to be the major enzyme in the provision of UDP-glucose from sucrose, with the plasmalemma and with cellulose and callose synthesis (Amor *et al.*, 1995). Immunolocalization demonstrates an up-regulation of sucrose synthase in differentiating tracheary elements of Zinnia (C.H. Haigler,

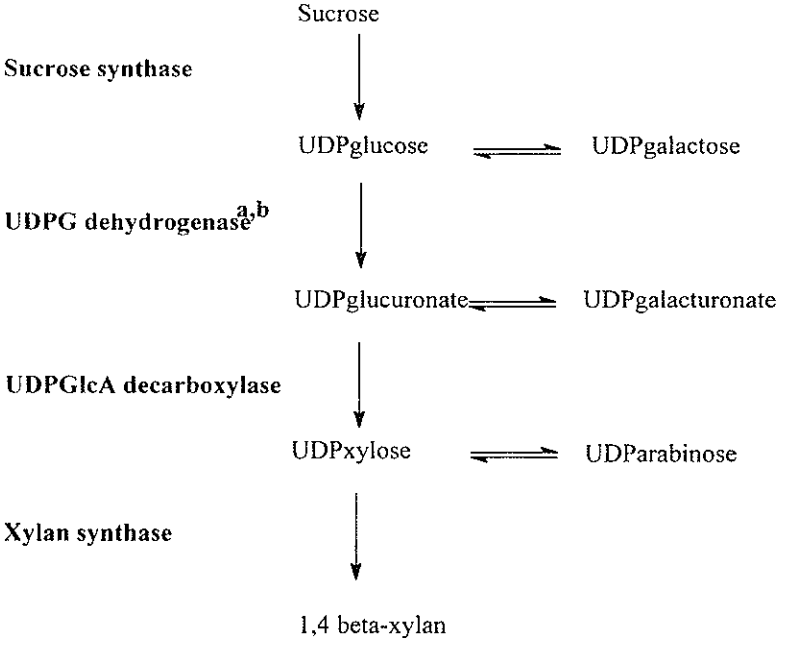

Figure 1. The biosynthetic steps to the xylan backbone. The enzymes involved in the synthesis of the xylan backbone and likely to be targets for genetic manipulation are shown in bold. There are two forms of the dehydrogenase, (a) a high specificity form with homology to mammalian UDPG dehydrogenase and, (b) a low specificity vascular-localized form with homologies to alcohol dehydrogenase. There are also three epimerases that interconvert UDP sugars between those used in pectin synthesis and those used in hemicellulose biosynthesis. All ensymes are cytoplasmic except the xylan synthase which is Golgi localized.

personal communication). In French bean cells, however, sucrose synthase declined in activity and it seems more likely that in these cells starch is a major source for wall polysaccharides (Robertson *et al.*, 1995a). UDP-glucose is converted to UDP-glucuronate by UDP-glucose dehydrogenase. The total detectable enzyme activity in plant tissues may reflect the participation of two enzyme systems. The 50 kDa form, which shows high sequence similarity to mammalian UDP-glucose dehydrogenase (Tenhaken and Thulke, 1996), and has a Km of 0.2 mM is most highly expressed in root tissues and epicotyls of soybean. A less specific 40 kDa form, which is an isoform of alcohol dehydrogenase, has a Km of 5.5 mM and has been isolated from French bean (Robertson *et al.*, 1996). There may also be contributions through the inositol pathway. The less specific UD-glucose dehydrogenase form was immunolocalized to developing xylem and phloem of bean hypocotyl. The product, UDP-glucuronic acid, can readily be converted to UDP-xylose by the action of a decarboxylase. There have been no reports of this enzyme being purified to homogeneity from plants. This is also true of the epimerases which interconvert the UDP-sugars to supply the precursors for both hemicellulose and pectin biosynthesis.

