ISSN 0717-3644
ISSN online 0718-221X

THE WOOD CELL WALL AT THE ULTRASTRUCTURAL SCALE – FORMATION AND TOPOCHEMICAL ORGANIZATION

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In memoriam of Dr. Walter G. KAUMAN

ABSTRACT

The macromolecular organization of the secondary wall of the cells from tree xylem is in large part responsible for the mechanical and physiological properties of wood. Modeling secondary walls of wood is difficult because information about their macromolecular architecture at the ultrastructural scale is missing. Numerous microscopic studies have provided views of the lignocellulosic composite material, but nanoscale distribution of the polymers and their interaction in muro is still not clearly understood. The intimate macromolecular organization of cell walls is defined during their differentiation. It is at the stage of wall thickening corresponding to secondary wall development that the topochemical organization and the interactions between cellulose, hemicelluloses and lignin are established.

Using the conjunction of the high resolution of transmission electron microscopy (TEM) and the specificity of immunological probes directed against the main cell wall polymers, we investigated the deposition of hemicelluloses and lignins from the early stage of cambium differentiation to the mature fiber and vessel walls in growing model plants of *Arabidopsis thaliana* and poplar. TEM examination of differentiating cells as well as various wood and wood –derived materials and genetic plant mutants brought multiple evidence of the lamellar sub-organization of the secondary walls. Immuno-gold labeling showed that two structurally different xylan types were deposited at different stages in the wall thickening. Similarly two different types of lignin molecules were shown to be differentially polymerized at different steps of the building of the wall, lignin molecules of the condensed type being first deposited at the earliest stage of secondary thickening before the non-condensed types. This process may be modified in response to environmental factors, as in tension wood.

The spatio-temporal relationships occurring between hemicelluloses, lignin and cellulose microfibrils (CMFs) during the secondary wall development suggest that xylans with less substituted chains would be more directly interacting with CMFs than those with higher substitution patterns. It also suggests that lignin molecules of the non-condensed type have a function in bringing cohesion between the lamellae of CMFs. A model of wall assembly during secondary thickening is proposed.

Keywords: Secondary plant cell wall; Xylan; Lignin; Immunolabelling; Assembly

INTRODUCTION

The secondary walls of woody plants are typically described as composite materials consisting of tight mixtures of polysaccharides, glycoproteins and polyphenols, organized around the crystalline and amorphous cellulose microfibril framework, that are deposited after cessation of primary wall expansion. Lignocellulosic secondary walls provide supporting structures and water conduction to the living plants and mechanical properties to wood materials. Like all living systems, the plant cell wall assembly is the result of synergistically and spatio-temporally controlled biosynthesis networks interacting to give rise

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Received: December 01, 2005. Accepted: April 13, 2006.

to supramolecular structures which are more complex than the simple summation of their elementary components. Such a complexity is the result of the action of the hundreds of genes that are implicated in the plant cell wall biogenesis (Somerville et al. 2004; Asperborg et al. 2005), the precise function of most of them remaining unassigned. Also, the precise localisation and function of each of the cell wall components is still not fully understood.

Describing the plant cell wall is complicated by the fact that plants have diversity of cells, each having its own function, with its own type of cell wall adapted to the function in the tissue. Primary walls show less compositional and organisational diversities according to cell specificity and have been globally classified in two types. To the difference of primary walls, variability is mostly important in the secondary walls whose various functions in the mature tissues are reflected in large variations of their composition between the different cell types (Brown et al. 2005). This is illustrated with the thick lignified secondary cell walls which constitute the major part of the xylem and of plant biomass. In this composite, cellulose represents 40 to 50%, and due to its supramolecular microfibril architecture, it endows the thick secondary wall with precise and specific anisotrope organization. Hemicelluloses and lignin are deposited around the cellulose microfibrils to form matrix substances. The interaction between cellulose and hemicelluloses has been largely investigated (Atalla et al. 1993; Tokoh et al. 2002a,b; Akherholm and Salmen 2001). Most of the data on the assembly of the cellulose/hemicelluloses networks were essentially obtained in vitro. It appears that pectic polysaccharides, glucomannans and glucuronoxylans differentially influence the assembly of cellulose microfibrils, altering cellulose ribbon formation and affecting the crystal structure (Tokoh et al. 2002b). Therefore, the concept of cellulose microfibril (CMF) may better be regarded as corresponding to cellulose coated with hemicelluloses (Albersheim 1975). Hemicelluloses not only affect cellulose structure but also are intermediates for CMFs crosslinking via polyphenol and lignin (Fry 1986; Newman 1992; Iiyama 1994), bringing cohesion to the growing secondary wall (Anterola and Lewis, 2002; Ha et al. 2002).

