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Differential localization of arabinan and galactan side chains of rhamnogalacturonan 1 in cambial derivatives

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Abstract. The development of pectin structural features during the differentiation of cambial derivatives was investigated in aspen (*Populus tremula* L. × *P. tremuloides* Michx.) using biochemical and immunocytochemical methods. Comparisons were also made between active and resting tissues. Active tissues, in particular cambial cells and phloem derivatives, were characterized by a high pectin content. Use of antibodies raised against arabinan side chains of rhamnogalacturonan 1 (LM6), as well as biochemical analysis, revealed an obvious decrease from the cortex to the differentiating xylem. Galactan side chains, detected with LM5 antibodies, were present mainly in the cambial zone and enlarging xylem cells. In contrast, they were totally absent from sieve-tube cell walls. Image analysis of LM5 immunogold labelling in the cambial zone showed a clustered distribution of galactan epitopes in the radial walls, a distribution which might result from the association of two different periodic processes, namely the exocytosis of galactan and wall expansion. Cessation of cambial activity was characterized by cell wall thickening accompanied by a sharp decrease in the relative amount of pectin and a lowering of the degree of methylesterification. The data provide evidence that the walls of phloem and xylem cells differ in their pectin composition even at a very early stage of commitment. These differences offer useful tools for identifying the initial cells among their immediate neighbours.

Key words: Cambium – Differentiation (cambial derivatives) – Pectin (immunolocalization) – *Populus* (pectins)

Abbreviations and definitions: FITC = fluorescein isothiocyanate; LM5 = antibody against β-(1,4)-galactosyl residues of RG1; LM6 = antibody against α-(1,4)-L-arabinan; PATAg = periodic acid-thiocarbohydrazide-silver proteinate; RG1 = rhamnogalacturonan 1

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Introduction

Despite the fundamental role of the cambium in wood formation, many questions about the biology of this meristem remain unanswered. One of the most important questions concerns the lack of suitable molecular markers to discriminate between the cambial cells and their issue which differentiate into either phloem or xylem.

It is known that cell wall thickness increases faster on the phloem side than on the xylem side, indicating early differences in the deposition of cell wall polymers. These differences have been confirmed by cytochemical observations performed on several tree species after sequential extraction of polysaccharides (Catesson 1994). A treatment solubilizing pectin polymers not engaged in Ca^{2+} crosslinking (boiling water, pH 5), extracted most of the cell wall material from the first xylem derivatives but very little from phloem derivatives (see Catesson 1994). However, the solubilization of pectic material often disturbed the wall structure, the remaining walls being swollen and distorted, which masked the possible real location of the pectin polymers. In contrast, antibodies raised against specific carbohydrate epitopes provide useful information on the organization of cell surfaces (Knox 1997). However, these tools have rarely been used on tree material. The antibodies JIM 5 and JIM 7 which recognize poorly and highly methylated homogalacturonan, respectively, have been used on the cambial zones of *Aesculus hippocastanum* (Chaffey et al. 1997), *Populus x euramericana* (Guglielmino et al. 1997), and *Populus tremula x tremuloides* (Follet-Gueye et al. 2000). Thus, homogalacturonan was shown to be more or less similarly localized in cells engaged either in the phloem or in the xylem pathway. Both antibodies stained the cell walls of phloem- and xylem-derivatives, the only difference being the precise wall areas associated with each antibody. In phloem derivatives, acidic pectins were present in the primary wall whereas they were restricted to cell junctions and the middle lamella on the xylem side.

The other constituent of the pectin material, i.e. the so-called rhamnogalacturonan 1 (RG1), has been examined using new immunological tools, LM5 and LM6: LM5 is known to recognize only the β-(1,4)-galactosyl residues present in RG1 side chains (Jones et al. 1997; Vicré et al. 1998; Willats and Knox 1999) and LM6 reacts specifically with α-(1,5)-L-arabinan (Willats et al. 1998). Immunological observations were complemented with biochemical analysis of carbohydrates extracted from different tissue fractions scraped from young hybrid aspen stem cuttings. This species represents a good material for investigating cambial functioning since it grows rapidly and can be easily transformed (Nilsson et al. 1997).

The aim of these studies was to obtain precise information on the changes occurring in the cell wall components before, during and after the irreversible orientation of the cambial cells towards phloem or xylem, and also to understand how these cells develop at the cessation of growth. The first biochemical and cytochemical data representative for one seasonal cycle are reported below.

Materials and methods

Plant material. Samples were collected from 1.5- to 3-m tall hybrid aspens (*Populus tremula* L. × *P. tremuloides* Michx.) grown at the Swedish University of Agricultural Sciences (Umea) as described by Nilsson et al. (1997). Different tissue fractions were scraped as illustrated in Fig. 1 and then frozen in liquid nitrogen and stored at −80 °C.

Isolation of cell wall fragments. Frozen tissues were ground in a mortar in liquid nitrogen, the powder was then suspended in 5 mM Mes-cysteine buffer (pH 6.0) and filtered after 15 min through cheesecloth. The next steps were carried out according to Goldberg et al. (1986). The cell wall fragments were suspended in water. These cell walls were intended for studies on both cell wall pectin methylesterases (PMEs) and pectins. The PMEs were first extracted with 1 M NaCl in 5 mM Mes-cysteine buffer (pH 6.0) and the cell walls further used for pectin analysis.