Much of our knowledge of polysaccharide biosynthesis has actually come from the study of wall biosynthesis in plant tissue cultures. While these have been used to study primary wall synthesis and its regulation during cell expansion, a number of systems have been developed to study secondary wall synthesis. The most common method of inducing differentiation-related changes is to increase the ratio of cytokinin to auxin and to raise the level of sucrose in the medium. In comparison with the intact plant, manipulating cell cultures to become xylogenic makes it possible to envisage more easily how different aspects of polysaccharide biosynthesis interact. Not only are there gross changes in the type of cell wall carbohydrate polymers synthesized but these are tightly regulated with respect to the metabolism of the necessary UDP-sugars required. Moreover, this is timed to coincide with the cytoskeletal changes involving microtubules which are required to deposit the newly formed polymers into the forming secondary wall. This is especially so in cells which display highly architectured arrays of secondary thickenings characteristic of xylem vessels and tracheids.

Early radiolabelling studies using autoradiography served to localize wall matrix polysaccharide biosynthesis with the Golgi. Since this early work, subcellular membrane fractionation followed by characterization of enzyme distribution has confirmed this and has recently been further supported by immunogold localization of nascent polysaccharides, but not as yet the enzymes themselves. Immunolocalization has served to define the subcellular localization of matrix polysaccharides in general. Freshour *et al.* have recently confirmed that xyloglucan and rhamnogalacturonan 1 and/or arabinogalactan proteins are among the first components laid down in the newly synthesized wall (Freshour *et al.*, 1996). Immunocytochemical studies using antibodies to secreted polysaccharides with specific sugar epitopes have demonstrated that different polysaccharides are synthesized in different types of Golgi. For example, in sycamore cell suspension cultures, which were high pressure frozen/ freeze substituted, it was seen that assembly of pectic polysaccharides involved *cis*-, *medial*- and *trans*- types of Golgi cisternae, whereas the synthesis of xyloglucan was confined to *trans*-Golgi cisternae and the *trans*-Golgi network (Moore *et al.*, 1991, Zang and Staehelin 1992). There has not been such an extensive study of xylan biosynthesis, but it can be shown to be Golgi-localized and the nascent polysaccharide is secreted into

the wall especially into secondary thickenings (Northcote *et al.* 1989; Bolwell, 1993).

Xylosyl transferases involved in secondary wall xylan synthesis have been described from French bean. Membrane fractions from bean hypocotyl and callus were shown to incorporate xylose from UDP-a-D-xylose into xylan. No lipid or proteinaceous intermediates were found and glycosylations were either stimulated by added dolichyl phosphate nor inhibited by compounds that usually prevent transfers involving polyprenylphosphate intermediates (Bolwell and Northcote, 1983). The French bean xylan synthases could be solubilized and two isoforms were purified. One, an M_r 40000 (XS2) form could be purified to apparent homogeniety. The relative recoveries of the two peaks of xylosyl transferases were dependent on the age at which the bean hypocotyls were harvested. At 8 days, the relative activity of the peak designated XS1 was greater than that of XS2, whereas between 10 and 12 days the relative activities were reversed. This is the period of maximum xylan synthesis, so that the M_r 40000 XS2 is likely to be the secondary wall-specific xylan synthase. It is likely, however, that both these forms were associated with xylan synthesis since the glucan backbone necessary for xyloglucan biosynthesis was not present in the reaction mix. Polysaccharide analysis showed that xylan was present as about 15% of the total hemicellulose at the peak of XS1 activity whereas xylans constituted about 60% of total hemicellulose at the peak of XS2 activity (Rodgers and Bolwell, 1992). Xylan synthase activity has also been examined in differentiated xylem cells of sycamore trees (Dalessandro and Northcote, 1981a). The Km of the synthase for UDP-D-xylose was 0.4 mM. Enzyme activity was not enhanced in the presence of detergent or EDTA, but was stimulated by Mg^{2+} and Mn^{2+}. Increased xylan production during differentiation of xylem cells was exerted by a six-fold increase in xylan synthase activity during the period of maturation of the cells. High levels of xylan synthase can also be detected in differentiating xylem of horse-chestnut (A.C.E. Gregory and G.P. Bolwell, unpublished data) and in flax (N. Carpita, personal communication).

