Study of Lignification by Noninvasive Techniques in Growing Maize Internodes

An Investigation by Fourier Transform Infrared Cross-Polarization–Magic Angle Spinning \(^{13}\)C-Nuclear Magnetic Resonance Spectroscopy and Immunocytochemical Transmission Electron Microscopy

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Noninvasive techniques were used for the study in situ of lignification in the maturing cell walls of the maize (Zea mays L.) stem. Within the longitudinal axis of a developing internode all of the stages of lignification can be found. The synthesis of the three types of lignins, \(p\)-hydroxyphenylpropane (H), guaiacyl (G), and syringyl (S), was investigated in situ by cross-polarization-magic angle spinning \(^{13}\)C-solid-state nuclear magnetic resonance, Fourier transform infrared spectroscopy, and immunocytochemical electron microscopy. The first lignin appearing in the parenchyma is of the G-type, the mixed GS dehydrogenative polymers (K. Ruel, O. Faix, J.P. Joseleau [1994] J Trace Microprobe Tech 12: 247–265). The specificity of the immunological probes demonstrated that there are differences in the relative temporal synthesis of the H, G, and GS lignins in the different tissues undergoing lignification. Immunochemical determination of the three types of lignin in transmission electron microscopy was possible thanks to the use of antisera prepared against synthetic H, G, and the mixed GS dehydrogenative polymers (K. Ruel, O. Faix, J.P. Joseleau [1994] J Trace Microprobe Tech 12: 247–265). The specificity of the immunological probes demonstrated that there are differences in the relative temporal synthesis of the H, G, and GS lignins in the different tissues undergoing lignification. Considering the intermonomeric linkages predominating in the antigens used for the preparation of the immunological probes, the relative intensities of the labeling obtained provided, for the first time to our knowledge, information about the macromolecular nature of lignins (condensed versus noncondensed) in relation to their ultrastructural localization and developmental stage.

Lignin is a complex phenyl propanoid polymer, which is integrated into the wall framework of vascular plant cells. This polymer is complex and heterogeneous with respect to the relative proportions of its three constituting monolignol units (Higuchi, 1990). Variations in lignin composition occur within plant species, tissues, cell types, and also according to developmental stage (Campbell and Sederoff, 1996). The ultrastructural heterogeneity of lignin in secondary walls may be more complex than has so far been demonstrated, because it may be influenced by various factors such as the nature of phenoxy radicals, their steady-state concentrations, the local pH, and the environment at the site of polymerization.

The molecular diversity of lignins is usually expressed in terms of the relative content of the three phenylpropane building units, resulting from the dehydrogenative polymerization of the three hydroxycinnamyl alcohols (Higuchi, 1985, 1990; \(p\)-coumaryl, coniferyl, and sinapyl). The co-polymerization of the three monolignol precursors gives rise to lignins with H, G, and S unit compositions (Yamamoto et al., 1989; Higuchi, 1990; Terashima et al., 1993). In spite of the tissue and cell wall heterogeneity, lignins from gymnosperms are predominantly composed of G units, whereas lignins from angiosperms consist essentially of the mixed GS-type. Large variations in the S to G ratio are observed between species. In grasses and cereals, however, the three types of units are always found with \(H < G < S\) (Lewis and Yamamoto, 1990). The dehydrogenative polymerization of monolignols via random coupling of transient free radicals leads to a variety of interunit substructures (Nose et al., 1995). The diversity of assembly of these substructures is the cause of an additional level of complexity in lignins, which lies in their macromolecular heterogeneity.

Because of the high II-electron density at the phenolic oxygen atom, the interunit linkage involving the phenoxy position has the highest frequency in lignins (Glasser and Glasser, 1980). This mode of coupling is the most representative of the noncondensed linkages. This is opposed to the couplings involving the C-3 or C-5 position of the phenolic ring and leading to the condensed linkages. All three of the monolignol precursors do not show an identical reactivity of their radical forms. Consequently, the H units tend to form more condensed linkages than the G lignins, and the S units tend to form more noncondensed substructures. These features are also influenced by the conditions of

Abbreviations: CP-MAS \(^{13}\)C-NMR, cross-polarization–magic angle spinning \(^{13}\)C-NMR; DHP, dehydrogenative polymer; FTIR, Fourier transform IR; G, guaiacyl; H, \(p\)-hydroxyphenylpropane; HRP, horseradish peroxidase; S, syringyl; TEM, transmission electron microscopy; z1, Zulauf method; zt, Zutropf method.