Pectin analysis. Pectins were extracted with boiling water as described in Goldberg et al. (1994) and the extracts lyophilized. Powders were suspended in water for subsequent analysis. Uronic acids were estimated according to Blumenkrantz and Asboe-Hansen (1973) using galacturonic acid as standard, and total carbohydrates according to Dubois et al. (1956) with galactose as standard. For sugar analysis, the samples were methanolyzed, methylsilylated, and fractionated by gas-liquid chromatography as previously described (Morvan et al. 1991). Degrees of methylation (DM) and acetylation (DA) were estimated according to Voragen et al. (1986).

Preparation of specimens for microscopy. Small pieces of tissues including the cambial zone and the youngest phloem and xylem layers were fixed either in 4% glutaraldehyde or in a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 2 h. Specimens were then washed, dehydrated, and embedded in LR White resin (London Resin Co., London, UK).

Immunolabelling of pectin epitopes. The procedure followed the same steps as previously described for galacturonan immunolocalization (Guglielmino et al. 1997). In this case, the sections were incubated for 4 h at room temperature with the cell culture supernatants of LM5 or LM6 hybridoma, diluted respectively 1/10, 1/4 (v/v) in Tris-buffered saline (TBS)-Tween (Sigma). Most semithin sections, treated with a goat anti-rat IgG coupled to fluorescein isothiocyanate (FITC), were observed by fluorescence microscopy. Some of them were observed with a confocal Laser Scanning Microscope using a TCS-4D confocal imaging system (Leica instrument, Heidelberg, Germany). A focal series was collected for each specimen. The focus step between the sections was generally 0.5 µm.

Ultrathin sections collected on nickel or gold grids were treated with a solution of goat anti-rat IgG conjugated with 10- or 15-nm colloidal gold complex (EMGAT 10 or 15; BioCell Cardiff, UK) diluted 1/30 in TBS. After washing, grids were stained either with 1% uranyl acetate or with periodic acid-thiocarbohydrazide-silver proteinate (PATAg; Thiéry 1967). Observations were made with a Philips 400 electron microscope (Eindhoven, The Netherlands).

For both light- and electron-microscopy, controls were prepared with pre-immune serum.

Image analysis. The pictures (13 × 18 cm^2) were scanned with an ARCUS II scanner (Agfa) at 150 pixels per inch. The images were analysed on an Indigo R-4000 Work Station (*SiliconGraphic*, Mountain View, Calif., USA) with Visilog 4.1.1 (Noesis, Saclay, France) under the C-interpreter with which the dedicated software was written. The particles were segmented using a tophat filter and reduced to their centroid. The local density of the particles was determined as follows: (i) calculation of particle primary and secondary weights, (ii) histogram treatment and (iii) image processing. The calculation of the primary weight (W) of the particles was as follows. An area of influence (AOI) the radius of which equalled 20 pixels was defined around of each of the particles. The distances (d_i) between the central particle of the AOI and the neighbours which were located inside the AOI were calculated. The primary weight of the central particle is given by: $W = \sum_{i=1}^{i=n} \frac{1}{d_i}$, where i is the rank of the particles and n the number of neighbours. Arbitrarily, when the number of particles located in the AOI is lower than 4, the primary weight of the central particle is 0. The secondary weight of the central particle of each of the AOIs was calculated step by step, as the mean of the weights of all the particles which were located inside the AOIs (Cibert et al. 1999). At each step, the weight of the central particle was arbitrarily multiplied by 10. After convergence, the secondary weights were converted to a grey level ranging between 10 and 254. The mean grey level of the particles (t0) was determined. This image was dilated once, closed three times and thresholded between 1 and 255.

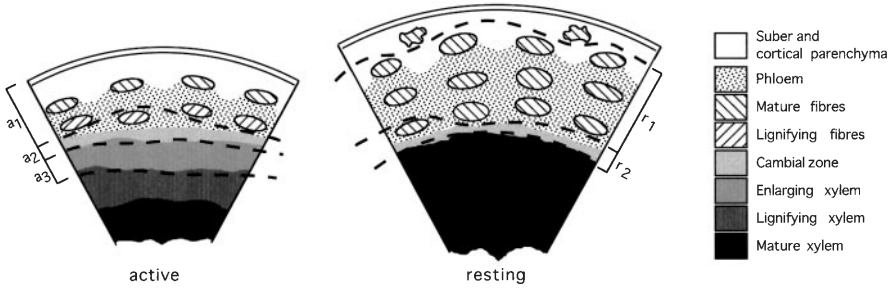

Fig. 1. Schematic transverse sections of active and resting hybrid aspen shoots showing the tissues collected for biochemical analysis. Three fractions (a_1, a_2, a_3) were scraped from active shoots and only two (r_1 and r_2) from resting shoots