Regulation of xylan and other matrix polysaccharide biosynthesis and turnover

Some general principles for regulation of cell wall polysaccharide biosynthesis are emerging and also apply to xylan biosynthesis. Fluxes in polysaccharide biosynthesis and metabolism can be measured by the flow and accumulation of radioactivity from labelled sugar substrates into specific polysaccharides and cellular compartments. Cell cultures and, in particular, suspension cultures can be manipulated in such a way that they offer an excellent model to study polysaccharide biosynthesis. Alternatively, another approach to gauging the rates of cell wall biosynthesis can be through assaying component enzymes and correlating their specific activities with pathway fluxes in relation to developmental events. In its most sophisticated form, this data can be combined into control analysis, which can be used to identify key regulatory reactions. This has not yet been applied to wall polysaccharide biosynthesis. Even without this rigorous analysis, clues exist as to the type of control mechanisms involved. Cell culture systems that have been particularly well studied for expansion growth are carrot and spinach, while bean, tobacco and most spectacularly, the differentiation of mesophyll cells of *Zinnia elegans* into tracheids have been used to study xylogenesis. These systems can be manipulated experimentally to study control mechanisms

involved in synthesis and modification of hemicelluloses. A more descriptive method has been to take successive scrapings through the cambial, xylem initial and differentiating layers during the spring growth period of a number of tree species such as sycamore and, most productively, Loblolly pine. The latter source has been used to clone the cognate genes of the enzymes of the whole lignification pathway and a number of vascular-specific proteins (Whetton and Sederoff, 1995).

Although it is generally assumed that the major controlling factor in the qualitative production of cell wall polysaccharides resides in the complement of the membrane bound synthases (Bolwell, 1993), the underlying enzymes involved in the supply of UDP-sugars, as substrates for the synthases, may affect the overall balance of cell wall polysaccharide biosynthesis. The enzymes responsible for the observed UDP-glucose dehydrogenase activity constitute, in combination, a key regulatory step since the reaction is thought to be committal as it is irreversible. UDP-glucose dehydrogenase increased in activity which correlated with the xylogenic like changes found in cell walls of induced French bean cells (Robertson *et al.*, 1995a). Correlated with these changes was an increase in the level of UDP-glucuronate decarboxylase. This enzyme also appeared to be under developmental control but to a lesser extent than the dehydrogenase (Robertson *et al.*, 1995a). However, it can influence flux since the product of the reaction, UDP-xylose can exert negative feedback control on the activity of the purified dehydrogenase (Robertson *et al.*, 1996). In sycamore and poplar the activities of the UDP-glucuronate decarboxylase and UDP-glucose dehydrogenase also increased during differentiation and the decarboxylase activity is always higher than that of the dehydrogenase which is probably rate limiting (Dalessandro and Northcote, 1981b). However, the dehydrogenase step can be bypassed using a direct route from glucose via *myo*-inositol to UDP-D-glucuronic acid (Loewus and Loewus, 1983). There is evidence for the coexistence of both pathways with changing importance of either route during plant development (Tenhaken and Thulke 1996). Thus, the decarboxylase also becomes an important control step for the production of UDP-xylose as a substrate for xylan synthesis. The epimerases do not appear to be under regulatory control to the same extent as the other enzymes of UDP-sugar metabolism.

The actual synthesis of cell wall polysaccharides is a Golgi-based process with the exception of the glucans, cellulose and callose, which are synthesized at the plasmalemma. All these glycosyl transferases remain rather poorly described but progress is being made in their characterization (Gibeaut and Carpita, 1994). When cells of French bean are induced to form secondary walls, arabinosyl transferase activity catalysed by an M_r 70,000 Golgi-localized enzyme is reduced indicating a cessation of pectin synthesis (Bolwell and Northcote, 1983 a,b; Rodgers and Bolwell 1992). Similarly, there was a loss of polygalacturonate acid synthase in sycamore cells on differentiation (Bolwell *et al.*, 1985). On the other hand, xylosyl transferase activity involved in xylan synthesis, which is probably catalysed by an M_r 40 000 protein (Rodgers and Bolwell, 1992), is seen to rapidly increase several-fold in cells grown in induction medium (Bolwell and Northcote, 1983 a,b). This increase in xylan synthase activity reflects that seen in differentiating sycamore (Bolwell *et al.*, 1985) and for enzymes responsible for the synthesis of other hemicelluloses such as glucomannan (Dalessandro *et al.*, 1986; Piro *et al.*, 1993). In cultures, the kinetics of appearance of these examples of glycosyl transferase activities correlates to the changes observed