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polymerization (Sarkanen, 1971; Terashima, 1989). Several important factors may influence the polymerization process: nature and activity of peroxidases (Van Huystee, 1987), rate of generation of hydrogen peroxide at the site of polymerization, pH, and template effect of the pre-existing polysaccharide framework already synthesized in the cell wall (Siegel, 1957; Higuchi, 1985). Most important, however, is the steady-state concentration of the phenoxy radicals (Sarkanen, 1971).

Another level of complexity in the biogenesis of the lignified plant cell walls concerns the ultrastructural distribution of lignins. A large uncertainty remains concerning the temporal aspects of lignification in a developing tissue, and the relation between the ultrastructural localization and the chemical nature of the deposited lignin. There is evidence that different compositions in monomer units may characterize different cell types and different cell wall localizations. Lignins found in the primary wall, secondary wall, middle lamella, and cell corners are all different (Hardell et al., 1980; Saka and Goring, 1985, 1988; Eom et al., 1987). The chemical nature of lignin also depends on the age of the cell (Terashima et al., 1993). Thus, when radioactive-labeled 3H-monolignol glucosides were administered to various angiosperm trees, the incorporation of radioactive-labeled 3H-monolignol glucosides into the wall was found in the order of the H, G, and S units in different stages of the cell wall formation. The incorporation of the H unit was the earliest and was limited to the cell corners and middle lamellae. The G units were deposited throughout the vessel and fiber walls. The S lignin predominated in the secondary wall of fibers (Terashima et al., 1986; Fukushima and Terashima, 1990). Similarly, in monocotyledons structural heterogeneity with respect to morphological region was shown to differ among fibers, vessel, and parenchyma cell walls (He and Terashima, 1990).

To better describe the relationship between lignin heterogeneity and cytological and ultrastructural localization, noninvasive methods are of particular interest. Contrasting reagents for the lignin used in TEM are not numerous and specific probes corresponding to the different lignin units do not exist. The nondestructive techniques that have been applied to study lignins in situ, such as CP-MAS 13C-NMR (Himmelsbach et al., 1983) or FTIR and Raman spectroscopy (Agarwal and Atalla, 1986) have insufficient resolution to prove the ultrastructural scale of the microfibrillar organization of the lignified plant cell wall (Ruel et al., 1979). Lignin distribution at the cytological level has been studied in the different morphological regions and cell types by various techniques such as UV microscopy photometry (Fergus and Goring, 1970) and a combination of selective bromination and TEM energy-dispersive x-ray analysis (Saka and Goring, 1988).

The administration of radioactive-labeled monolignol glucosides and the detection of their incorporation into the cell walls was utilized (Terashima et al., 1986; He and Terashima, 1990) for the localization of the H, G, and S lignins. The technique allowed visualization of the progressive deposition of lignin in specific morphological regions of the differentiating tissues. From further improvement of the technique it was deduced that lignin in the middle lamella was of the condensed type (Fukushima and Terashima, 1990; Terashima et al., 1993). Such a conclusion about the macromolecular features of lignin may be interpreted as the consequence of the polymerization of monolignols within the pectin gel of the middle lamella. To our knowledge, this is the only reference to the condensed versus noncondensed type of lignin obtained by an in situ observation. In spite of this improvement in its specificity, microautoradiographic observation does not provide a resolution that can allow observation of lignin at the ultrastructural scale of the microfibrillar organization of the lignified cell wall (Ruel et al., 1979). Another drawback of this technique is that it can only be performed on living plant material in the active phase of lignification.

In an attempt to obtain probes that could be specific for the different types of lignin and that could be used at the ultrastructural level in TEM, we prepared novel immunogold probes by raising antibodies against pure H-, G-, and mixed GS-types of lignins that correspond to the most common types of lignins found in nature. Since there are no known pure extracted lignins, the antigens used in this study were synthetic lignins (DHPs) prepared by in vitro dehydrogenative polymerization of the corresponding monolignols (Ruel et al., 1994). In the present study the three probes were applied to a developing internode of maize used as a model to gain insight into the lignification of the different tissues during cell differentiation. The relative distribution of the three types of lignin is discussed with reference to the biochemical analysis data obtained by CP-MAS 13C-NMR and FTIR spectroscopy. Because of the macromolecular structure of the DHPs used as antigens (i.e., the relative proportion of noncondensed structures), indications on the nature of the interunit linkages of the lignins labeled by the probes could be obtained. This approach represents a novel in situ analytical and ultrastructural determination of native lignin deposition during the course of cell differentiation.

**MATERIALS AND METHODS**

**Biological Material**

The maize (*Zea mays* L.) inbred line W 401 was grown in a field trial at Tienen (Belgium) by the Zeneca Seeds Society. The plants were harvested 5 d after anthesis. The fourth internode from the top of the culm was used for electron microscopy studies.