Table 1. Analysis of carbohydrates solubilized with boiling water from cell walls of active (a_1, a_2, a_3) and resting (r_1, r_2) aspen shoots. a_1 and r_1, cortex plus non-functional phloem; a_2 and r_2, cambium plus recent phloem; a_3, enlarging xylem. DM, degree of methylation; DA, degree of acetylation. Assays for uronic acid and neutral sugar estimations were repeated 4–6 times and SD calculated. Only one assay was performed for DM and DA estimations due to the very low pectin amounts recovered from the isolated cell wall fragments, but the values obtained with similar assays performed with commercial pectins as controls gave reproducible data and have been taken as a guarantee for the reliability of the data

	a_1	a_2	a_3	r_1	r_2
Solubilized material [mg(g cell walls)$^{-1}$]	102	300	161	38	49
Neutral sugars [mg(g cell walls)$^{-1}$]	44 ± 2	192 ± 7	88 ± 7	32 ± 2	34 ± 3
Uronic acids [mg(g cell walls)$^{-1}$]	33 ± 3	96 ± 5	73 ± 3	10 ± 1	15 ± 1
DM (%)	22	27	26	11	10
DA (%)	23	33	42	42	46

From the resulting binary image, the areas and the grey means of the blobs were measured. The blobs were selected if their area and their grey mean were more than 19 pixels and t0, respectively. The boundaries of the selected blobs were superimposed on the original image.

Results

Time course of anatomical changes. The material studied in the present paper was harvested at two different times of the first growth year of aspen cuttings. Figure 1 summarizes the anatomical structure of active and resting shoots and localizes the different tissue fractions scraped from the shoots and used for biochemical analyses. Three fractions were collected from shoots with an active cambium. The first one (a_1) contained the unlignified cortical parenchyma and mature phloem. In the phloem, the outer ring of fibres alone was lignified. The following ring of secondary fibres was still differentiating. Many companion cells outside this ring were degenerating or already dead, showing that the corresponding sieve tubes were no longer functional. The second fraction (a_2) contained several phloem layers, mostly undifferentiated, together with the cambial zone, a meristematic region about 10–20 cells thick. An equivalent number of layers of fragile, enlarging future xylem cells scraped away from the lignifying zone was present in the third fraction (a_3).

Only two fractions, the first containing cortical parenchyma and the older phloem cells (r_1), the second the overwintering phloem and the cambium (r_2), were isolated from resting shoots. The cortical parenchyma was characterized by the presence of large clumps of lignified cells. The phloem was somewhat thicker than in active material, with one or two rings of lignified secondary fibres. The 10–20 cell layers between the innermost fibre ring and the first xylem cells (fraction r_2) comprised phloem derivatives overwintering in a more or less advanced degree of differentiation, the cambial initials and, against the xylem, the ''boundary layer cells'' as defined by Barnett (1992).

Cell walls were then isolated from the five tissue fractions and further treated with boiling water, in order to solubilize the pectin polymers not interconnected by calcium bridging.

Composition of hot-water extracts from active and resting materials. Table 1 reveals that during the period of activity the cell walls in the differentiating zones contained 30% (phloem side) and 16% (xylem side) of polymers soluble in boiling water. In contrast, during the rest period, these compounds were relatively less abundant, due to the presence of thick, cellulosic, sometimes lignified, cell walls in the tissue fractions. In all cases, the extracted compounds were nearly exclusively polysaccharides. The large amount of neutral sugars reflected the presence of rhamnogalacturonan 1 (RG1) but some hemicellulosic compounds may also have been solubilized together with pectic material. Only very low amounts of RG1 remained in the cell walls after the boiling-water treatment whatever the lignin content. For instance, analyses of r_1 and r_2 residual cell walls which differed appreciably in their lignin content showed that, in both cases, the galactose + arabinose/rhamnose ratios, which characterized the presence of side chains and in turn that of RG1 molecules, were similar and relatively low (around 2) while these ratios were much higher (about 10) for the water-soluble pectins extracted from both tissue fractions. The polysaccharides extracted with boiling water were both acetylated and methylated. The degree of methylation was lower in resting tissues than in active ones which suggests *in-muro* enzymatic demethylesterification at the end of the active period. In contrast, the polymers extracted from resting bark fractions were much more acetylated than those obtained from active tissues. The presence of some acetylated glucuronoxylan-like compounds in resting tissues containing fibre clumps might explain the high degree of acetylation. Table 2 shows the monosaccharide composition of the extracted polymers. The relatively high galacturonic acid/rhamnose ratio indicates the presence of homogalacturonan in all extracts. The proportion of xylose and glucose increased in resting tissues whereas the proportion of galacturonic units decreased. The presence of glucose, xylose and glucuronic acid reveals that some hemicellulose-like compounds had been solubilized together with the pectins. The ratio pectins/hemicelluloses appeared to shift at the end of the active period, the cell walls then containing more cellulose and hemicelluloses. Another striking feature was the decreasing gradient in arabinan developing from the outer cortical layers towards the

Table 2. Sugar compositions of pectins solubilized with boiling water from cell walls of active (a_1, a_2, a_3) and resting (r_1, r_2) aspen shoots. a_1 and r_1, cortex plus non-functional phloem; a_2 and r_2, cambium plus recent phloem; a_3, enlarging xylem. Data as mole percent

	a_1	a_2	a_3	r_1	r_2
Arabinose	42	29	19	39	21
Rhamnose	5	5	10	5	4
Xylose	4	4	11	10	7
Mannose	1	1	2	4	2
Galactose	6	23	14	9	20
Glucose	2	4	2	11	16
Galacturonic acid	34	25	31	16	22
Glucuronic acid	6	9	11	6	8

xylem. Moreover, in active as well as in resting shoots, ageing of phloem cells was characterized by divergent changes in galactan and arabinan: the former increasing while the latter decreased. All these data revealed that RG1 branches undergo important remodeling during the differentiation processes of cambial derivative cell walls. However, because the tissue fractions were somewhat heterogeneous (Fig. 1), immunological probes were then used to check and to confirm the preliminary biochemical information.