between the induced and non-induced cell walls. The cell walls found in the French bean system which have undergone xylogenic-like changes in composition are also reminiscent of those found in differentiated Zinnia cells (Ingold *et al.*, 1988). Similarly, in the Zinnia system, xylosyl transferase activities have also been correlated to the increase in xylan synthesis (Suzuki *et al.*, 1991). Furthermore, these observations made in French bean cells have also been made in developing bean hypocotyls which would tend to confirm that cell culture systems provide a valid model for xylogenic differentiation (Bolwell and Northcote, 1983b). Other systems such as flax which accumulates high levels of xylan may be more amenable to study of the biosynthesis of these hemicelluloses (N. Carpita, personal communication).

Cellulose synthesis has not been measured directly due to the difficulty in measuring this enzyme activity, but there is known to be increased cellulose deposition in Zinnia (Taylor *et al.*, 1992; Suzuki *et al.*, 1992). Xylan deposition and assembly appears to be coupled to this such that perturbation of cellulose biosynthesis leads to a cessation of xylan deposition. The polymers, which are exocytosed into the extracellular compartment, have to be deposited at defined points along the plasmalemma to give rise to the characteristic architecture of the secondary cell wall during xylogenesis. Use of the Zinnia system in the presence of inhibitors of cellulose biosynthesis suggests that the secreted polymers assemble in a self-perpetuating cascade (Taylor and Haigler, 1993). Normal secondary cell wall thickenings contain cellulose, xylan and lignin as well as specific proteins. When cells were treated with either 2,6 dichlorobenzonitrile or isoxaben at concentrations that inhibit cellulose biosynthesis at the sites of secondary thickening, then xylan and glycine rich proteins could not be detected immunologically. At lower inhibitor concentrations where some cellulose synthesis occurred, xylan and glycine-rich proteins could be detected between thickenings but were not assembled, indicating that a whole population of components were required to allow self-assembly (Taylor and Haigler, 1993). Once the thickenings are established, it is only then that lignin deposition occurs.

In contrast to the extensive study of turnover of primary wall xyloglucan, there is little known about possible turnover of xylans by endogenous plant-derived enzymes. However, an extensive study is commencing to explore the complex changes in the glycan structures of the wall in Zinnia (Stacey *et al.*, 1996). Changes in the secretion and turnover of pectins, xyloglucan and the arabinogalactan epitopes on AGPs, show that a rhamnogalacturonan appears around the time of determination, while a specific AGP appears later and accumulates in secondary thickenings. The fucose-containing epitope on xyloglucan disappears just before the onset of secondary thickening. Such studies may reveal which polysaccharides are targetted for the generation of possible modulatory signals. A fucosidase appears to be one hydrolase that is activated. There may some involvement in the production of oligsaccharin signals as feedback or monitoring signals for the cells as to the stage on the differentiation pathway that has been reached. Indeed, this may be true for xylan. Transformed tobacco cells undergoing tracheid formation have an extractable xylanase present in their cell walls which is absent from control cultures and could be involved in generating fragments for feedback signals during morphogenesis. Additionally, xylanase treatment of plant tissues induces ethylene production (Fuchs *et al.*, 1989), which is a known signal for xylogenesis. Turnover of xylans may prove to be important for developmental regulation.