Stalk samples were dissected into nodes and internodes and the nodes were discarded. The upper internodes were used for the study for CP-MAS 13C-NMR spectra; 5-mm-thick sections were dissected at various distances from the base of the internode. For FTIR spectra of parenchyma, handmade thin sections (0.5 cm in thickness) were cut at 1, 2, 4, 7, and 9 cm from the base of the internode. For FTIR spectra of vascular bundles, handmade dissections were performed under a magnifying lens for separation of the tissues.
DHPs

The three DHPs used in this study were gifts from Prof. O. Faix (Hamburg, Germany). They were synthesized from coniferyl alcohol, an equimolecular mixture of coniferyl alcohol and sinapyl alcohol, and p-coumaryl alcohol. Solutions of the hydroxycinnamic alcohols in phosphate buffer were pumped into a solution of HRP in the same buffer, and hydrogen peroxide was simultaneously added over a period of 120 h, according to the zt, as described in Ruel et al. (1994). The DHPs were characterized as described in Faix (1986).

Antibodies

Each polyclonal antiserum was raised in two New Zealand rabbits injected intradermally. The first injection was diluted with Freund's complete adjuvant. Booster injections were done with the antigen diluted in incomplete Freund's adjuvant every 2 weeks. Blood samples were collected alternately, 1 week after each injection, and rabbits were bled after 6 months.

Electron Microscopy

Fixation and Embedding

Samples were cut just after harvesting and immediately fixed in a freshly prepared mixture of 0.3% glutaraldehyde and 4% paraformaldehyde in a 0.05 M phosphate buffer. Samples dehydrated up to ethanol 70% were then embedded in London Resin White (hard mixture) and polymerized for 24 h at 50°C.

Immunocytochemical Labeling

Thin sections floating on plastic rings were labeled according to the protocol described in Ruel and Joseleau (1993) with a few modifications: BSA was replaced with 5% nonfat dried milk in both TBSS00 (0.01 M trisphosphate buffer, pH 7.5, containing 0.5% NaCl) and Tris buffer (0.01 M, pH 7.5). The antisera were diluted 1/30 for the anti-H and anti-G antisera and 1/50 for the anti-GS. The second-ary marker was protein A (PA10, or PA2) (Janssen Pharmaceuticals, Geel, Belgium) diluted 1/20 in the anti-GS. The secondary marker was protein A (PA10, or PA2) (Janssen Pharmaceuticals, Geel, Belgium) diluted 1/20 in the Tris buffer containing 0.5% fish gelatin plus 0.02% PEG or Tris buffer (milk 0.5%). After rinsing in Tris buffer, sections were fixed in 2.5% glutaraldehyde, rinsed in water and transferred to carbon-coated copper grids. Poststaining was in 2.5% aqueous uranyl acetate. Observations were performed with an electron microscope (400T or CM200-cryo, Philips) operating at 80 kV.

The Specificity of Antisera

Preincubation of GS antiserum with G homopolymer was carried out to suppress any possible competitive labeling of homoguaiaacyl lignin. The specificity of polyclonal antibodies was assayed by an affinity test in electron microscopy in which the antigens were embedded in London Resin White (no aldehyde fixation), and thin sections subjected to the antisera were examined by electron microscopy. The affinity was revealed by complexation with a secondary marker conjugated to gold colloid (protein A-gold), as described above.

Comparison of Labeling Intensities

For each antiserum the dilution corresponding to the optimal labeling was determined by applying a series of dilutions of the antisera to thin sections of the antigens. This was also done with a series of ultrathin sections of the same plant material. The intensity of the labeling was estimated for each dilution by manual counting of the number of gold grains per surface unit.

Immunocchemical Controls

Several control experiments were performed for each polyclonal antibody used in this study. The controls included the omission of the primary antibody from the immunolabeling experiments or the substitution of the primary antibody with the nonimmune serum, and incubation with the primary antibody that had first been saturated with the corresponding antigen. All of the tests resulted in no detectable gold deposits.

Lignin Analysis

Lignin analysis was performed by the acetyl bromide method (Iiyama and Wallis, 1988). Finely ground tissues (inferior 180 μ) were oven-dried at 60°C. The sawdust (10 mg) was placed in screw-cap tubes, and a solution of 25% (w/w) acetyl bromide in acetic acid (5 mL) containing perchoric acid (70%, 0.2 mL) was added. The tightly sealed tubes were placed in an oven at 70°C for 30 min. The resulting solution was transferred to a flask containing 2 mL sodium hydroxide (10 mL) and acetic acid (25 mL). The volume was adjusted to 100 mL with acetic acid. The lignin content was measured at 280 nm using a standard curve established with bamboo lignin (a gift from Professor T. Higuchi, Wood Research Institute, Kyoto, Japan).

