The Pattern of Distribution of Pectin, Peroxidase and Lignin in the Middle Lamella of Secondary Xylem Fibres in Alfalfa (Medicago sativa)

S. G. WI, A. P. SINGH†, K. H. LEE and Y. S. KIM*

College of Agriculture and Life Sciences, Chonnam National University, Yongbong-dong 300, Buk-gu, Gwangju, 500-757 Korea

Received: 11 September 2004 Returned for revision: 3 November 2004 Accepted: 3 January 2005 Published electronically: 14 February 2005

INTRODUCTION

Lignin is a constituent of the secondary xylem, and its incorporation into the cellulose framework of cell walls confers strength and stability to conductive tissues as well as to the entire plant body. Cell wall lignification begins in the middle lamella region (Kutscha and Schwarzmann, 1975; Takabe et al., 1981, 1986; Joseleau and Ruel, 1997; Terashima, 2000; Donaldson, 2001) and since this part of the cell wall is crucial to cell adhesion, the stability and strength of tissues will depend upon the concentration and pattern of lignin distribution in this region. Examining the extent and pattern of lignification of middle lamella during the normal course of xylem development, therefore, is important for understanding features of abnormal lignification which may be caused by abiotic factors, such as nutrient deficiency (Downes et al., 1991) and drought (Donaldson, 2001).

Although aspects of lignification such as polymerization of monolignols deposited into cell walls are reasonably well understood (Nose et al., 1995; Higuchi, 1997; for reviews, see Terashima, 2000; Donaldson, 2001), information on micro-distribution of lignin within the middle lamella and the secondary wall is only just beginning to emerge (Daniel et al., 1991; Singh and Daniel, 2001). For example, observations using transmission electron microscopy (TEM) have provided clear differences between hardwoods and conifers with reference to the micro-distribution of middle lamella lignin, hardwoods showing much greater heterogeneity in lignin concentration (Daniel et al., 1991; Singh and Schmitt, 2000) as compared with conifers. In addition to being of interest from the perspective of cell wall formation, this feature has a profound influence on the fracture behaviour of cell walls during thermo-mechanical pulping, resulting in the surface characteristics of fibres which are widely different in soft- and hardwoods (Singh and McDonald, 2000; Singh et al., 2003b). Recognizing the urgency for making greater use of non-woody agricultural plants for fibre production, it is important to also characterize lignin distribution in these plants.

This paper provides evidence of marked heterogeneity in the micro-distribution of middle lamella lignin in alfalfa (Medicago sativa) stems. Since the middle lamella consists largely of pectic polysaccharides (Minor, 1991), and as pectins are widely regarded to be the intercellular glue which plays a role in cell adhesion (Jarvis et al., 2003), the development of cambial derivatives into fibres has been followed to see if the distribution of pectin in the middle lamella is also irregular. In addition, the distribution of hydrogen peroxide (H₂O₂) and peroxidase was also investigated because of its importance in the lignification process (Takabe et al., 1981; Nose et al., 1995; Kim et al., 2002). The observations presented lead to the proposal that the inhomogeneity in lignin distribution in the middle lamella of the secondary xylem fibres in alfalfa may be related to the irregular pattern of pectin distribution.

MATERIALS AND METHODS

Plant material

Alfalfa (Medicago sativa L.) was grown under field conditions in the nursery of the Forest Experiment Station,...
Chonnam National University, Korea. The seventh internode from the top was used for all experiments. The samples were taken in triplicate in July and December 2002, and were prepared and examined.

**Pectin and lignin detection**

For pectin detection, small pieces of alfalfa stems ($1 \times 1 \times 4$ mm) from the cambial region were fixed for 4 h in a mixture of 0-05 % ruthenium red (w/v) and 3 % glutaraldehyde (GA) (v/v) in 50 mM cacodylate buffer (CB) (pH 7-4). After washing in the same buffer, the samples were post-fixed in 1 % osmium tetroxide (w/v). After dehydration in a graded series of acetone they were embedded in Spurr’s low viscosity resin (Spurr, 1969). Ultra-thin sections were stained with uranyl acetate and lead citrate and then examined with a Jeol 1010 TEM.

For lignin detection, small pieces of alfalfa stems from the cambial region were fixed for 4 h in a mixture of 2 % GA and 4 % paraformaldehyde (PA) (v/v) prepared in 50 mM CB (pH 7-4). After washing in the same buffer and dehydration in a graded series of ethanol, the specimens were embedded in London Resin White (London Resin Co., London, UK). Ultra-thin sections (80 nm) were stained with 1 % KMnO4 (w/v) (prepared in 0-1 % sodium citrate) (Donaldson, 1992) to contrast lignin in fibre cell walls. Subsequently, the sections were examined with a Jeol 1010 TEM.