The influence of hemicelluloses on the type of lignin deposited in the vicinity of CMFs and the tight links that associate lignin and hemicelluloses are such that the relative distributions in the wood secondary wall follow parallel patterns. This was early demonstrated at the ultrastructural scale by transmission electron microscopy (TEM) and scanning TEM (STEM) (Ruel et al. 1978; Ruel et al. 1979), thus confirming the most accepted model from Kerr and Goring (1975). Recent production of lignin–specific antibodies allows one to visualise the topochemical distribution of lignin epitopes at the ultrastructural level relative to CMFs in wood and in growing wood cell walls (Joseleau and Ruel 1997; Burlat et al. 2000). It thus appears that due to the relationship between hemicelluloses and lignin, visualisation of one provides information about the localisation of the other. In this presentation lignin topochemical distribution in various wood and wood fibres is considered relative to secondary wall biogenesis.

Materials and Methods

Cytochemical staining for polysaccharides in electron microscopy

The polysaccharides from the walls were contrasted on ultra-thin sections by the periodic acid-thiocarbohydrazide-silver proteinate (PATAg) method (Thiery, 1967) modified for secondary walls by Ruel et al. (1999). Briefly, the periodate oxidation was carried out with a 5% solution of periodic acid in water for 90 min (instead of with a 1% solution for 30 min), thiocarbohydrazide was 48 h and silver proteinate 30 min, in the dark.

Preparation of antisera

The xylan specific antiserum was prepared from an arabinoxylan from esparto grass in which the arbinofuranosyl side chains were stripped off by selective hydrolysis with oxalic acid. The unsubstituted structure of the xylan was checked by methylation analysis and 13C nmr spectroscopy.

Lignin specific antisera were prepared from various synthetic lignin polymers (DHPs) as described in Ruel et al. (1994) and Joseleau and Ruel (1997). New Zealand rabbits were immunized by injection intradermically of a mixture of the antigens of interest (ca 1 mg) in phosphate buffer saline (PBS) with Freund's complete adjuvant. Booster injections were done with the antigen diluted in complete Freund's adjuvant every 2 weeks. One week after the last injection, blood was collected then centrifuged.

Dot-blot Immunoassays

All the immunoassays were carried out in semi-micro disposable cuvettes of 0.5 ml, in order to reduce the amounts of antiserum (Ruel et al., 2004). Dots of the antigen and of the substrates to be tested were deposited (1µl) at varying concentrations on strips of positively charged nylon membrane (Boehringer; 8 x 50mm). The strips were soaked in PBS then blocked in PBS-non-fat dry milk (5% v/v). Incubation at various dilutions in PBS-milk (3%) was carried out at room temperature 3h then overnight at 4ºC. The membrane strips were rinsed in PBS and incubated with Protein A diluted in PBS-0.1% fish gelatin (pA-G20, BioCell; 5h, room temperature). After rinsing in PBS, then water, the strips were fixed in 2% glutaraldehyde and rinsed in water before silver enhancement (SEK 250 BioCell).

Immunocytochemistry

Two specific polyclonal antibodies against lignins were prepared and characterised as described in Joseleau and Ruel, (1997). They were directed respectively against: mixed guaiacyl/syringyl (GS) lignin polymer rich in non-condensed inter-unit linkages (8-O-4) (defined as predominantly C-O-C linkages of the aryl-alkyl type) and guaiacyl lignin polymer enriched in condensed inter-units (defined as predominantly C-C linkages) (Higuchi, 1990; Tanahashi and Higuchi, 1990; Joseleau and Ruel, 1997).

All comparative immunolabelling experiments were carried out in parallel in order to keep the same experimental conditions (dilutions of antibodies, times of contact etcÉ).