CP-MAS 13C-NMR Spectroscopy

Samples of the maize internode were cut at various distances from the intercalary meristem. The samples were washed with hot water and extracted with ethanol:toluene (2:1, v/v) in a Soxhlet apparatus (Prolabo, Grenoble, France).

Natural-abundance CP-MAS 13C-NMR spectra were recorded at ambient temperature on a spectrometer (MSL 200, Bruker Instruments, Inc., Bilerica, MA) operating in a magnetic field of 4.7 teslas. Transfer of polarization between protons and carbon nuclei, at the frequency of 200 MHz, involves the dipolar interaction between the two spin systems. Chemical shifts were referenced to tetramethylsilane via hexamethylbenzene as an external standard (aliphatic peak at 17.4 ppm). Each spectrum was acquired with 3000 to 4000 scans. For dipolar dephasing, the same technique was applied, allowing a decoupling delay of 40 μs for carbon-proton contact. This gave spectra in which nonquaternary 13C resonances were eliminated.
FTIR Spectroscopy

Spectra were recorded on an FTIR spectrometer (model 1700 X, Perkin-Elmer). For collecting parenchyma spectra, hand-cut thin sections were mounted in the sample card, and the selected parenchyma area was centered in the laser beam. Transmission spectra were recorded between 4000 and 600 cm⁻¹. For the spectra of young vascular bundles, the tissues were suspended in ethanol and ground in liquid nitrogen. The ground material was then mounted in the sample card as above. For the more mature vascular bundles, the standard KBr pellet technique was used.

RESULTS AND DISCUSSION

Lignin Analysis (CP-MAS ¹³C-NMR and FTIR Spectroscopy)

During the temporal development of a maize internode all of the stages of cellular differentiation and lignification are spanned from the base to the top of the internode (Joseleau et al., 1976; Scobbie et al., 1993). Chemical analysis of the basal, middle, and top region of a 10-cm-long internode of maize showed that the lignin content gradually increased upward, as the distance from the intercalary meristem increased. Thus, lignin determination by the lignin content of 5% at the base of the internode, 6.5% in the middle part, and 9.5% in the top part. The progressive lignification within one internode is accompanied by changes in the relative proportions of the H, G, and S units spanned from the base to the top of the internode (Iiyama and Wallis, 1988) gave a lignin content of 5% at the base of the internode, 6.5% in the middle part, and 9.5% in the top part. The progressive lignification within one internode is accompanied by changes in the relative proportions of the H, G, and S units found between the base and the top of the internode, as indicated by in situ detection by CP-MAS ¹³C-NMR spectroscopy recorded from crude samples. This technique provided fingerprint indication of the predominating lignin types that are present in the different parts of the internode (Fig. 1).

Assignment of the principal signals of lignin in the region of the spectrum between 110 and 160 ppm (Bardet et al., 1985; Manders, 1987; Almendros et al., 1992) (Table I) suggested that the nature of the lignin deposited in the early lignifying tissues near the intercalary meristem differed from that of the fully lignified tissues of the top of the internode. This suggests that the activities of monolignol-synthesizing enzymes are not uniform as the internode extends. Thus, in the basal region signals corresponding to units H and G are visible, whereas S is also present but does not predominate. Conversely, in the top region lignin of the H-type is only weakly represented compared with G, and the S-type is predominant as shown by signals at 153, 136, and 57 ppm. Between the base and the top, the relative intensities of the signals at 153 and 148 ppm (Fig. 1), respectively, assigned to oxygen-linked carbons in etherified S and G units (Table I), reflect the more active synthesis of S lignin as maturation of the internode increases. The part of the spectra between around 105 and 60 ppm changes dramatically from the basal to the top part of the region. This corresponds to the enhanced deposition of polysaccharides, particularly of the cellulose in the top part, the signals of which tend to dominate over the signals of the other wall polysaccharides.

For FTIR spectra, parenchyma and vascular bundles were analyzed separately. Only a few IR spectral features allow a distinction between G and S nuclei, the most intense bands in young tissues being those of polysaccharides (Séné et al., 1995). G and S nuclei were assigned on the basis of the FTIR spectra recorded from crude samples. This technique provided fingerprint indication of the predominating lignin types that are present in the different parts of the internode (Fig. 1).

Figure 1. CP-MAS ¹³C-NMR spectra of maize sections from the base (B) and from the top (T) of the internode. In the more mature tissues situated at the top of the internode, characteristic signals of the S nuclei become conspicuous.

