CELL WALL MODIFICATIONS IN WOODY STEMS INDUCED BY MECHANICAL STRESS*

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ABSTRACT

The first example of modified wall architecture in woody cells examined was in poplar trees where wounding caused wall thickenings of xylem fibres differentiating at the time of wounding. These fibres close to a wound also displayed slightly higher lignin content and an inhomogeneous lignin distribution as revealed by UV-microspectrophotometry. Additionally, the lignin in the middle lamella and the S_2 layer of modified fibres contained more guaiacyl units than fibres of normal wood. These wound response mechanisms are assumed to contribute to an increased resistance. Secondly, in a pine seedling displaying stem bending, extensive compression wood formation was observed. Autofluorescence confirmed that the lignin content in the compression wood tracheids was distinctly higher than in normal tracheids. Immunolabelling of galactan for the first time clearly demonstrated that the bulk of galactan is localised in the outer S2 wall regions.

Keywords: wood; mechanical stress; wall thickening; compression wood; lignin; galactan; *Populus* sp.; *Pinus radiata*.

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INTRODUCTION

The lateral meristem cambium, and also cambium derivatives in early developmental stages, respond to various stresses by producing secondary xylem tissues with altered characteristics, which are directed to enhancing the capability of plants to withstand the effect of stressful conditions. Cell wall modification by way of increased thickness or compositional changes is a common mechanism operating in plants to respond to wounding, and to mechanical and physical stresses. The present paper deals with two examples of cell wall modifications that can occur when the cambium or the cambium-adjacent differentiating xylem is stressed by mechanical injury or by bending of the stem.

Responses of xylem tissue to mechanical injuries have been variously described on the macroscopic as well as on the microscopic level (e.g., Shigo 1984; Schmitt & Liese 1990, 1992, 1993; Liese & Dujesiefken 1996; Pearce 2000). The most prominent responses revealed for angiosperm trees wounded during the season were reactions of parenchyma cells, such as synthesis of phenolic compounds with subsequent secretion into neighbouring fibres and vessels, tyloses formation, and suberisation. There is also evidence that xylem cells differentiating at the time of wounding and without secondary wall layers, were capable of redifferentiation into parenchymatic tissue with frequently dividing cells thus contributing to the formation of a callus (Frankenstein *et al.* 2005). However, little is known about those differentiating xylem cells which had already started with deposition of secondary wall material. This paper is focused on modifications of the secondary wall layer in these cells, with special emphasis on the lignin topochemistry.

Compression wood is a type of reaction wood that is produced in gymnosperms in response to factors that force stems or branches to bend or lean. The compression wood produced varies in its severity depending apparently on the magnitude of the stresses imposed. The characteristic features which are typical of severe compression wood, such as rounding of tracheids, presence of intercellular spaces, thicker cell walls, greater lignification of the outer region of the S_2 layer, reduced lignification of middle lamella, absence of an S_3 layer, and the presence of helical cavities in the S_2 layer, have been well characterised (Timell 1986). The anatomy of mild compression wood cells, which develop features that are intermediate between severe compression wood and normal wood, has also been characterised in detail (Donaldson *et al.* 1999, 2003; Singh & Donaldson 1999; Singh *et al.* 2003).

Higher lignin content and the presence of (1→4)-β-D-galactan in cell walls are generally regarded to be the two most characteristic compositional features of all grades of compression wood (Timell 1986). Most of the galactan is removed during delignification, and it appears that lignin and galactan may be chemically linked (Mukoyoshi *et al.* 1981). In the present paper we used the anti-(1→4)-β-D-galactan monoclonal antibody LM5 (Jones *et al.* 1997) as a marker for identifying mild and

severe compression wood cells, and to examine the pattern of galactan distribution in compression wood cell walls.

Both selected phenomena are examples of an enormous variety of stress reactions in woody tissue, but represent characteristic modifications of the chemical composition mainly in the secondary wall layers.

MATERIAL AND METHODS

Plant Material

To study cell wall modification due to wounding, rectangular wounds of 10 × 10 cm were made on 7 July 2002 and 23 June 2003 by removing the bark from stems of four mature poplar trees (*Populus* sp.) using a saw and a chisel. Samples were collected from the lateral edges of wounds 2, 4, 8, 10, 17, 62, and 95 weeks after wounding. The wound tissue, the adjacent modified xylem, and the unaffected xylem were removed with a chisel and razor blades. For galactan localisation, approximately 1-year-old *Pinus radiata* D. Don seedlings were collected in the Whakarewarewa Forest, Rotorua, New Zealand. Selected seedlings had a stem of 5 mm diameter and showed extensive compression wood.