Immunodetection of pectic polymers. No meaningful labelling could be observed on control sections treated with preimmune serum, either at the light- or the electron-microscope level.

At the light-microscope level, LM6 staining decreased from the outer to the inner part of the stem during cambial activity (Fig. 2A). The gradient across the cambial zone was even more obvious when the

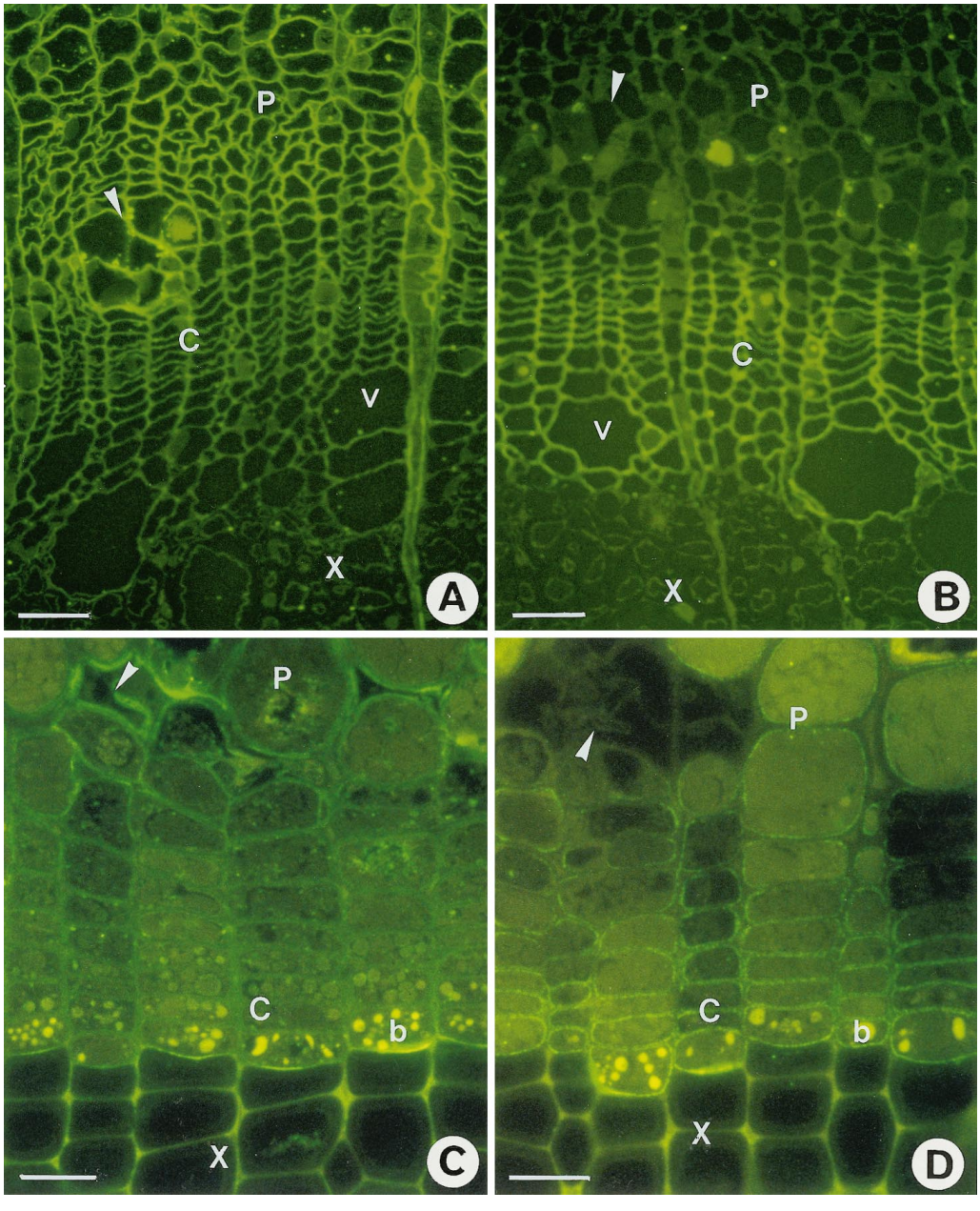

Fig. 2A–D. Immunofluorescent localization of RG1 side chains on transverse sections of active (**A**, **B**) and resting (**C**, **D**) hybrid aspen shoots. **A**, **C** Localization of arabinan with LM6 antibody. During the active period (**A**), the labelling decreases from the phloem (*P*), where the sieve-tube walls (*arrowhead*) are strongly reactive, to the cambial zone (*C*). Labelling disappears on the xylem side (*X*) where only the youngest enlarging vessels (*V*) are stained. In resting shoots (**C**), arabinan labelling is much weaker in the phloem and the cambium, except in sieve-tube walls (*arrowhead*). The primary walls of the first xylem cells are stained. **B**, **D** Localization of galactan with LM5 antibody. The staining is restricted to the cambial zone and enlarging xylem cells when the cambium is active (**B**). Note the absence of reaction in the sieve tubes (*arrowhead*). During the rest period (**D**), the walls of the cambial cells are labelled, except the middle lamella of the radial walls. A faint reaction can be seen in the walls of the youngest phloem parenchyma cells and in the first xylem cells. There was no labelling of the sieve tube walls (*arrowhead*). Note that the vacuolar contents of the border layer cells (*b*) give an unspecific reaction with both antibodies. Bars = 40 μm (**A**, **B**), 20 μm (**C**, **D**)

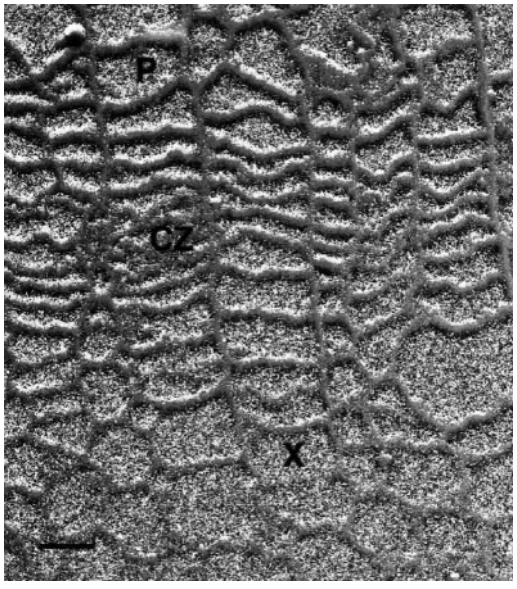

Fig. 3. Observation of LM6 staining with a confocal microscope. Superimposition of successive images demonstrates the existence of a decreasing arabinan gradient from the phloem to the xylem, *P*, phloem; *CZ*, cambial cells; *X*, xylem

sections were observed with the confocal microscope, ruling out possible autofluorescence artifacts (Fig. 3). These observations fit well with the data reported in Table 2. During the rest period (Fig. 2C), the staining was very faint in the cambial zone except in the tangential primary walls common with the first xylem cell layer. In the xylem proper, at least in the late wood, cell junctions and the unlignified pit membranes between vessels and contact cells were reactive. With LM5, during the period of cambial activity (Fig. 2B), fluorescent labelling was found in an area of 15–25 cells located between the mature phloem and the lignifying xylem cells. The labelling decreased rapidly in the walls of very young