Table I. CP-MAS ¹³C-NMR assignments for the principal signals of the H-, G-, and S-types of lignins

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<thead>
<tr>
<th>ppm</th>
<th>Lignin</th>
<th>Assignment</th>
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<tbody>
<tr>
<td>153-152</td>
<td>S</td>
<td>C3, C5 in β-O-4</td>
</tr>
<tr>
<td>148-147</td>
<td>G</td>
<td>C3-etherified</td>
</tr>
<tr>
<td>136-135</td>
<td>S</td>
<td>C4 in β-O-4</td>
</tr>
<tr>
<td>128</td>
<td>H</td>
<td>C2, C6</td>
</tr>
<tr>
<td>116-115.5</td>
<td>G</td>
<td>C5</td>
</tr>
<tr>
<td>105-104</td>
<td>H</td>
<td>C3, C5</td>
</tr>
<tr>
<td>57-56</td>
<td>G, S aromatic</td>
<td>OCH₃</td>
</tr>
<tr>
<td>31-32</td>
<td>Alkyl</td>
<td>CH₂</td>
</tr>
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</table>
of lignin. In the spectra of the following sections (1 and 2 cm), peaks at 810, 870, and 1270 cm$^{-1}$ revealed that lignin early synthesized in parenchyma cell walls is of the G-type. At 5 cm from the base the appearance of a band of low intensity at 840 cm$^{-1}$ was indicative of S nuclei beside the bands at 810 and 870 cm$^{-1}$ of the G nuclei. Lignin of the S-type was confirmed by the absorption band at 1325 cm$^{-1}$. The same peaks revealing the increasing proportion of S units were present in the spectrum of the most mature section of the internode (at 9 cm from the base). The progressive synthesis of S nuclei in the maturing parenchyma cells of the 5- and 9-cm sections was again confirmed by the presence of more clearly defined bands at 1510 and 1595 cm$^{-1}$ in which the band at 1595 cm$^{-1}$ was of higher intensity (Evans, 1991; Faix, 1991). The intensity of the band at 1235 cm$^{-1}$, assigned to phenolic hydroxyls, increased with maturation of the tissue. It must be noted that all of the above signals of lignin were relatively weak.

In comparison, the spectra given by the vascular bundles gave stronger lignin-absorption bands. In this case the spectra of the youngest tissues, isolated from sections at 0.5 to 1.0 cm from the base of the internode, showed bands of S nuclei at 840 and 1325 cm$^{-1}$, together with a slightly positive ratio of the intensities of the bands at 1600 and 1510 cm$^{-1}$. The presence of predominating S lignin was obvious in all of the lignifying vascular bundles examined in the upper sections of the internode, the intensity of the bands becoming stronger beyond 2 cm from the intercalary meristem.

**Immunogold Labeling of Lignins of Types H, G, and GS in Transmission Electron Microscopy**

Three DHPs were synthesized in vitro by polymerization of the corresponding monolignols catalyzed by HRP in the presence of hydrogen peroxide. Thus, a G homopolymer (G-DHP), an H homopolymer (H-DHP), and a mixed GS polymer (GS-DHP) were obtained, respectively, from the polymerization of coniferyl alcohol, of $p$-coumaryl alcohol, and of a mixture of the coniferyl and sinapyl alcohols in which sinapyl alcohol predominated (Faix, 1991; Ruel et al., 1994). The mode of polymerization of the DHPs according to the zt (Sarkanen, 1971) by dropwise addition of the monolignols to the medium containing the peroxidase generally favors the production of the uncondensed type of linkages, i.e. lignins enriched in $\beta$-O-4 structures. This affects the conformation of the resulting macromolecules (Sarkanen, 1971). This is as opposed to the zt of polymerization, in which batch addition of the monolignols that generally leads to macromolecules enriched in condensed linkages. Polymerization of $p$-coumaryl alcohol has the highest tendency of the three monolignol precursors to form condensed linkages, whereas the polymerization of coniferyl alcohol may lead to either condensed or noncondensed interunit linkages, with the predominating type of macromolecule being influenced by several factors controlling the polymerization (Terashima, 1989). In particular, the Zutropf polymerization of the G-DHP used for the preparation of our immunoprobe is considered to give a polymer enriched in noncondensed subunits (Sarkanen, 1971; Terashima, 1989). This is even more the case for sinapyl alcohol, which polymerizes in a pattern favoring $\beta$-O-4 linkage formation.

The mixed GS-DHP fraction used in this study, which contained 75% S units, was characterized by a significant proportion of noncondensed units, as shown by its low proportion of free phenolic hydroxyls (O. Faix, personal communication).