Light- and Electron-microscopy

For conventional light-microscopy, poplar samples (10 × 10 × 8 mm) were fixed for 3 days in a phosphate-buffered solution of 3.7% formaldehyde, dehydrated in a graded series of propanol, and embedded in glycol methacrylate (Technovit 7100). Transverse sections 6 μm thick were cut with a rotary microtome, and stained for 1.5 h with a standard Giemsa solution. *Pinus radiata* samples were prepared as described above, except that they were dehydrated in a graded series of ethanol. Transverse sections were cut at a thickness of 10 μm and stained as described above.

For transmission electron microscopy (TEM), the poplar samples from the same series were reduced to a final size of 3 × 3 × 8 mm, fixed for 1 day in a mixture of 5% glutaraldehyde and 8% paraformaldehyde, washed in 0.1 M sodium cacodylate buffer (pH 7.3), dehydrated in a graded series of acetone, and embedded in Spurr´s epoxy resin (Spurr 1969). Ultra-thin (80–100 nm) transverse sections were either double-stained with uranyl acetate and lead citrate, or stained with potassium permanganate, according to Donaldson (1992). The samples were examined with a Philips CM 12 TEM at an accelerating voltage of 60 or 80 kV.

UV-microspectrophotometry

For UV-microspectrophotometry, the poplar samples were processed as described for TEM. Semi-thin 1-μm transverse sections were mounted on quartz slides, immersed in a drop of non-UV absorbing glycerol, and covered with a quartz cover

slip. An ultrafluar objective 32:1 and a 100:1 objective were used. Scanning UV-microspectrophotometry was carried out using a ZEISS UMSP 80 microspectrophotometer equipped with an Osram high-pressure xenon lamp, an ultrafluar quartz condenser, and a scanning stage, enabling the determination of image profiles at a constant wavelength of 280 nm using the scan programme APAMOS (Automatic-Photometric-Analysis of Microscopic Objects by Scanning, Zeiss). This wavelength represents the typical absorbance maximum of lignified cell walls. The scan programme digitises rectangular tissue portions with a local geometrical resolution of 0.25 μm^2 and a photometrical resolution of 4096 grey scale levels, converted into 14 basic colours representing the measured absorbance intensities (Koch & Kleist 2001). Additionally, specimens were analysed by point measurements with a spot size of 1 μm^2. The spectra were taken at wavelengths ranging from 240 to 400 nm in 2-nm steps using the programme LAMWIN (Zeiss). These point measurements for a semiquantitative determination of the lignin content were automatically repeated 50 times at each spot for individual wall layers, ie., compound middle lamella, the S_2 layer of the secondary wall, and cell corners.

Immuno-labelling and Confocal Microscopy

Pinus radiata sections were prepared as described above and fixed on microscope slides. The sections were blocked with 50 mM PBS containing 3% nonfat milk (pH 7.4) for 1 h at 24°C. A 50-ml droplet of anti-(1→4)-β-galactan monoclonal antibody LM5 (Jones *et al*. 1997) in blocking buffer (diluted 1:10) was then placed on the sections. The sections were incubated for 18 h at 4°C, washed with the blocking buffer, and then incubated for 2 h at 24°C with the fluorescent secondary antibody Alexa Fluor®660 (Invitrogen New Zealand Ltd., Auckland), diluted 1:500 in the blocking buffer. Sections were washed with water, dried, mounted in immersion oil, and viewed on a confocal laser scanning microscope (Leica TCS NT). Control sections were prepared with the primary antibody omitted. For immuno-fluorescence, samples were illuminated with an excitation wavelength of 647 nm. The emission passing through a long-pass filter (LP 665) was recorded. Lignin autofluorescence was imaged from the same areas of the sections. An excitation wavelength of 488 nm was used and the emission passing through a long-pass filter (LP 515) was recorded. Stacks of 10 confocal optical sections were taken for both immuno-fluorescence and lignin autofluorescence, and projections were compiled for imaging. The immuno-fluorescence images were digitally coloured red.