Influence of xylans on pulping: present processing solutions

The pulp and paper industry today presents itself as a worthy candidate for biotechnological intervention. The environmental and economic costs of the chemical delignification process, and the strict government regulations concerning industrial pollution are putting increasing pressure on pulp and paper industries for renewed innovation. In both kraft and bisulphite pulping processes substantial chemical and energy expenditure is required to convert lignocellulosic material to pulp. This process consumes lots of energy and generates large quantities of byproducts (chlorinated organic compounds) which have adverse impacts on the environment (Welker and Schmitt, 1997, Onysko, 1993, Eriksson, 1993). Tough government regulations regarding AOX (absorbable organic halides) concentration in pulp and waste paper sludges, have led to a shift away from traditional bleaching methods using molecular chlorine. Alternatives to the traditional bleaching methods are elemental chlorine free bleaching (ECF) or total chlorine-free bleaching (TCF) of kraft pulps. The former method substitutes chlorine dioxide for molecular chlorine as bleaching agent, the latter method replaces chlorine entirely by using alternative chemical delignifying agents like oxygen, sodium hypochlorite, ozone and hydrogen peroxide. Benefits have been realized from the reduction in chlorine (Cl_2) usage in the pulping process (Axegard *et al.*, 1992; Buchert *et al.*, 1994). These new bleaching methods, however, have their limitations. Many of the alternative delignifying agents (oxygen, ozone and hydrogen peroxide) used in TCF bleaching process are not very selective, and there are high costs associated with their implementation.

The primary goal of wood processing is to eliminate lignin, which causes pulp discolouration without damaging the polysaccharide component of the wood. The initial stages in the kraft pulping process involve debarking and chipping of wood logs. This is followed by alkaline extraction of lignin using high temperature and pressure conditions. During the alkali extraction phase, part of the xylan as well as lignin is dissolved in the pulping liquor. When the alkali concentration decreases, substantial amounts of xylan as well as lignin are solubilized and they precipitate on to the surface of the cellulose microfibrils forming a chemically resistant lignin–xylan complex (xylans covalently bonded to lignin) on the outer surface of the microfibrils (Iversen and Wannstrom, 1986; Buchert *et al.*, 1996; Visser *et al.*, 1994.; Viikari *et al.*, 1996). Glucomannan does not precipitate together with lignin (Buchert *et al.*, 1996). The formation of the lignin–xylan complex physically restricts the passage of high molecular weight lignin out of the pulp fibre, forming an effective barrier to the extraction of lignin both in soft and hard woods (Wang *et al.*, 1997). Recently, the behaviour of xylan during kraft pulping was studied by radio-trace techniques (Imai *et al.*, 1997). Labelled *myo*-inositol was fed to growing stems of *Magnolia kobus* and allowed to metabolize for a long period. This resulted in the almost selective labelling of xylan in mature cell walls. The labelled wood meal was subjected to kraft pulping and the radioactivities of the black liquor and treated wood meal were determined at various stages of the pulping. The results showed that (i) xylan was dissolved rapidly during the heat-up period and the dissolution was almost completed before the pulping temperature reached 170°C, (ii) 70–80% of the total xylan was dissolved during the pulping and (iii) redeposition of xylan on pulp fibers took place soon after the pulping temperature reached 170°C.

One approach to tackle this problem is biopulping/biobleaching in which biological agents are used in the delignification of lignocelluloses, biobleaching of chemical pulps, and in effluent treatment. Enzymatic treatments using xylanases [endo-1,4-beta-D-xylanase (E.C-3.2.1.8)] from bacterial and fungal origin have been effectively employed to depolymerize the hemicellulose backbone, to enhance delignification (Biely *et al.*, 1992). Partial hydrolysis of insoluble xylan by xylanases (which cleave 1,4-β-xylosidic bonds) renders the pulp more permeable (promotes softening and swelling of fibre structure) facilitating the removal of residual lignin and chemical pulp bleaching (Buchert *et al.*,1995, Wang *et al.*,1997). Xylanases are generally used for the treatment of brown stock pulp or oxygen delignified pulp. The enzyme is supplied as a liquid concentrate and it is mixed with the pulp as it is pumped to brown stock storage (Paice *et al.*, 1995). Owing to high temperatures and alkalinity of the pulp, the enzymes are usually applied after pH adjustment to between 5 and 7, and cooling of the pulp to 40–50°C (Buchert *et al.*,1995; Prasad *et al.*, 1996). A resident time of between 1 and 2 hours is required in the brown stock tower (Paice *et al.*,1995). Although xylanase pre-bleaching is used world-wide, the chemical savings that can be achieved in the subsequent bleaching steps is limited to a ceiling at around 25% (Reid and Paice, 1994). This is not the case using fungal delignification using a variety of special white rot fungi which have been shown to replace 72% of the chemicals needed to bleach kraft pulp (Fujita *et al.*,1991). Ligninolytic fungi which cause white rot of wood produce extracellular oxidative enzymes, the best characterized of which are laccase (polyphenol oxidase), lignin peroxidases (ligninase) (LiP) and manganese peroxidases (MnPs) (Hammel *et al.*,1995). White rot fungi (i.e *Trametes versicolor*) have been used successfully in the pre-treatment of thermomechanical, post-treatment of mechanical pulps and in the biobleaching of kraft pulps (Paice *et al.*,1995). The positive benefits of enzymatic and fungal pre-treatments include reduced operating costs, reduced organopollutants, improved pulp brightness, increased pulp viscosity, reduced Kappa number, and increased fibre yield. Kappa number is a measure of residual lignin content of pulps. Overall, there are significant improvements to paper quality, from the reduced requirement for non-selective bleaching chemicals, milder reactions and better delignification of the pulp (Viikari *et al.*, 1994, Viikari *et al.*, 1996).