phloem parenchyma cells and fibres. No staining was observed in the walls of mature sieve-tube members and companion cells. On the xylem side, cessation of LM5 labelling seemed to occur rather abruptly, at a time when vessels were still enlarging. This might explain the apparent discrepancy between the cytochemical and biochemical data, the former showing an increased labelling from phloem to xylem derivatives, and the latter indicating a lower relative amount of galactan in xylem cell walls. This discrepancy results from the composition of the two analyzed tissue fractions, a_2 and a_3 respectively. The a_2 fraction contained mostly cambial cells and immature phloem cells all reacting with LM5, while the a_3 fraction comprised enlarging xylem and some lignifying cell walls unlabelled by the antibody. This may explain the lower value found for galactose content in this fraction. The LM5 labelling was much fainter during the rest period (Fig. 2D). It occurred in the inner part of the walls in the cambial cells and the first phloem derivatives in which the middle lamella was conspicuously devoid of label. No reaction was seen either in the sieve-tube members or in the companion cells. On the xylem side, some staining could be observed in the primary walls of recently formed cells.

At the electron-microscope level, LM6 immunolabelling results were difficult to interpret. As observed with the light microscope, cell wall staining tended to decrease from the phloem to the xylem. However, a rather heavy, unspecific deposit of gold particles was observed over most cytoplasmic structures in parenchyma and cambial cells whatever the experimental procedures used (presence or absence of milk in the preincubation buffer, presence or absence of tween in the incubation medium). In contrast, satisfactory images were obtained with LM5 (Fig. 4). With this antibody, Golgi apparatus and Golgi vesicles were labelled in the cambial cells and in their immediate derivatives both towards the xylem and towards the phloem (Fig. 4A,C). In the cambial zone, only a few scattered gold particles were seen in the nascent cell walls and labelling intensity increased in parallel with cell wall maturation. (Fig. 4B,C). The gold particles tended to be restricted to the plasmalemma-cell wall interface in the older radial walls (Fig. 4B) where they often appeared grouped in clusters (Fig. 4C). The staining remained rather strong in cells committed to the xylem pathway, at least to within a short distance of the young secondary walls where it disappeared (Fig. 4E). On the phloem side, as already observed at the light-microscope level, labelling decreased rapidly in the walls of future parenchyma cells and fibres, and disappeared from the walls of future sieve tubes as they differentiated (Fig. 4D).