RESULTS AND DISCUSSION

Wound Response

Light-microscope and TEM investigations of poplar xylem revealed wound-induced cell wall modifications within xylem cells differentiating at the time of

wounding. This modified xylem zone became obvious between xylem laid down prior to wounding and tissue laid down after wounding (Fig. 1) and was characterised by fibres with unusually thick walls (Fig. 2). Electron microscopy showed that, compared with unaffected xylem cells, these fibres deposited additional secondary wall material leading to extremely thick walls (Fig. 3, 4). Cell corner regions within the modified xylem consisted predominantly of material with high electron density (Fig. 4). Schmitt *et al*. (2000) observed the formation of a tangential band of thick-walled fibres between modified and regular xylem after insertion of a pin into beech xylem. Probably, these modified fibres also developed in the zone of undifferentiated xylem.

To semiquantitatively determine the lignin distribution and composition within individual wall layers, the poplar tissues were examined by scanning UV-microspectrophotometry at a constant wavelength of 280 nm (Fig. 5–8). In addition, point measurements were made with a spot size of 1 μm and a wavelength range of 240 to 400 nm (Fig. 9, 10). Both methods have been proven to be reliable with a high spatial resolution (e.g., Bland & Hillis 1969; Scott *et al*. 1969; Fergus & Goring 1970a, b; Musha & Goring 1975; Bauch *et al*. 1976; Bucciarelli *et al*. 1999; Fukazawa 1992; Okuyama *et al*. 1998; Grünwald *et al*. 2001; Grünwald, Ruel, Kim, & Schmitt 2002; Grünwald, Ruel, & Schmitt 2002; Koch & Kleist 2001; Takabe 2002; Koch & Grünwald 2004).

The distribution pattern of lignin in unaffected xylem fibres and vessel elements in an earlywood region is shown in Fig. 5. The fibres were characterised by thin S_2 wall layers with low and uniform absorbance values of around Abs_{280nm} 0.09, and increasing absorbance values in compound middle lamella regions (Abs_{280nm} 0.16 to Abs_{280nm} 0.23). Latewood fibres in unaffected xylem showed slightly higher absorbance values in their wider S_2 and in cell corners, particularly in the compound middle lamella regions (Abs_{280nm} 0.23 to Abs_{280nm} 0.35) (Fig. 9). The highest absorbencies of Abs_{280nm} 0.35 to Abs_{280nm} 0.67 were found in some cell corners between latewood vessel elements and adjacent fibres. Regarding the lignin type, representative point measurements (Fig. 9) resulted in mean absorbance maxima at wavelengths 270/272 nm for the S_2, indicating a low content of predominantly syringyl-type lignin. Cell corner regions showed the highest lignin concentrations at a wavelength of 278 nm therefore mainly composed of guaiacyl-type lignin, which corresponds well with earlier studies (Fergus & Goring 1970a, b; Musha & Goring 1975; Fukazawa 1992). Point measurements of middle lamella regions displayed only a slightly higher maximum absorbance at 270/272 nm compared to the maximum absorbance of the S_2. The middle lamella regions and the S_2 therefore had the same lignin composition. A higher relative abundance of guaiacyl lignin moieties has been reported variously (e.g., Fergus & Goring 1970b; Musha & Goring 1975; Terashima, Fukushima, & Takabe 1986; Terashima, Fukushima,