For immunization of rabbits each of the DHPs was used without conjugation to a carrier. The three resulting polyclonal antisera were essayed by use of an electron microscopy affinity test. This test is used for insoluble substrates that cannot be conveniently assayed by ELISA (Ruel and Joseleau, 1993; Ruel et al., 1994). Each DHP preparation was embedded in London Resin White and thin sections were exposed to the antisera. The affinity was revealed by the application of a secondary marker conjugated to gold colloid (Fig. 3).

No cross-reactivity was observed between anti-H and the G and GS antisera. Only a slight cross-reactivity could be detected between the anti-GS(zt) and anti-G(zt) antisera. To suppress this slight competitive labeling of homoguaiacyl lignin, the GS(zt) antiserum was first incubated with the G(zt) homopolymer before being used for thin-section labeling. It is important to note that the anti-G(zt) did not label the mixed, noncondensed GS(zt) antigen. Only a very weak labeling was observed when the GS(zt) (i.e. condensed) antiserum was applied on the GS noncondensed antigen. It can therefore be concluded that the specificity of the GS(zt) antiserum is directed against the noncondensed,
mixed GS lignin. It is worth noting that the absence of cross-reactivity between the G and GS antisera and the H-antiserum excludes the possibility of interference due to entrapped residual HRP in the antigen preparation, since all three of the antigens were polymerized with the same HRP.

Lignification of Parenchyma Cell Walls

Of the three antisera used, the anti-GS antiserum gave the most significant, although discrete, labeling of the parenchyma at the base of the internode, whereas the presence of the mixed GS-type lignin in the more lignified parenchyma cell walls of the upper part of the internode was well demonstrated by the stronger labeling given by the GS antiserum (Table II). This was particularly clear in the pluristratified parenchyma cells (Fig. 4). It is interesting to note that the anti-H antiserum gave a poor labeling in the first stages of lignification of the internode, about 2.0 to 2.5 cm from the base, but that the labeling became more significant at the top of the internode. This result is somewhat surprising, since it is generally admitted (He and Tarashima, 1990; Terashima, 1993) that p-coumaryl alcohol

Table II. Relative intensities of immunolabeling in the walls of the different tissues of the maize maturing internode

| Antisera | Parenchyma | Fiber | Tracheid | Vessel | Phloem | Cell Corner
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<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Mature</td>
<td>Young</td>
<td>Mature</td>
<td>Young</td>
<td>Mature</td>
</tr>
<tr>
<td>Anti-H</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Anti-G</td>
<td></td>
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<td></td>
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<tr>
<td>Anti-GS</td>
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</table>

The results concern cell corners (tricellular junctions) between fibers. Trace. The number of plus signs refers to the intensity of the labeling. No labeling.
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is incorporated during cell wall development, and that it is especially localized in the middle lamella. On the other hand, considering that p-coumaric acid esterifies directly to maize lignin (Ralph et al., 1994), and the fact that these p-coumarate groups are incorporated in abundance during the later stages of lignification, it could be that our H probe had some affinity for these groups. This needs to be verified in future work. A surprisingly low labeling was observed with G-antiserum at any stage of lignification of the parenchyma. This observation is in apparent contradiction with the preceding FTIR spectra of parenchyma, which demonstrated that the G lignin appeared in the lignifying cells before the S units. The lack of significant labeling by the anti-G probe used in the present study may be attributed to the fact that the anti-G probe is preferentially directed toward noncondensed G lignin. This observation correlates with the results reported by Terashima et al. (1993) showing that the early formed G lignin is a condensed polymer. Application of the GS antiserum preincubated with the G antigen resulted only in a slight weakening of the labeling. This suggests that the lignin of ground parenchyma is essentially a mixed GS lignin in which the proportion of G nuclei is low.

Lignification in Fibers

Using the immunological probe for H units, a faint labeling of H lignin was visible at the earliest stage of fiber lignification. The intensity of the labeling increased with maturation, and in the most lignified fibers a uniform distribution of gold particles was observed in the S2 layers (Fig. 5). This supports the proposal that H lignin forms early in fibers and is then actively deposited during secondary thickening, as demonstrated by microautoradiography studies (He and Terashima, 1990). The labeling was also observed in the tricellular junction between fibers, suggesting that lignin of the H-type is present in this region. With the anti-G probe, no significant labeling in the younger fibers from the base of the internode was observed. However, G units could be detected in the mature fibers from the upper parts of the internode. In these cells the labeling appeared heterogenous in the polylamellar cell walls (Fig. 6B), in agreement with earlier observations of the uneven distribution of lignin in polylamellate structures (Parameswaran and Liese, 1982). It must be noted...
that the labeling provided by our G probe was consistently weak in most cell types, except vessels of the metaxylem (Table II).