Despite obvious advantages to deployment, biological bleaching of kraft pulps has some limitations. The large-scale capacity of the pulp and paper, the costs of scaled-up enzyme production and purification, makes practical utilisation difficult. This is further complicated by sensitivity of enzymes to processing, limited diffusion, and insufficient knowledge of the optimal enzyme concentration and the control of reactions catalysed by these enzymes. Also, certain carbohydrate portions present in lignin carbohydrate complexes appear resistant to xylanase (Watanabe *et al.*, 1996; Wang *et al.*, 1997). Xylanases could soon become a major industrial enzyme handled in the high volumes required by the pulp and paper industry. This prospect has raised concern regarding the allergenic properties of xylanases (Wong and Sadler, 1992). A problem with the fungal delignification is that some white rot fungi have been shown to switch from selective to simultaneous degradation over time (Otjen and Blanchette, 1986). Different strains of the same species can show considerable variation in their delignification abilities (Blanchette *et al.*, 1992).

Genetic engineering of lignification in tree species: a paradigm for xylan manipulation

Opportunities now exist in the field of molecular biology to tackle problems of variability in processing. Recent advances in genetic engineering have offered the pulping industry the potential to go one step further, to make directed improvements to wood quality; by altering wood characteristics, the basic raw material. This has already been achieved through the directed manipulation of lignin biosynthesis (Halpin *et al.*, 1994) which has become a paradigm for manipulation of gene expression in plants. The improved chemical extractability of lignin in cinnamyl alcohol dehydrogenase (CAD) antisense tobacco plants supported a role for the use of this technology in the pulp and paper making process. A reduction in kappa number and a significant reduction in chemical and thermal energy usage during the bleaching process was reported from simulated Kraft pulping experiments carried out by Centre Technique du Papier (Schuch, 1996). These results have lately been confirmed in CAD down-regulated transgenic poplar. Alkaline pulping experiments on three-month-old poplar trees showed a reduction in kappa number (~20%) of the pulp without affecting the degree of cellulose degradation (Baucher *et al.*, 1996). More recently using sense and antisense RNA strategies, the activities of a second lignin specific enzyme, cinnamoyl CoA reductase (CCR, EC;1.2.1.44) have been suppressed in tobacco plants (O'Connell *et al.*, 1997; Piquemal *et al.*, 1997). A reduction in lignin content (45%) and an alteration in lignin composition was observed in the most CCR reduced line (1.2% residual CCR activity). Pilot-scale pulping experiments on transgenic tobacco stems showed a reduction in Kappa number, increased cellulose fibre yield, and improved fibre characteristics (increased fibre length, width, curl, flexibility and reduced coarseness) when compared with the control. Improvements observed in fibre characteristics as predicted lead to marked improvements in paper strength properties (O'Connell *et al.*, 1997). It is clear that the molecular/genetic engineering approach will play a central role in the future in making refinements to the pulp and paper making process. It is now theoretically possible to genetically engineer a tree to produce wood that is good for pulping, structural use and biomass conversion. Genetic transformation of shorter rotation commercial tree species such as poplar is now done routinely and genetic transformation of Eucalyptus and conifers has been achieved using Agrobacterium and biolistic DNA delivery systems (Merkle *et al.*, 1991; Walter, 1994, O'Malley *et al.*, 1996; Machado *et al.*, 1997). Advancements in somatic embryogenesis technology, coupled with the development of automated micropropagation systems, are allowing for the rapid multiplication of high value genotypes (Gupta *et al.*, 1993).