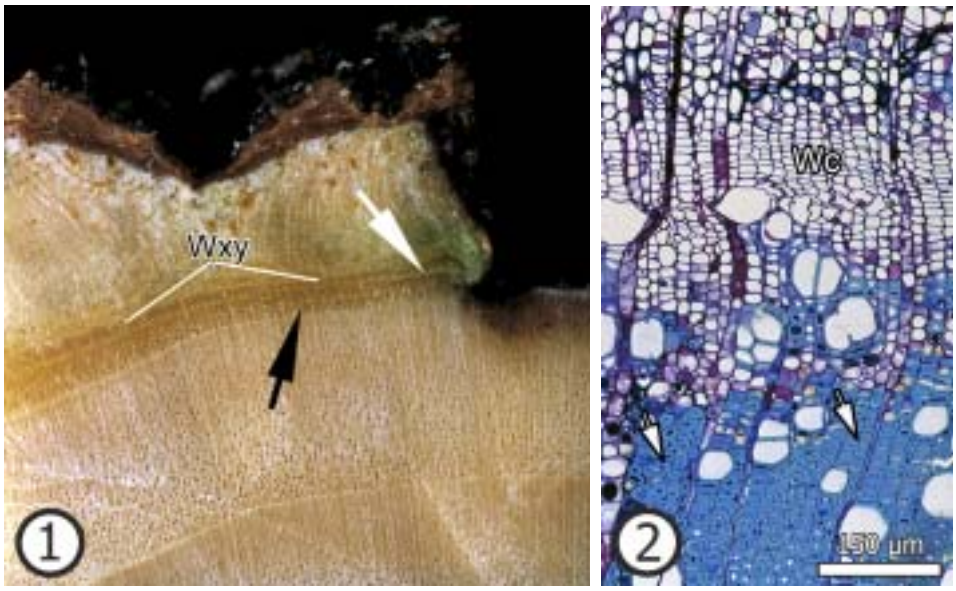

FIG. 1–Callus formation at the lateral wound edges; black arrow indicates the position of the transition zone, white arrow indicates the position of the cambium at the time of wounding, wound xylem (Wxy) formed after wounding.

FIG. 2–Transverse section through the modified transition zone between xylem laid down prior to and after wounding containing fibres with wound-induced wall thickenings (arrows), wound cambium (Wc); light micrograph, Giemsa staining.

Tsuchiya, & Takabe 1986; Terashima *et al.* 1993). Our results for secondary wall and cell corner regions of unaffected poplar xylem were in good agreement with the cell wall composition typical for many hardwoods (e.g., Fergus & Goring 1970a, b; Musha & Goring 1975; Terashima, Fukushima, & Takabe 1986; Terashima, Fukushima, Tsuchiya, & Takabe 1986; Terashima *et al.* 1993; Donaldson 2001; Donaldson *et al.* 2001; Grünwald, Ruel, Kim, & Schmitt 2002; Koch & Kleist 2001; Takabe 2002).

The influence of wounding on the lignin distribution in walls of modified xylem cells is shown in Fig. 6–8. Already after a short wound response period, the secondary walls of many UV-scanned modified fibres showed several concentric sub-layers with absorbance values varying between Abs_{280nm} 0.09 and Abs_{280nm} 0.48, the lowest lignin content being always in the layers adjacent to lumen (Fig. 6). In comparison, unmodified hardwood fibres were characterised by a uniform lignin content across the entire secondary wall layer (e.g., Saka & Goring 1988; Koch & Kleist 2001). As compared with unaffected references, higher absorbance values were regularly recorded in the compound middle lamella between fibres (Abs_{280nm}

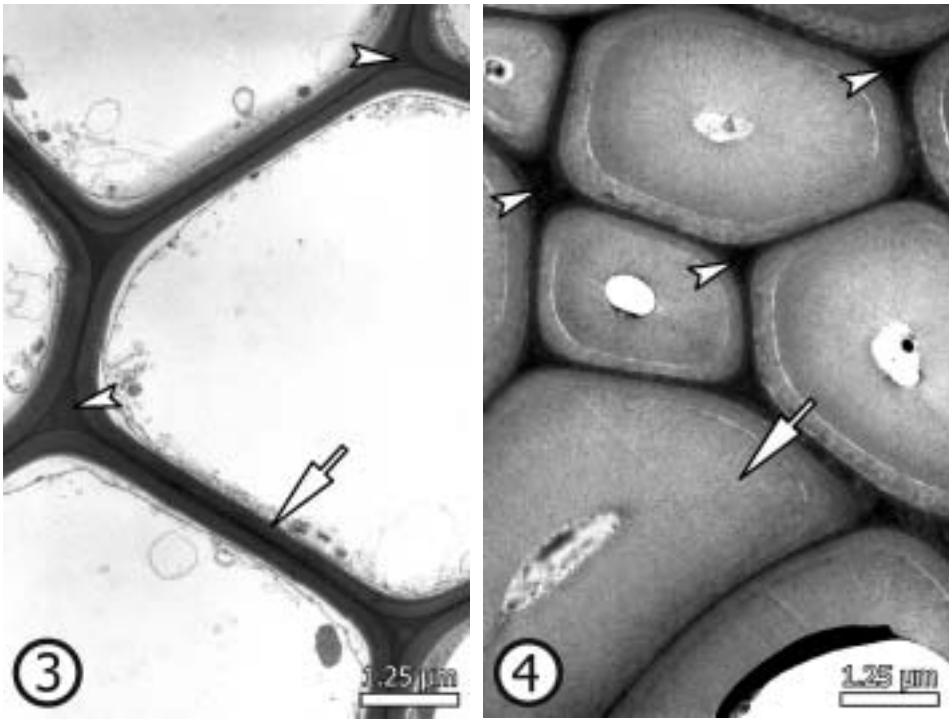

FIG. 3–Transverse section through unmodified poplar xylem; typical thin-walled fibres (arrow), with less electron dense material in cell corners (arrowheads); TEM, uranyl acetate, and lead citrate staining.