The clustered distribution of gold particles observed on many cell walls in the cambial zone was checked by image analysis. The particles were numbered and their local density determined. As shown in Fig. 5, a clustered distribution of the gold particles was present along the radial walls, close to the plasma membrane. These clusters did not seem to be associated with any specific cytoplasmic structure. The separation between consecutive clusters was measured. The two-dimensional spatial frequencies of this distribution were multiples of ca. 0.6 μm, a value substantially higher than the threshold of the distance used in the software (Webb and Dorey 1990). The periodicity of the spatial distribution might reflect the periodicity of a physiological process occurring at the plasmalemma-cell wall interface. This periodicity could not be recognized in the tricellular junctions between xylem derivatives as the increasing size of clusters led to their becoming contiguous.

During the rest period (Fig. 6), LM5 labelling was present more or less throughout the tangential walls of the cambium while it was distributed on each side of the radial walls, leaving a narrow unstained margin on the inner side of the walls. A similar, lateral distribution was also observed in phloem derivatives where labelling decreased progressively. No gold particles were observed on lignified cell walls. Most sieve tube-companion cell complexes overwintering in an undifferentiated state were unlabelled, except for some of the youngest ones. No Golgi activity was observed at that stage of the seasonal cycle.

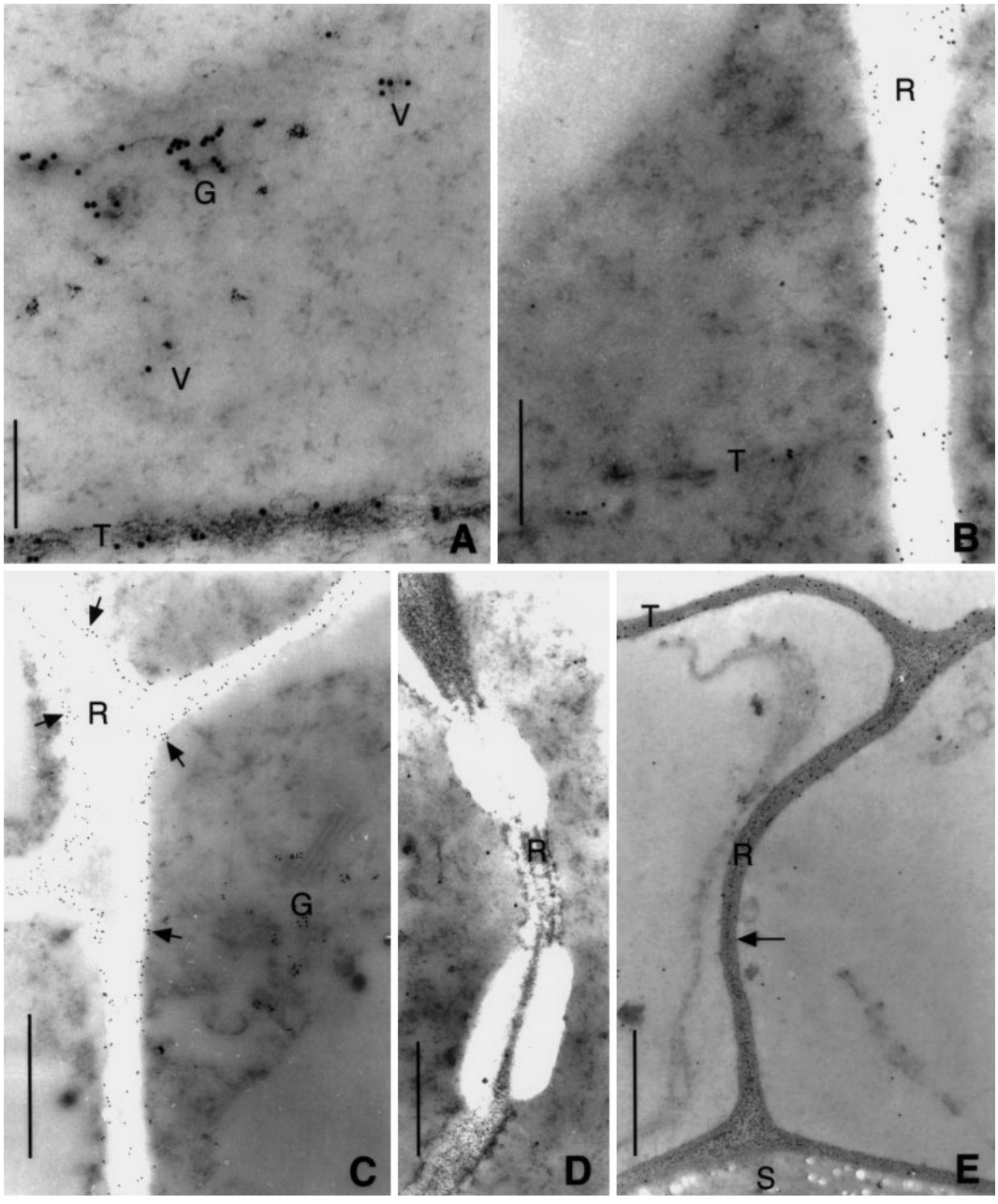

Fig. 4A–E. Immunogold localization of galactan in the cambial zone of hybrid aspen shoots during the active period at the electron-microscope level. **A** Cambial cells, PATAg counterstaining: gold particles are present on the Golgi stacks (*G*) and vesicles (*V*) and on the tangential wall (*T*). Bar = 0.25 μm. **B** Cambial cells: nascent tangential wall (*T*) with a very few gold particles; radial wall (*R*) with most particles distributed along each side. Bar = 0.5 μm. **C** Cambial cells: labelling of the Golgi apparatus (*G*) and of the walls; note the distribution in clusters (*arrows*) of the particles along the inner part of the radial wall (*R*). Bar = 1 μm. **D** Differentiating sieve plate with callose platelets unstained with PATAg; the radial wall (*R*) is not labelled. Bar = 0.5 μm. **E** Xylem derivatives, PATAg counterstaining; the gold labelling ceases in the primary wall (*arrow*) before the beginning of secondary wall (*S*) formation. Bar = 1 μm

Discussion