Immuno-gold labelling in TEM was done on ultra-thin transverse sections (50 nm) floating downward in plastic rings passed on 50 ml drops of reagents deposited on parafilm. The sections were first treated with 0.15M glycine in Tris-HCL buffer 0.01M, pH 7.6, containing 500 mM NaCl, for blocking remaining aldehyde functions. This was followed by 2 min rinse (x5) on TBS500. Protein-protein interactions were blocked for 30 min by incubating on 5% (w/v) non-fat dried milk in TBS500. The sections were then incubated on each antiserum diluted 1/50-1/100 in the blocking buffer. Incubation time was 3h at room temperature followed by one night at 4ºC. After 4 washes (2 min each) in TBS500 followed by 3 rinses in Tris-HCl buffer (0.01 M Tris-HCl, pH 7.4-7.6), the sections were floated on the secondary marker [protein A-gold (pA 5), (BioCell)] diluted 1/25 in Tris-HCl buffer containing 0.2% fish gelatin for 90 min at room temperature. They were washed 5 times (2 min each) in Tris-HCl buffer and 3 times in H2O. The sections were then post-fixed in 2.5% glutaraldehyde in H2O and washed 3 times in H2O. At this stage, the diameter of the 5nm gold particles was further enhanced using a silver enhancing kit from Amersham. Finally, thin sections were transferred on carbon-coated copper grids and post-stained in 2.5% aqueous uranyl acetate. Observations were performed at 80 kV with a Philips CM 200-Cryo.

RESULTS AND DISCUSSION

In order to investigate wood cell wall development, the secondary wall thickening of fibres was visualised in actively growing young poplar trees. Transmission electron microscopy showed characteristic steps in the assembly of the secondary wall during the formation of the S2 layer of fibres. In parallel, xylans and lignin depositions were visualised by immuno-gold labelling.

Preparation of immunological probes for xylans and lignins

A difficulty in choosing appropriate probes for the visualization of matrix polymers is their

polydiversity and polymolecularity. This is true for hemicelluloses which vary in the nature and frequency of the substitution patterns a, as well as for lignins whose variability concerns the monolignol composition and the nature of the inter-unit linkages. In both cases the consequences on the conformation of the polymers and on their capacity of association among themselves and with cellulose may be important. Taking into account the structural polydiversity of xylans due to the side chains substituting the xylopyranosyl backbone, an antibody raised against the β-O-4-linked xylopyranosyl backbone common to all xylans from land plants was prepared (Joseleau et al., unpublished data).

The biosynthesis of lignin involving three monolignols and their polymerisation by radical coupling catalysed by wall associated peroxidases, gives rise to several monomer combinations. Therefore, in order to localise in planta the major epitopes of lignins at the resolution of electron microscopy, we produced antibodies directed against various structural motives found in condensed and non-condensed lignin types (Ruel et al. 1994; Joseleau and Ruel, 1997; Ruel 2003). These immunological probes proved their specificity and selectivity in demonstrating that the sub-layering disclosed in the secondary walls of hardwood vessels by chemical staining with KMnO4 actually corresponded to variations in lignin structures. They also demonstrated differences in the patterns of the distribution of lignins between fibres and vessels.

Lamellar organization of the secondary wall

In the developing xylem of young poplar stems the investigation of the microstructure in the nascent secondary wall showed that at the very early stage of formation of the S2 layer, CMFs were first laid down as single individualized microfibrils. Interestingly at this early stage of S2 formation the loose microfibrils showed an orientation towards the lumen, contrary to the general high degree of parallelism observed in the mature wall (Fig. 1a). As the thickening of S2 progresses inwards, the first deposited CMFs aggregate together and gain a parallel arrangement whereas the newly synthesized CMFs are still unorganised and loose (Fig 1b). In this process the aggregated CMFs form lamellae that, in the later stages of S2 formation, associate into a coherent secondary wall in such a way that lamellae are no longer visible in the mature secondary wall (Fig. 1c).

Indirect demonstration of the lamellar fundamental organization of the wood cell walls was evidenced by the release of lamellae under the action of enzymes on mechanical pulp fibres (Sigoillot et al., 1997). Removal of a part of the xylans with a xylanase resulted in separation of lamellae within the secondary wall. A similar result was obtained when the lignin-specific manganese peroxidase was used (data not included). These results clearly confirm that lamellae constitute a basic entity in the secondary wall architecture, and they also demonstrate the role of both a certain type of xylan and a certain type of lignin structure play in the cohesion of the lingo-cellulosic complex that maintain CMF lamellae in a coherent association.

Similarly, the mechanical effects of refining treatments results in the release of lamellae about 100 to 200 nm in thickness. This shows that the junction between lamellae remains a weak point in the assembly of the secondary wall.