FIG. 4–Transverse section through modified xylem of the transition zone; fibres with distinct secondary cell wall thickenings (arrow); cell corners contain material with extremely high electron density (arrowheads); TEM, potassium permanganate staining.

0.55 Abs_{280nm} 0.68) and in the cell corners (Abs_{280nm} 0.81 up to over Abs_{280nm} 0.94) (Fig. 6). After prolonged wound response (62 to 95 weeks), thick-walled fibres often showed increased absorbance values and an inhomogeneous lignin distribution. Within the thickened secondary wall, absorbance values varied between Abs_{280nm} 0.09 and Abs_{280nm} 0.42 (Fig. 7). Absorbance values for the compound middle lamella ranged from Abs_{280nm} 0.23 to Abs_{280nm} 0.81 and in cell corner regions from Abs_{280nm} 0.42 up to Abs_{280nm} over 0.94. The secondary wall of vessels showed absorbance values between Abs_{280nm} 0.23 and Abs_{280nm} 0.42. The UV-absorbance in compound middle lamella regions ranged from Abs_{280nm} 0.48 to Abs_{280nm} 0.68 and from Abs_{280nm} 0.55 to Abs_{280nm} 0.94 for the cell corners (Fig. 7, 8). The lumina of many ray parenchyma cells were filled with accessory phenolic deposits (Fig. 7). These deposits were also observed in the lumina of numerous fibres, especially in tissues close to the wound surface (Fig. 8). As compared to lignin, the spectra of