The results on the localization of pectin-associated carbohydrates presented here are the first to be reported for the cambium and vascular tissues. Immunolabelling images were complemented by biochemical analysis, allowing a more precise comparison between the different tissues of the stem and between samples from active or resting trees.

During the active period, cambial cell walls have a high pectin content, which agrees with previous data obtained on *Populus tremuloides* (Simson and Timell 1978). A high pectin content seems to characterize actively dividing (Schaumann et al. 1993; Dolan et al. 1997) or elongating (Goldberg et al. 1986) cells. In hybrid aspen, we detected both homogalacturonan and RG1 with arabinan and galactan side chains. Moreover, to our knowledge, this is the first immunocytochemical

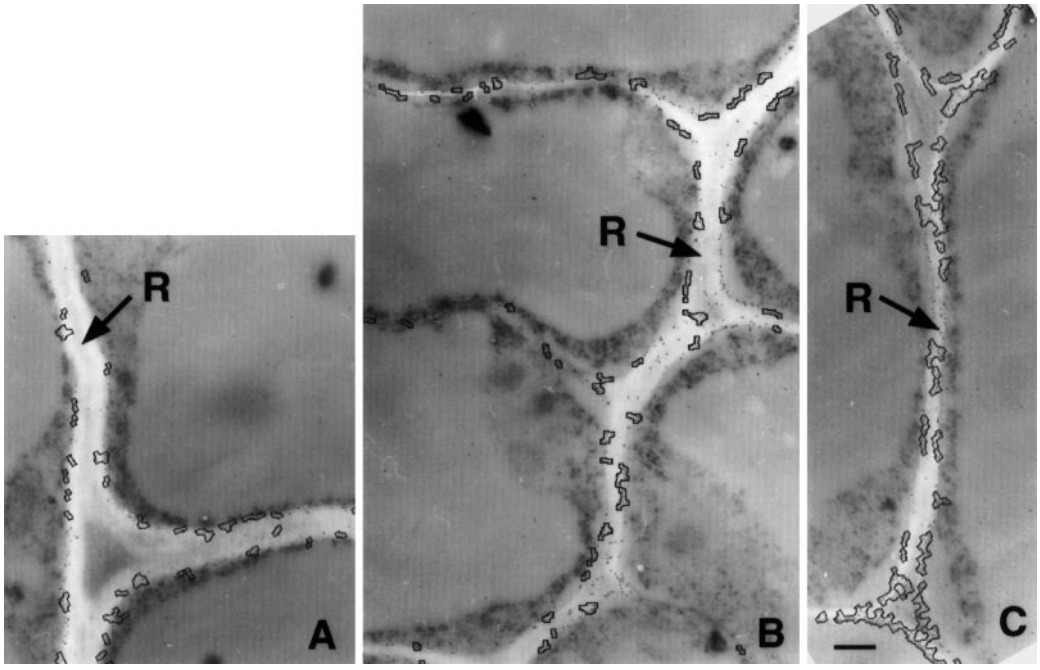

Fig. 5A–C. Clustered distribution of galactan epitopes (outlined) as revealed by image analysis of immunogold-labelled ultrathin sections of aspen shoots. **A** First phloem derivatives; **B** cambial cells; **C** xylem derivatives. *R*, radial wall. Bar = 0.5 μm

study to demonstrate that arabinan becomes prevalent at a very early stage in cells committed to differentiate into phloem. More than 20 years ago, Dalessandro and Northcote (1977) observed that in sycamore and poplar, cambial derivatives retained throughout growth a full capability for the interconversion of UDP-sugars and concluded that cell wall differentiation was controlled by the relative activity of the different polysaccharide synthetases. In hybrid aspen shoots, activation of arabinan synthases would characterize the derivatives entering the phloem pathway. Our data revealed also the development of an important remodelling of pectin side chains throughout the course of phloem derivative differentiation. In contrast, galactan synthesis occurred in the whole cambial zone and in enlarging xylem derivatives. On the phloem side, galactan present in the first derivatives disappeared progressively during differentiation, the walls of maturing sieve tube-companion cell complexes being totally devoid of labelling. The absence of labelling in differentiated phloem and xylem cell walls could result either from the masking of galactan epitopes by newly deposited cell wall components (lignin, cellulose, hemicelluloses...) or from *in-muro* hydrolysis of galactan chains. Rearrangement of RG1 side chains have been described in other materials. For instance, Hervé du Penhoat et al. (1987) showed that the galactan chains associated with pectin polymers underwent remodelling processes during maturation of hypocotyl cell walls. In this case, high-molecular-weight, probably highly branched, galactans were progressively replaced by small unbranched polymers.