Since G lignin has been shown to be present in developing maize internode (Gaudillère and Monties, 1989), and this was confirmed by the preceding FTIR and CP-MAS $^{13}$C-NMR analyses, the weak labeling was ascribed to the higher affinity of the G probe for the noncondensed lignin. Consequently, we suggest that G lignin in most cell types comprises a significant portion of the condensed linkages. This contention may be supported by the fact that in an acidic medium having a pH similar to that in secondary walls, coniferyl alcohol gives β-5 dimers, i.e. condensed dimer (Yoshida et al., 1994). Similarly, the acidic pectic environment in the middle lamella may also facilitate the formation of condensed G lignin in this area (Terashima, 1989). In the vessels of metaxylem the intensity of the labeling remained about the same all along the maturing internode, suggesting that in vessels of metaxylem noncondensed G lignin is regularly synthesized, in addition to the condensed form described by other authors (Saka and Goring, 1985).

Labeling with the anti-GS antiserum demonstrated that GS lignin was already synthesized in fibers at the earliest stage of lignification (Fig. 7, A–C). The intensity of this labeling was the strongest given by any of the three antisera all along the fibers within the internode, indicating that the more methoxylated monolignol precursors are incorporated in the fibers as soon as lignification proceeds. Moreover, the strongly positive labeling provided by our GS(zt) probe suggests that noncondensed GS lignin is abundant in fibers. This is in agreement with the anti-GS labeling that polymerization of secondary wall lignin within the template of cellulose microfibrils likely favors the formation of the extended macromolecule of noncondensed lignin (Siegel, 1957), rather than that of the bulky macromolecule of condensed lignin (Higuchi, 1990; Terashima et al., 1993). It is noteworthy that the intensity of the labeling provided by the GS antiserum was only slightly affected by preincubation with the G antigen (Fig. 7D). This supports the idea that the lignin depicted by the GS probe corresponds to a true mixed-type polymer. This result also agrees with the only slight affinity of anti-GS for the pure G homopolymer (Fig. 3).

For immunological control of the specificity of the labeling of lignin in maize tissue sections with the various lignin antisera, several experiments were performed. Omission of the primary antibody resulted in the complete absence of labeling (Fig. 8B). Substitution of any of the lignin antibodies with nonimmune serum gave negative labeling (Fig. 8C). Preincubation of the different antibodies with the different corresponding antigens abolished the labeling of maize cell walls (Fig. 8D).

**Figure 6.** Reactivity of fibers toward anti-G antiserum. A, Heterogenous distribution of the labeling in fibers from the top of the internode. In the polylamellar fiber bordering the lacuna (Lc, upper part of the micrograph) the more recently deposited layer is almost not labeled (arrowheads). In the fiber of the lower part of the micrograph, only the first deposited outer layer (OL) shows a significant presence of the homoguaiacyl polymer. The tricellular junction (TC) is slightly labeled. B, Detail of a vessel of metaxylem. The presence of the G units is strongly underlined by the anti-G serum. G lignin is deposited throughout the secondary wall, although with less intensity over the last deposited inner layers (IL). Magnifications: A, ×17,500; and B, ×12,950.

Lignin in the Cell Corners Is a Primarily Condensed Polymer and Differs from Lignin of the Cell Walls

There have been several indications that lignification starts in the cell corners (Fergus and Goring, 1970; Saka and Goring, 1985) and then proceeds in the middle lamellae. The only consistent labeling obtained in the cell corners with the three antisera was provided by the anti-H probe. That was the case in both the youngest and the oldest tissues of the internode. Therefore, no labeling in this region could be ob-

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Figure 7. Early synthesis of GS lignin in fibers and vessels. The labeling is done with anti-GS antiserum with Protein A-gold (10 nm). A, Very young fibers give a positive labeling with anti-GS. The younger fibers are less densely labeled (arrows), the vessel wall (V) is strongly reactive, and the tricellular junction (TC) gives a negative response. B, At an early stage of maturation, sclerenchyma walls show a positive reaction to the anti-GS antiserum. The tricellular junction is totally negative. C, Three fibers near the lacuna at the base of the internode. The secondary thickenings (arrows) show a heavier labeling for the mixed GS lignin than the compound middle lamella-primary wall. The fiber bordering the lacuna is less labeled than the two other fibers. Again, the lignin in the tricellular junction is unreactive to the anti-GS antiserum. D, Three fibers of the top showing a polylamellar organization. The GS lignin shows a concentric distribution. The preincubation of the anti-GS antiserum with the G antigen has only faintly affected the intensity of the labeling. This is an indication that the mixed GS lignin type is actively synthesized in these tissues. Magnifications: A, ×18,550; B, ×16,800; C, ×19,600; and D, ×18,200.