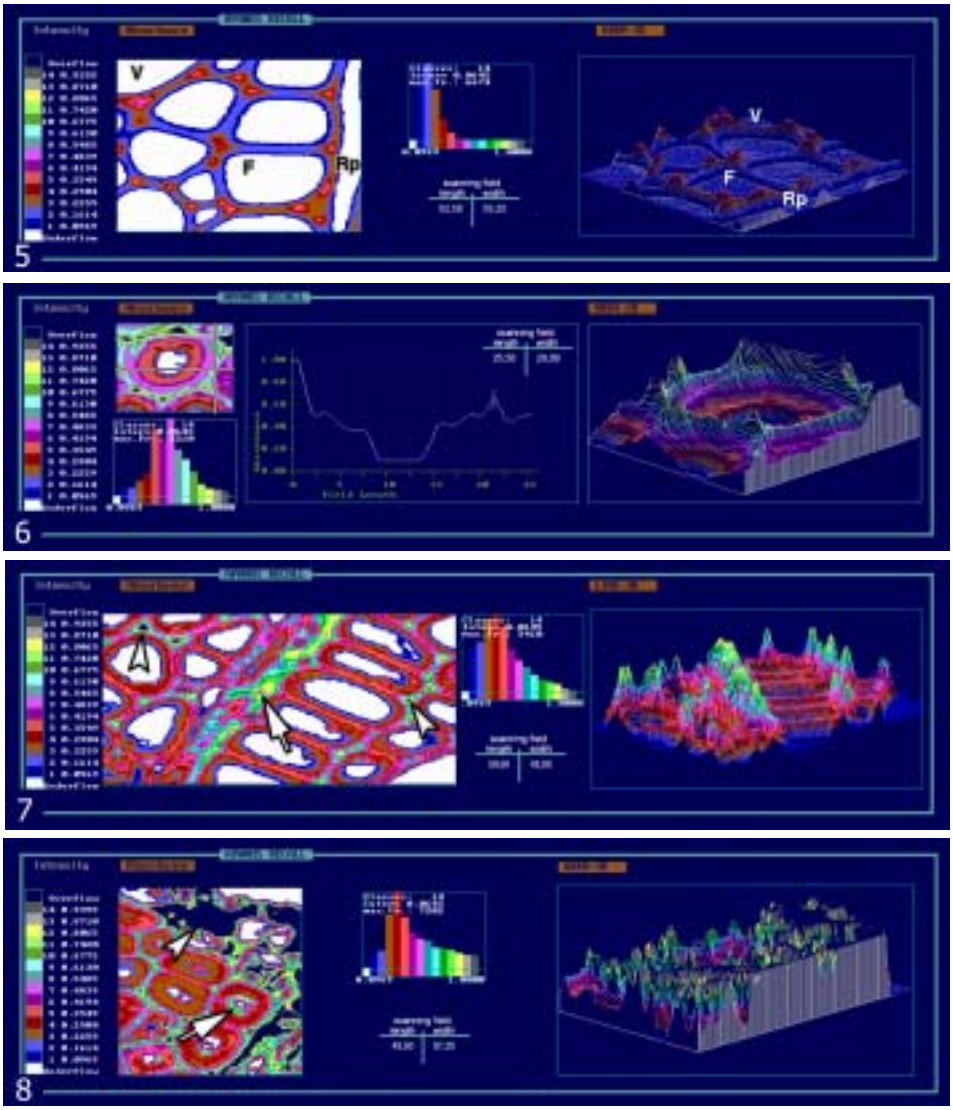

FIG. 5–UV-micrograph and 3D UV-microscopic scanning profile of unaffected xylem. The coloured pixels represent different UV-absorbance values within the fibre wall layers at 280 nm wavelength; vessel (V), fibre (F), ray parenchyma (Rp).

FIG. 6–UV-micrograph and 3D image profile of a fibre showing extreme secondary wall thickenings and increased lignin contents.

FIG. 7–UV-micrograph and 3D image profile of modified xylem; fibres showing wall thickenings; ray parenchyma cells are filled with accessory phenolic compounds (arrows) and extremely high lignin concentrations in cell corner regions (arrowheads).

FIG. 8–UV-micrograph and 3D image profile of a modified xylem directly adjacent to the degenerated tissue on the wound surface (arrowhead); some thick-walled fibres are filled with accessory phenolic compounds (arrows).

these accessory phenolic deposits in fibres and ray parenchyma cells generally had higher absorbance values (up to Abs_{280nm} 1,38), and their absorbance maxima displayed a bathochromic* shift to a wavelength of 284 nm to 286 nm and a slower decrease of the absorbance with increasing wavelength. Therefore, any influence of the condensed accessory phenolic deposits on the UV-measurements is likely to be negligible.

The spectra recorded from modified fibres indicated that wounding induced an alteration in lignin composition, being restricted mainly to secondary wall and compound middle lamella regions, whilst cell corner lignin mostly remained unchanged. Within thickened secondary walls, the maximum absorbance shifted slightly towards higher wavelengths (272–274). The same effect became evident for middle lamella regions (274–276), indicating an increase in the amount of guaiacyl moieties (Fig. 10). The UV-scannings and UV-spectra of all thickened wall layers showed a distinctively higher average lignin content with highly variable maximum values.

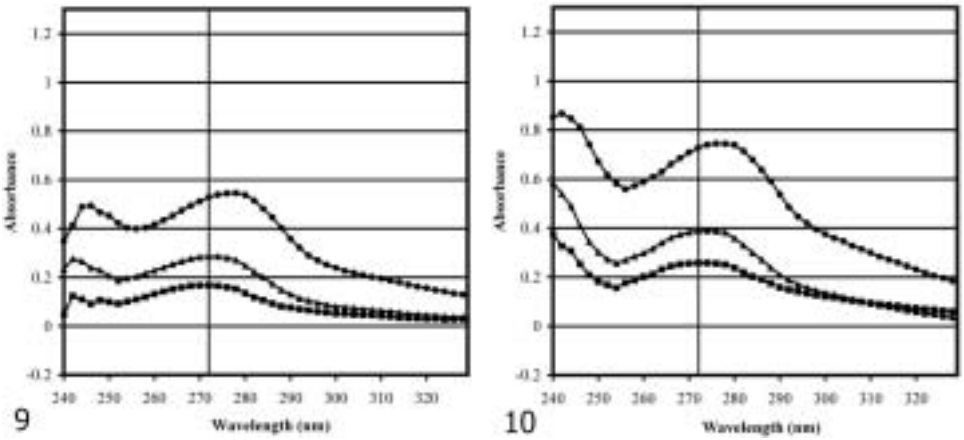

FIG. 9 and 10–Representative UV absorbance spectra of fibre wall layers of controls (9) and of fibres within the transition zone (10): S_2-layer (-■-), compound middle lamella (-▲-), cell corner region (-●-); vertical line indicates a wavelength of 272 nm.