**Hydrogen peroxide ($H_2O_2$) localization**

$H_2O_2$ was detected by the cerium chloride ($CeCl_3$) method, as described by Bestwick et al. (1997). In brief, small pieces ($1 \text{mm}^3$) of tissues from the cambial region of alfalfa stems were incubated in freshly prepared 50 mM MOPS (3-(N-morpholino) propanesulfonic acid) buffer, pH 7-2, containing 5 mM $CeCl_3$ for 1 h. Subsequently, the samples were fixed in a mixture of 1·25 % (v/v) GA and 1·25 % (v/v) PA in 50 mM CB, pH 7-2 for 1 h. After washing in CB, samples were post-fixed for 1 h in 1 % osmium tetroxide (prepared in CB), dehydrated in a graded ethanol series, and embedded in London Resin White. Ultra-thin sections were examined with a TEM at 80 kV without post-staining. To confirm the specificity of $CeCl_3$ staining for $H_2O_2$, samples were incubated for 20 min in 50 mM MOPS, pH 7-2 containing either 1 mM sodium azide (to inhibit peroxidase) or 25 mg mL$^{-1}$ bovine liver catalase (to decompose $H_2O_2$). They were then transferred to $CeCl_3$ solution and incubated for 1 h and processed for TEM as described above. Some samples were fixed without the $CeCl_3$ treatment. After post-fixation in OsO4, samples were processed for TEM as outlined above.

**Immunogold labelling for peroxidase**

Peroxidase was localized using the indirect immuno-gold labelling on ultra-thin sections (Kim et al., 2002). The antibody against horseradish peroxidase type III (approx. mol. wt 44 000; Sigma, USA) was obtained from Research Genetics, Inc. (Huntsville, AL, USA). After being dissected, the specimens were immediately fixed for 2 h at 4 °C in a mixture of 0-1 % GA (v/v) and 4 % PA (v/v) prepared in 50 mM CB, pH 7-4. They were then washed in the buffer, dehydrated in an ascending ethanol series, and embedded in London Resin White. Ultra-thin sections, mounted on uncoated nickel grids (300 mesh), were incubated in the anti-peroxidase serum (primary antibody) diluted in PBS buffer containing 0-05 % Tween. The sections were then labelled with goat-anti-rabbit antiserum (secondary antibody) conjugated to colloidal gold (10 nm particle size). The sections were examined with a TEM after counterstaining with 4 % uranyl acetate. Control samples were treated as described above, except that either the primary antibody was omitted from the incubation solution or preimmune rabbit serum was used instead of the primary antibody.

**RESULTS**

Alfalfa stems provided a suitable material for the study of secondary xylem formation, as the secondary xylem in this plant develops rapidly and the extent of xylem produced is sufficient for examining stages in xylem cell development, including cell wall lignification. In all cases, the developing and mature fibres examined were part of the xylem tissues nearest the cambium. The focus of the work was to examine fibres; vessels were not examined. Whereas size difference formed the basis for recognizing fibres from vessels in the early stages of xylem development, cell size and cell wall thickness distinguished these cell types at more advanced stages of their differentiation.

**Pectin and lignin distribution**

Pectins, which were well contrasted with ruthenium red, were found to have a unique pattern of distribution in the middle lamella of cambial and developing fibre cells. The pectin distribution was inhomogeneous and also highly variable in the extent of inhomogeneity, as shown in Figs 1 and 2. Figure 1 shows the pattern of pectin distribution in the middle lamella between adjoining cells at a stage prior to the onset of lignification. The distribution of pectin, which appears as dense fibrils, in the middle lamella is irregular, with the cell corner region of middle lamella appearing less dense than other regions of this wall. Figure 2 shows the pattern of pectin distribution in the middle lamella at an early stage of secondary wall formation. A large part of the cell corner middle lamella appears lucent; in contrast, the rest of the middle lamella is markedly dense.

The staining of sections with KMnO4 revealed the pattern of lignin distribution in the middle lamella, which was also irregular, as shown in Figs 3 and 4. The lignification of the middle lamella in the cells shown in both Figs 3 and 4 appears to be complete (Fig. 4) or nearly complete (Fig. 3). Marked inhomogeneity in lignin distribution is apparent in differentiating (Fig. 3) as well as maturing fibres (Fig. 4), as judged by the presence of discrete electron-lucent regions in some parts of the middle lamella, being most pronounced in cell corners (Figs 3 and 4).
Localization of hydrogen peroxide

The distribution of hydrogen peroxide in the middle lamella was also distinctly inhomogeneous, as shown in Figs 5 and 6. Figure 5 shows a highly irregular pattern of CeCl₃ deposits, dense deposits being present in some parts and absent in others. In Fig. 6, the cell corner region of the middle lamella contains a discrete lucent region where only a few CeCl₃ particles can be spotted. In the remaining middle lamella, the distribution of CeCl₃ is patchy, some areas showing greater aggregation of particles than others.