served with the two other probes at any stage of lignification. This confirmed that a specific lignification occurs in the cell corners, and that the first to be synthesized is an H lignin (Terashima, 1989). Moreover, the absence of methoxylation at C-3 and C-5 of the aromatic ring of the p-coumaryl monolignol makes this unit more susceptible to forming condensed interunit linkages than the substituted ring of the G and S precursors. From that it can be deduced that our anti-H probe has a good affinity for unsubstituted H lignin. This being valid for in vitro as well as in vivo. Therefore, it may therefore be inferred that lignin in a cell corner is a condensed lignin. Recent results obtained with immunoprobes directed against condensed DHPs confirmed the presence of condensed G lignin in cell corners (J.P. Joseleau, V. Burlat, and K. Ruel, data not shown).

CONCLUSION

The developing maize internode is a convenient model for the investigation of lignification in noninvasive lignified plant cell walls,
because it offers several degrees of complexity relative to the diversity of cell types and tissues and to the heterogeneous structure of lignins (Scobbie et al., 1993). In this actively growing material maturation of lignin globally corresponds to an increase in synthesis of methoxylated monolignols (Joseleau et al., 1976; Higuchi, 1990). This trend of progressive methoxylation accompanying maturation was verified by CP-MAS $^{13}$C-NMR spectroscopy in the samples in which all of the tissues were mixed. However, this only corresponds to an averaged estimation of the lignin composition at different stages of development in the maize internode. Higher resolving techniques such as FTIR spectroscopy and immunogold TEM, because they can be applied to specific tissues and specific ultrastructural areas of the cell walls, respectively, showed that the general concept of increased methoxylation with maturation is not representative of the temporal evolution of lignin in all of the tissues. When parenchyma cell walls begin to lignify, G monomers are synthesized first. The appearance of S nuclei in the spectra of the maturing tissues was visible in the upper half of the internode. This may correspond to the biphasic thickening of the wall associated with the laying down of structural carbohydrate, followed by a slow increase of lignification (Scobbie et al., 1993). Lignification appears to be controlled differently in vascular bundles, where S units are synthesized from the beginning of lignification.

The novel immunological probes that were used in this study allowed visualization of the progression of lignin deposition during maturation in each cell type, and characterization of structural variations in the lignin macromolecule as lignification proceeded. Our results show that all tissues do not undergo lignification simultaneously and at the same rate (Table II), indicating that lignification is a spatial and temporal phenomenon, which varies according to tissues and cell walls. Conducting and supporting tissues such as fibers, vessels, and tracheids showed a precocious lignification of their secondary walls in comparison with the walls of the parenchyma. In tracheids of protoxylem and in vessels of metaxylem, the earliest synthesized lignin of the secondary walls was found to be already highly heterogeneous with significant proportions of H units mixed with GS units, but lower proportions of homopolymeric G lignin. This ultrastructural heterogeneity of lignin in the secondary walls could not be demonstrated without the proper probes for TEM.

The conjunction of analytical techniques, such as FTIR spectroscopy and CP-MAS $^{13}$C-NMR, with an ultrastructural analysis by immunocytochemical methods in TEM has the potential to provide in vivo new insights into the biosynthetic pathways involved in the complex lignification of plants. The new markers of lignins that were used in this study allowed refined correlations between the chemical nature of lignins and their ultrastructural distribution. An interesting aspect of these immunological probes is their capacity to differentiate between macromolecular features of condensed and noncondensed lignins. Although the epitopes that are recognized by the polyclonal antisera are not precisely known, it remains that the fact that two different antisera label different zones of the cell wall demonstrates that the nature of lignin in these zones is definitely not identical. The immunolabeling brings evidence that the cell wall microenvironment influences lignin polymerization mechanisms differently according to the cell type, the tissue, and the wall layer in which the synthesis occurs.

ACKNOWLEDGMENTS

We wish to thank Prof. O. Faix (Institut für Holzchemie und chemische Technologie des Holzes, Hamburg, Germany) for the gift of DHPs, and Dr. P. Balis (Zeneca Seeds Society, Tienen, Belgium) for the gift of the maize stalks. We acknowledge the assistance of Mr. A. Rousseau (Centre d'Etudes Nucléaires de Grenoble) in recording the solid-state NMR spectra.
LITERATURE CITED


Study of Lignification by Noninvasive Technique

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