Mechanical Stress Response

The choice of a *Pinus radiata* seedling displaying stem bending proved valuable in our study for examining the pattern of distribution of galactan within compression wood cell walls by immunolabelling. There was an abundance of compression wood in the stem, and it was possible to compare normal and compression wood

* Shift of a spectral band to lower frequencies (higher wavelengths), it is also referred to as a red shift

within the same year of growth (Fig. 11). In Fig. 12-A the lignin concentration in cell walls of large areas of normal and compression wood is compared at a low magnification, based on lignin autofluorescence of the cell walls. Higher brightness levels indicate higher lignin concentration. Greater brightness was associated with the region of secondary xylem containing compression wood cells; in contrast, the region containing normal wood cells displayed a much reduced brightness. Confirmation of this feature was obtained by observations made at higher magnifications, which also revealed other cellular features characteristic of compression wood, such as the presence of rounded tracheids and intercellular spaces. As shown in Fig. 12-B, the pattern of immuno-localisation of galactan closely corresponds to the pattern of autofluorescence in the secondary xylem, with the compression wood region being brightly fluorescent for galactan. The cell walls in the normal wood region showed only a slight fluorescence. Interestingly, a narrow band of strongly fluorescing xylem for galactan (Fig. 12 A, B) showed only marginally greater autofluorescence relative to the normal xylem present on either side of this band. As for other xylem tissues, rays which showed strong autofluorescence in localised places lacked fluorescence for galactan, and autofluorescing resin canal epithelial cells also lacked fluorescence for galactan. Phenolic extractives present in ray parenchyma cells and in resin canal epithelial cells were likely contributors to observed autofluorescence of these tissues. Control sections did not label with the secondary antibody (data not shown).

The patterns of cell wall autofluorescence and galactan immunofluorescence are more clearly resolved in the images in Fig. 13-A and -B, which were produced from the same section. The region of the cell wall in the compression wood zone

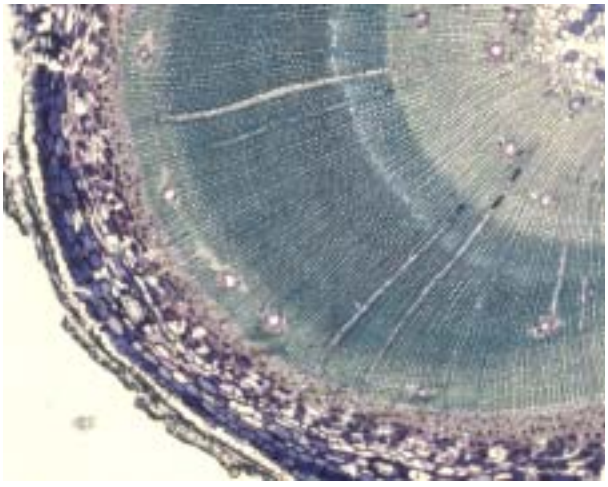

FIG. 11–Transverse section through a stem of a 1-year-old *P. radiata* seedling giving an overview of the variability of wood structure caused by mechanical stresses.

(Fig. 13-A), which showed strong autofluorescence forming a continuous band along the compound middle lamella, corresponded to an outer region of the S_2 layer (S_{2L}) where lignin concentration was greatest, as determined by UV microscopy (Yumoto *et al*. 1983). This region of the S_2 layer showed strong fluorescence from immuno-labelled galactan (Fig. 13-B), the brightness intensity being greatest in

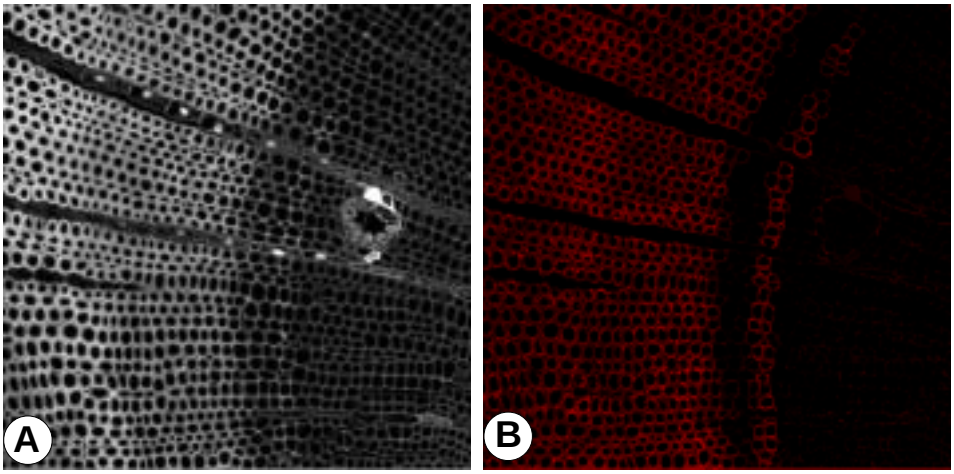

FIG. 12–Confocal micrographs of a transverse stem section of a 1-year-old *P. radiata* seedling. A: Lignin autofluorescence. B: Immunofluorescent localisation of the LM5 epitope. LM5 labelled the cell walls of compression wood cells.