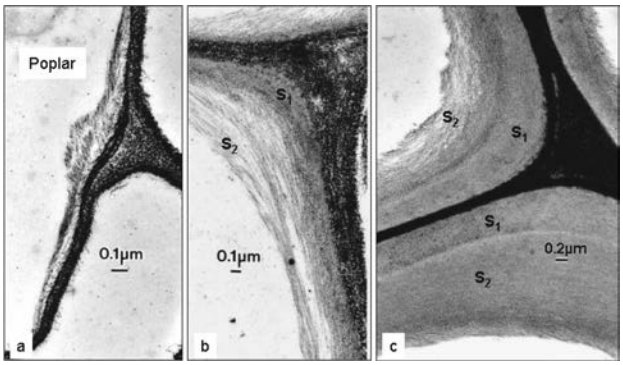

Figure 1*:* Assembly of fibre wall in developing secondary wall of poplar. a: early stage of deposition of CMFs in S1; b: cellulose microfibrils and lamella of microfibrils are visible in internal S2; c: upper part, fibre with incomplete and loose S2; bottom part, mature fibre with compact S2.

Probing xylan distribution in developing secondary wall by immunolabelling

Various approaches have been implemented to understand of the ultrastructural role of hemicelluloses in wood secondary walls. Specific removal of xylans by endo-xylanases and visualisation of the residual CMF skeleton showed that a first consequence of xylan removal was the release of lamellae of microfibrils (Ruel et al. 1976; 1977). In another approach, the specificity of enzymes complexed to colloidal gold was used to probe hemicelluloses (Vian et al. 1983; Ruel and Joseleau 1984; Joseleau et al. 1992). Immuno-cytochemical labelling constitutes a more precise way to visualise hemicelluloses in relation with the variations in their primary structures (Reis et al. 1994). We recently prepared a polyclonal antibody (antiX-l) with specificity directed against the linear β -1,4-xylopyranosyl backbone of xylans (Ruel et al., unpublished). Its used on sections of developing poplar xylem showed that unsubstituted xylans chains were early deposited in the incipient secondary wall, and predominantly distributed in the S1 and S3 layers of the secondary wall of mature fibres and vessels (Fig.2b). Such patterns of distribution provided with our antibody are different from the even distribution of xylans given by the anti-glucuronoxylan antibody applied on Japanese beech wood (Awano et al. 1998) which depicts the distribution of 4-Omethyl-substituted xylans. Altogether our results of immunolabelling show that xylans were unequally accessible to a single type of polyclonal antibody. They also showed that xylans are not deposited at the same stages during wood cell wall thickening and consequently they are not homogeneously distributed in wood secondary walls.

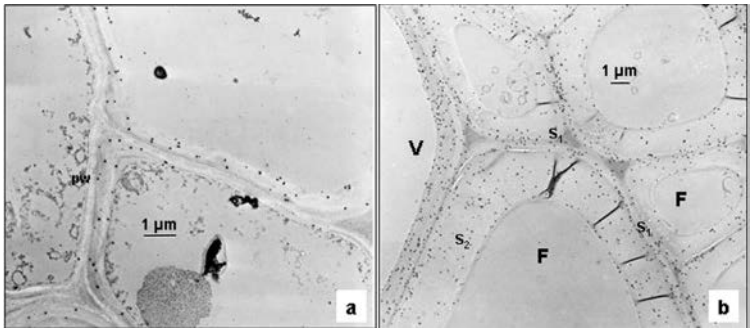

Figure 2: *Immuno-gold labelling of xylans in developing poplar stems. a: At the onset of secondary thickening xylans with unsubstituted backbone are deposited within the loose framework of the incipient S1 layer. Note that the primary wall (PW) is almost void of gold particles. b: mature fibres (F) in which S2 is less intensely labelled. Xylans appear more homogeneously distributed in S1, S2 and S3 layers of the vessel (V).*

Topochemical variations of their distribution appear related to variations in their primary structures. The difference of orientation of CMFs in S1 and S2 may also be a factor that could influence their respective immunolabelling. In any case the results show that the interrelation between xylans and the other wall polymers is of two kinds, whether they directly bind cellulose microfibrils or whether they cross-link lamellae of microfibrils. This view is consistent with the idea that xylans deposition occurs in two distinct phases during secondary wall assembly (Awano et al. 2002).

Probing lignin deposition by Immunolabelling

The use of antibodies with specificities directed against condensed guaiacyl lignin and non-condensed guaiacyl-syringyl lignins, respectively, revealed a higher labelling of condensed epitopes in the loose CMFs areas with virtually no labelling of non-condensed epitopes (Fig.3a,b). On the other hand non-condensed substructures appeared to predominate in the more compact and CMF-ordered zones of S1 and outer S2 (Fig 3c,d). Finally, when secondary thickening is completed, in a mature fibre secondary wall, both lignin types can be seen throughout the S2 layer.