Prospects for cloning genes of xylan biosynthesis

Full understanding of the regulation of hemicellulose biosynthesis requires molecular probes for analysis of transcript levels. Acquisition of cDNAs also allow the potential for genetic modification of important crop plants. They also allow the cloning of the cognate genes and analysis of the promoter structure and regulation. Compared with many other pathways, the identification of genes involved in hemicellulose biosynthesis has been slow. Very few have been cloned. However, the cDNA coding for one of

the possible enzymes controlling the biosynthesis of UDP-glucuronate, the high specificity UDP-glucose dehydrogenase, has recently been serendipitously cloned from soybean by an antibody screening procedure (Tenhaken and Thulke, 1996). The sequence was found to be highly homologous to that from bovine liver, being identical to 61% and homologous to more than 77%. The sequence contains a cofactor binding site for NAD and the various motifs of the enzymes are totally conserved, so a similar three-dimensional structure of the plant dehydrogenase would be expected to be similar to that of the bovine enzyme. Northern blot analysis was used to show that the gene was expressed highly in root tips and lateral roots and to a lesser extent in the epicotyl and expanding leaves. Expression in the main root, hypocotyl and in mature leaves was much lower. From this, Tenhaken and Thulke conclude that UDP-glucose dehydrogenase plays an important role in providing hemicellulose precursors in roots and expanding leaves. The low expression of the gene in other parts of the plant can be explained either by a low demand for UDP-glucuronic acid derived sugars in these relatively well-differentiated cells or by the possibility that the inositol oxidation pathway is utilized in these tissues. Obviously the expression of the genes involved in the alternative pathway needs to be examined to make a direct comparison, and this has been made possible by the cloning of inositol-1-phosphate from tomato (Gillaspy *et al.*, 1995). In the related French bean, the low specificity UDP-glucose dehydrogenase is highly expressed in differentiating xylem of the hypocotyl.

Acquiring protein sequence not only allows searching the Arabidopsis ESTs and accessing cDNA clones through that route, but also the possibility of cloning homologous genes from other species by a PCR approach through design of oligonucleotide primers. Work is ongoing by the authors to obtain sequence information from purified xylan synthase in order to adopt this approach. Other targets are the xylem-specific, low specificity, UDP-glucose dehydrogenase, UDP-glucuronate decarboxylase and possibly UDP-xylose epimerase. Cloning of these will probably not arise in the same serendipitous way as the high specificity UDP-glucose dehydrogenase as there are no microbial or animal homologues in the database to allow identification. Cloning thus demands purification to homogeneity in sufficient amounts to obtain protein sequence which allows design of oligonucleotides of relatively low redundancy. This does not require large amounts, due to modern methods of protein microsequencing. These proteins are at various stages of purification in the authors' laboratory and some sequence has already been obtained. Incidentally, this approach proved successful for the cloning of xyloglucan modifying enzymes. Endoxyloglucan transferase, (EXT) was cloned and the cDNA sequenced (Okazawa *et al.*, 1993) from several plants following its purification from Vigna angularis or bean (Nishitani *et al.*, 1992). In the five plant species, the amino acid sequence of the mature proteins was conserved in the range of 71 to 90% throughout their length. The consensus sequence for N-linked glycosylation and four cysteine residues were conserved in all five species.

Prospects for xylan manipulation

An opportunity now exists to use the genetic engineering approach for the manipulation of xylans in wood. A dilemma arising from the use of xylanases to enhance delignification, is that reprecipitation and binding of hemicellulose to the outer surface of the microfibrils is generally believed to have a positive effect on yields and inter-

fibre bonding in paper making. A reduction in inter-fibre bonding in paper making has been consistently observed after treatment of pulp with xylanases (Viikari *et al.*, 1994, 1996). This problem may be circumvented by the molecular approach. By the mechanism of gene silencing, it is theoretically possible to manipulate hemicellulose biosynthesis, xylan being the most appropriate target, in a directed and controlled fashion. The optimal level of reduction of xylans, which would be beneficial to the pulping process without being detrimental to the tree, can be ascertained. By combining these genetically engineered traits the levels of both lignin and hemicelluloses may be optimized in a given tree. Advances in molecular marker technologies will also allow the genes underlying quantitative traits to be identified and characterized. This will greatly improve the ability of breeders to combine naturally occurring traits so that the tree can be tailored to the requirements of the end user.