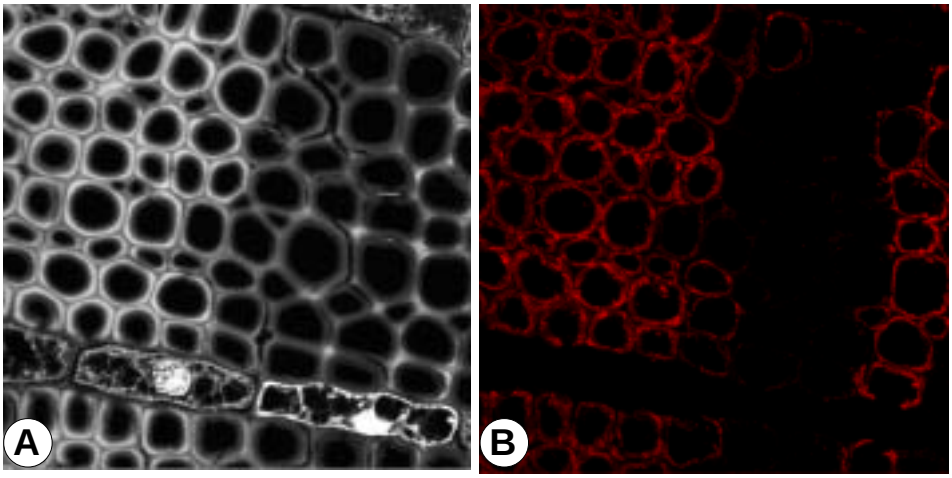

FIG. 13–Confocal micrographs of a selected area from the same section as shown in Fig. 12. A: Lignin autofluorescence. B: Immunofluorescent localisation of the LM5 epitope. Normal wood cells are not labelled. Compression wood cells are most intensely labelled in the outer S_2 cell wall region. The compound middle lamella is not labelled.

cell corner regions where lignin also occurred in highest concentrations in a range of compression wood (Donaldson *et al*. 1999; Singh & Donaldson 1999; Yumoto *et al*. 1983). The compound middle lamella regions in compression wood were not labelled with the anti-galactan antibody. The secondary xylem region, which is weakly autofluorescent and showed cellular morphology characteristic of normal secondary xylem, lacked any fluorescence for galactan. Autofluorescing ray parenchyma cells lacked any fluorescence for galactan in both normal and compression wood regions. Ray parenchyma in the sapwood of *P. radiata* is not lignified, which suggests that the observed autofluorescence of these cells is due to other factors, such as the presence of phenolic extractives.

The formation of a secondary wall in the compression wood, as in the normal wood, is a highly co-ordinated process, involving many gene products and metabolic processes. A recent statistical analysis of expressed sequence tags (ESTs) from secondary xylem-forming zones of *Pinus taeda* L. (loblolly pine) has provided evidence that certain genes are specific to compression wood development (Pavy *et al*. 2005). However, with functions known for only a tiny fraction of all the genes that may be involved in wood formation, our knowledge of the regulation of cell wall formation in compression wood is still rudimentary. Rather, specific localisation of galactan in the outer S_2 cell wall region, which becomes most highly lignified, suggests that galactan plays an important role for the cell wall architecture of the highly specialised cell walls of compression wood tracheids.

CONCLUSIONS

It can be concluded that walls of xylem cells in angiosperm and gymnosperm trees may deviate from the regular architecture due to responses to various stress factors. For example, in poplar wounding induced an increased wall thickness and a modified lignin topochemistry, restricted mainly to the secondary wall and compound middle lamella regions of xylem fibres differentiating at the time of wounding. These wound responses are assumed to contribute to an increased resistance. In pine seedlings displaying stem bending, galactan was present largely in the compression wood, being concentrated in the region of the S_2 layer, which is most highly lignified. Although it has been speculated earlier that galactan in compression wood may be associated largely with the S_1 and outer S_2 regions of the secondary wall, the present work is the first to demonstrate using the immunolabelling technique that the bulk of galactan is associated with the outer layers of the secondary wall.

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