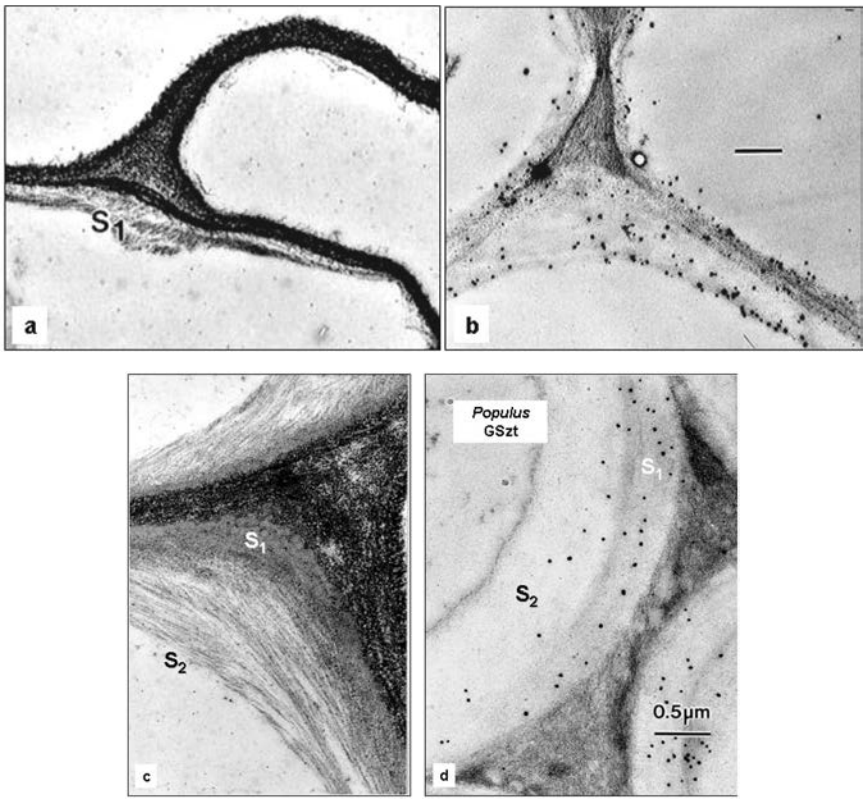

Figure 3: *Immuno-gold labelling of lignin epitopes in developing secondary walls of poplar. In (b) condensed lignin epitopes are concentrated in the loose texture of the last deposited inner S2, as illustrated in control (a). In (d) non-condensed epitopes are concentrated in the more compact areas of S1 and outer S2, see control in (c).*

Genetically modified plants are useful tools for understanding the cell wall assembly

Antisense down-regulation of genes involved in the biosynthesis pathways of cell wall polymers can induce dramatic changes in the cell wall architecture. A particularly interesting alteration in wood

cell walls was observed in cinnamoyl-CoA-reductase (CCR) down-regulated plants (Boudet 1998; Ruel et al. 2001). The transformed plants not only had a 50% reduction in total lignin content, but were also characterized by less non-condensed units than the normal plants. At the ultrastructural level the fibres and vessels of the modified plants were characterized by an inability to complete the assembly of their secondary wall (Chabannes et al. 2001; Ruel et al. 2002). This resulted in loose CMFs lamellae that were in the incapability to associate together to yield a coherent S2 layer. In the microscopic phenotype the zone of loose CMFs in the inner part of the wall coincided with the absence of non-condensed epitopes as evidenced by immunolabelling (Fig.4).

This observation further underscores the mutual influence that exists between CMFs and lignin for the development of a normal cell wall structure as was already observed with *Arabidopsis thaliana* mutants exhibiting collapsed xylem phenotype (Turner and Somerville 1997).