Image analysis of micrographs taken from immunogold labelled galactan confirmed that these polymers were present in cell wall specific microdomains (plasmalemma-cell wall interface) and revealed an apparent clustered distribution of gold particles in this area. This distribution is considered not to be an artifact of the specimen preparation because (i) similar results were obtained regardless of whether glutaraldehyde or paraformaldehyde was used as the fixative; (ii) we have previously observed that protein A or specific IgG used as secondary antibodies delivered the same images for pectin distribution; (iii) patchy labelling was also observed on semithin sections treated with LM5 and FITC; however, not when the sections were treated with LM6 and FITC. Moreover, the clustered appearance of LM5 staining was confirmed by calculations taking into account the local density of galactan residues on the section surface. This distinctive distribution might result from the association of periodic exocytosis processes and cell wall expansion since according to the theorical model of Kellershohn et al. (1996) and Prat et al. (1996), wall growth would result from the sum of the local two dimensional periodic tread milling processes.

The cessation of cambial meristematic activity was characterized by a thickening of the cambial walls, especially the radial walls as already observed in other trees species (Larson 1994). This thickening was accompanied, in hybrid aspen, by a sharp decrease in the relative amounts of pectin. This apparent decrease, occurring in all tissue fractions during the transition from activity to rest, most probably results from an increased synthesis of both hemicelluloses and cellulose as previously suggested for another poplar species (Viëtor et al. 1995). The synthetic pathways of polysaccharides then changed drastically with the cessation of production and growth of cambial derivatives. Further-

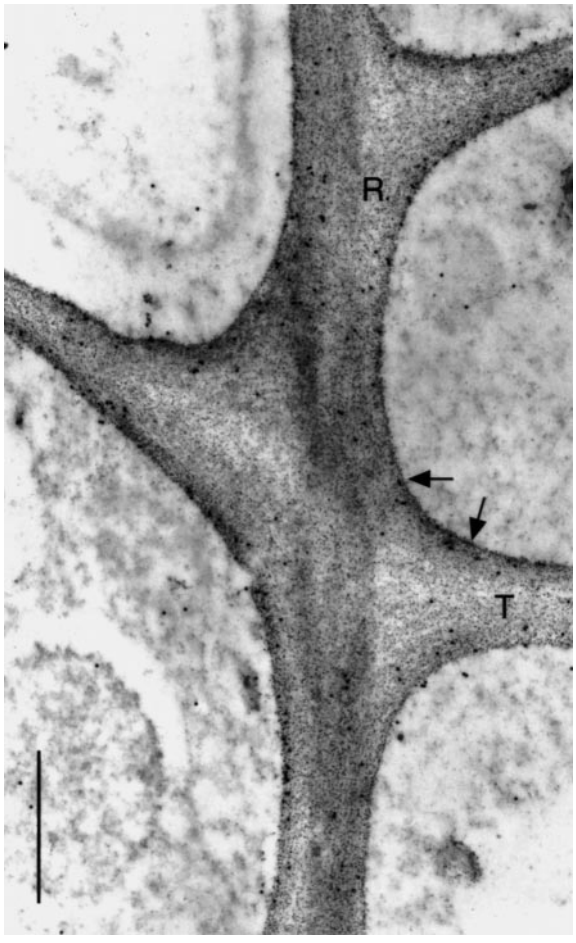

Fig. 6. Immunogold localization of galactan in the cambial zone during the resting period. PATAg counterstaining. In radial walls (*R*), gold particles are often localized in the inner part of the wall, leaving a narrow unlabelled band (*arrows*) along the periplasmic space. *T*, tangential wall. Bar = 1 μm

more, the galacturonic units were obviously less esterified during the rest period and this can be related to the high pectin methylesterase activity detected in active cambial cells (Micheli et al. 2000). According to Zhang and Staehelin (1992), only highly methylated pectins are exported into the cell wall. In hybrid aspen, these polymers would be progressively demethylated throughout the active period giving rise to low-methylated pectins which will remain as such after the cessation of growth. A decrease in the degree of methylation was also observed in other materials when the cells ceased to divide (Dolan et al. 1997; McCann et al. 1993; Schaumann et al. 1993), or to elongate (Goldberg et al. 1986; McCann and Roberts 1994; Li et al. 1995).

In conclusion, the pectin fraction can be considered as an interesting marker of the early steps of cambial differentiation for at least two reasons: (i) actively dividing cells are characterized by a high content of pectins whose structure develops differently on each side of this meristematic zone; (ii) cessation of division is accompanied by an obvious demethylation of the galacturonic units, modifications, and relative decrease of pectin synthesis processes. All these data suggest a strict molecular control of pectic enzymes during cell differentiation.

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