Immuno-cytochemistry of peroxidase

The localization of peroxidase, as indicated by the location of gold particles, in the xylem initial and developing xylem is shown in Figs 7 and 8. In the xylem initial shown in Fig. 7, at a stage prior to the onset of lignification, only a few gold particles are present in the cell walls. Gold particles are more abundant in the cytoplasm where they appear to be preferentially associated with the dictyosome and vesicles. In the developing xylem undergoing lignification of the middle lamella and the secondary wall, gold particles are present in both the middle lamella and the secondary wall, with a greater concentration in the former (Fig. 8). In the cell
corner middle lamella, gold particles are mainly associated with the dense regions. The lucent and less-dense regions contain fewer or no gold particles (Fig. 8). In the controls run to test the specificity of the secondary antibody used for peroxidase, there was no labelling of cell walls (not illustrated).

**DISCUSSION**

It is apparent from the observations provided that lignin distribution in the middle lamella of the secondary xylem fibres in alfalfa stems is inhomogeneous. This was demonstrated in this work by a combination of staining and immunocytochemical techniques. Staining with KMnO₄, which has been widely used to contrast lignin in plant and wood cell walls (Maurer and Fengel, 1990; Donaldson, 1992; Singh *et al.*, 2003a), provided evidence that lignin in the middle lamella is not uniformly distributed, but is patchy, with a greater concentration in some areas than others. There were also regions in the middle lamella which did not stain with KMnO₄, indicating the presence of little or no lignin in such regions.

Using ruthenium red for staining pectic polysaccharides, it was possible to clearly observe the pattern of distribution of pectin fibrils in the cambial derivative cells undergoing expansion growth as well as in the fibre initials, which had
initiated secondary wall formation after completing extension growth. Pectins are widely regarded to be the intercellular glue which, in addition to playing a role in cell adhesion (Jarvis et al., 2003) and in determining the porosity of the cell wall, have important functions in cell growth and differentiation (Ridley et al., 2001; Knox, 2002). However, it is not known whether pectin architecture in the middle lamella influences the lignin distribution in cell walls, and the intention was to undertake this by examining the pattern of distribution of pectin and that of the subsequently deposited lignin in the middle lamella, where much of the pectin in the cell wall is located and where lignification is initiated (Donaldson, 2001). The distinct inhomogeneity in the distribution of both pectin and lignin, often in corresponding regions of the middle lamella, suggests a relationship between these cell wall components.

Additionally, the combined use of cytochemical and immunocytochemical techniques used produced evidence of a close correspondence between the pattern of lignin distribution in the middle lamella and that of the distribution of H$_2$O$_2$ and peroxidase. That is, they are all distributed irregularly, and the pattern of distribution of peroxidase and H$_2$O$_2$ in the middle lamella of lignifying cells is reminiscent of the pattern of inhomogeneous distribution of lignin in maturing and mature xylem cells.

Peroxidases have varied functions including control of cell growth using H$_2$O$_2$ as an electron acceptor (Pedreira et al., 2004). Therefore, the presence of peroxidases in the middle lamella prior to the onset of lignification is not surprising. However, a dramatic increase in gold labelling in lignifying middle lamellae is consistent with a role of peroxidase in cell wall lignification (Higuchi, 1997; Kim et al., 2002) via oxidative coupling of monolignols. Peroxidases are also known to bind to pectic molecules in cell walls (Penel et al., 1999; Schweikert et al., 2000; Cathala et al., 2001), and since the middle lamella consists largely of pectic polysaccharides (Minor, 1991), the location of peroxidase and H$_2$O$_2$ is likely to match pectin distribution in lignifying cells. Thus, the observations presented here showing irregular distribution of peroxidase and H$_2$O$_2$ further support the suggestion that inhomogeneity in the pectic architecture may be related to the irregular pattern of the deposited lignin. Once initiated at the sites of pectins, peroxidases and H$_2$O$_2$, lignin polymerization could continue, penetrating the pores within the pectic architecture and possibly following the alignment of the pectin fibrils.

Why should the initial pectin distribution be so irregular is a puzzling question. Pectin remodelling during cambial derivative differentiation (Follet-Gueye et al., 2000) may be only a part of the story which can explain the establishment of this unusual pattern of pectic architecture in the developing xylem of alfalfa.

These and earlier observations of lignin inhomogeneity in cell corner middle lamella of other plant species, such as birch (Daniel et al., 1991) and rubberwood (Singh and Schmitt, 2000; Singh et al., 2003b) suggest a fundamental difference between hardwoods and softwoods in the pattern of expansion of cambial derivatives, as lignin in the middle lamella of normal softwood tracheids is more or less homogenously distributed.

In addition to being of interest from the point of view of cell wall development, the fundamental knowledge gained on the pattern of lignin microdistribution in alfalfa, in particular, and other angiosperms such as hardwood species, in general, is also relevant from the perspective of fibre utilization. For example, it has been shown that the pattern of lignin distribution in the middle lamella of wood cell walls has a profound influence on the surface characteristics of high temperature thermo-mechanical fibres (Singh et al., 2003b). Thus, there is need to better understand the control mechanisms related to pectin distribution in the walls of expanding secondary xylem initials, and how the pectic architecture may influence initial lignin deposition (Mort, 2002).

ACKNOWLEDGEMENTS

This work was supported by grant no. R01-2003-000-10073-0 from the Basic Research Program of the Korea Science and Engineering Foundation to YSK.

LITERATURE CITED