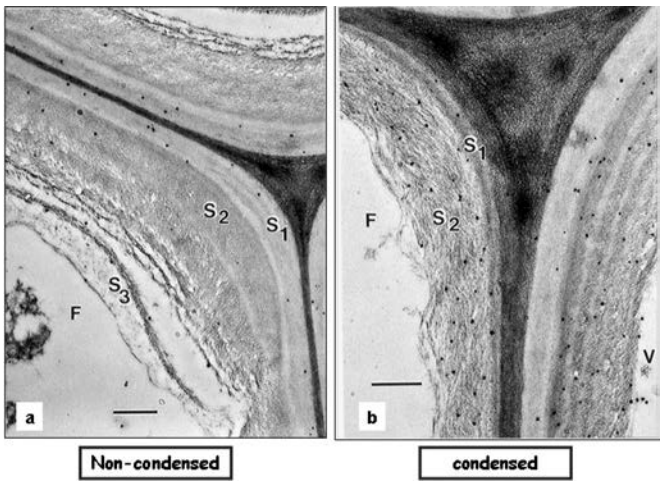

Figure 4: Impact of genetic modification on CCR down-regulation results in a characteristic secondary wall phenotype consisting of an incompletely assembled S2 layer. As in maturing cell walls, lignin condensed epitopes are associated with the open areas whereas non-condensed epitopes are more abundant in the more compact areas. Bar: 0.5µm.

Proposed dynamic model for the assembly of the lignified secondary wall

Examination of the mode of deposition of xylans and lignins in the differentiating walls of poplar showed the high degree of mutual relations between hemicelluloses and lignin during secondary wall assembly. The respective molecular diversity of xylans and of lignins corresponds to specific localisations in the cell wall and specific functions in the final secondary wall cohesion. The results suggest that lignin deposition during secondary wall thickening is subjected to a spatio-temporal control that affects the type of lignin. Since lignin types are largely defined by the mode of polymerisation of monolignols, it may be deduced that the loose environment constituted by the newly synthesized CMFs induces a mode of polymerisation giving rise to condensed linkages, in contrast to the more ordered and tighter space in the lamellae that would favour the endwise polymerisation leading to non-condensed type of lignin (Roussel and Lim 1995). Interestingly the first deposited condensed lignin is more likely to interact with the hemicelluloses coating the CMF surface. These could be the site of lignin carbohydrate complexes and therefore serve as anchors for the polymerisation of lignin. Lignin–hemicellulose covalent attachment constitutes a fundamental element of secondary wall assembly necessary for the lamellar organization of CMFs. This was evidence with the irx3 mutant of *A. thaliana* in which the glucuronoxylan – lignin complex remained in spite of the absence of cellulose scaffold (Ha et al. 2002). On the other hand, our results suggest that as the space between the loose microfibrils becomes narrower because of

the first deposited condensed lignin, these conditions favour the polymerisation of non-condensed lignin. The latter may interact with the already present condensed type of lignin and act as cross-linking molecules. The preceding sequence of events is summarized in the proposed schematic representation in Fig 5). Such associations between the two types of lignins can be established via hydrophobic bonds between the aromatic rings, with the consequence that as secondary thickening and lignification proceed, water is expelled and the hydrophobicity of the wall is increased.

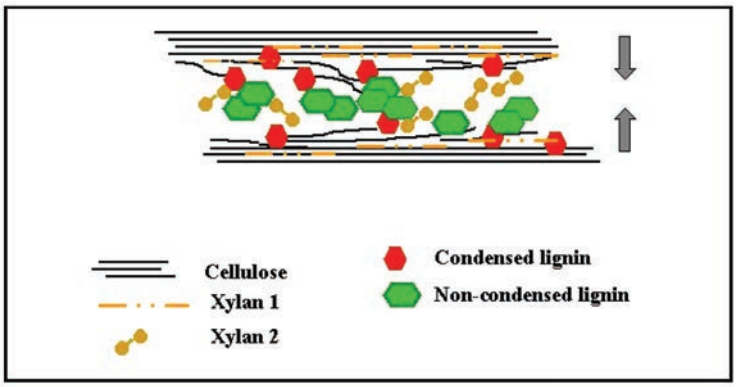

Figure 5: *Proposed schematic representation of polymer assembly during the secondary wall formation.*

CONCLUSIONS

Our view of secondary wall formation is consistent with Atalla and Agarwal's results (1985) suggesting the preferential alignment of aromatic rings parallel to CMFs, and supported by molecular dynamic simulations showing that β-O-4 guaiacyl chains were collinear with hemicellulose and cellulose microfibrils (Faulon and Hatcher 1994).

Taken together such a succession of coordinated events leading to chemical and physical links between lignin, hemicellulose and cellulose microfibrils, to build a consolidated secondary wall is likely to be of the types of self-assembly in natural system discussed by Philp and Stoddart (1996).

ACKNOWLEDGEMENTS

Some of the results were acquired in the framework of European program AIR QLK 5-2000-1493.

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