An opportunity therefore, exists for understanding the relatively short pathway to matrix polysaccharides and its manipulation. Manipulating cell wall polysaccharide composition has been previously limited to engineering extracellular enzymes. There is a future opportunity to test the feasibility of engineering walls through modification of biosynthetic processes. This would have important general implications in addition to the production of a new valuable resource. The relatively short pathways leading to the pool of UDP-sugars for hemicellulose biosynthesis is an obvious target. However, the formation of UDP-glucuronate from UDP-glucose is a complex step with at least three possible enzyme systems. A high specificity and specific UDPglucose dehydrogenase (Tenhaken and Thulke, 1996), a low specificity, high Km but vascular-specific dehydrogenase (Robertson *et al.*, 1996) and the inositol pathway (Loewus and Loewus 1983). The decarboxylase and UDP-xylose epimerase steps are likely to be less complex enzymatically. Down-regulating these steps by antisense may lead to less hemicellulose in the walls without being detrimental to plant. This is an approach that may lead to improved cellulose extraction. A more direct way to manipulate hemicellulose biosynthesis would be to target the polysaccharide synthases themselves. The chances of manipulating the actual polysaccharide synthases entirely rests on the cloning of the cognate genes which has not been achieved as yet.

Nevertheless, the technology has been applied with success to engineering lignin for improved cellulose extraction. In comparison with this work carried out on reducing lignification, tobacco is the model plant of choice for the development of the technology and studying of the effects of manipulation on fibre quality in the first instance, due to faster production of experimental material. The likely tree species to be manipulated first would be Eucalyptus as a tropical species and poplar as a temperate species. In these, xylan would be the most desirable target. In Gymnosperms, the equivalent hemicellulose target would be glucomannan which although it does not pose the same re-precipitation problems as xylan may still influence cellulose extraction. The feasibilty of manipulating glucomannan biosynthesis in loblolly pine is at present unknown. GDP-mannose is produced by a separate pathway and the extent of the characterization of the biosynthetic system would appear to be more limited than xyloglucan or xylan biosynthesis. Both partial sense and antisense could be used to generate xylose deficient wall polysaccharide plants. Manipulating the dehydrogenase and decarboxylase step should lower the pool of UDP-xylose and affect xyloglucan and xylan levels. Expression of the dehydrogenase is a likely factor in overall control of flux, so antisense may down-regulate matrix polysaccharides, in

general, while relatively increasing cellulose. Antisense manipulation of xylan synthase should affect xylan content only. If the cDNAs are derived from sources other than tobacco initially, they can still be tested since heterologous antisense manipulation has been carried out with success on the phenylpropanoid pathway in lignification by a number of groups. The availablity of various antibody or cDNA probes would allow verification of down-regulation. Transformants with reduced lignification have also been tested with success with paper companies. Incidentally, one study involving down-regulation of a particular step in the lignification pathway also affected hemicellulose levels. The effect of O-methyl transferase (OMT) cDNA modulation on cell wall composition and ultrastructure was recently analysed using antisense technology (Vailhe *et al.*, 1996). Antisense cDNA expression inhibited OMT activity by 92%, whereas sense constructs led to either 98% inhibition or overexpression of OMT activity. OMT depleted stems showed decreased hemicellulose content, but unchanged lignin content. This suggests that altering the hemicellulose content transgenically is feasible and a worthwhile goal to pursue. A number of companies are conducting field trials with transgenic Poplar, Eucalyptus and Loblolly pine. The feasibility of all this still requires absolute identification of the cognate genes of hemicellulose biosynthesis, but the rewards to generate new resources is evident. There is much to be learnt about the consequences of manipulating one component of the wall on the synthesis of other components. However, this is empirical science which could yield new material with high impact upon economic and environmental factors.

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