

Arnaud Day · Katia Ruel · Godfrey Neutelings
David Crônier · Hélène David · Simon Hawkins
Brigitte Chabbert

Lignification in the flax stem: evidence for an unusual lignin in bast fibers

Received: 26 November 2004 / Accepted: 29 January 2005 / Published online: 21 June 2005
© Springer-Verlag 2005

Abstract In the context of our research on cell wall formation and maturation in flax (*Linum usitatissimum* L.) bast fibers, we (1) confirmed the presence of lignin in bast fibers and (2) quantified and characterized the chemical nature of this lignin at two developmental stages. Histochemical methods (Weisner and Maüle reagents and KMnO_4 -staining) indicating the presence of lignin in bast fibers at the light and electron microscope levels were confirmed by chemical analyses (acetyl bromide). In general, the lignin content in flax bast fibers varied between 1.5% and 4.2% of the dry cell wall residues (CWRs) as compared to values varying between 23.7% and 31.4% in flax xylem tissues. Immunological and chemical analyses (thioacidolysis and nitrobenzene oxidation) indicated that both flax xylem- and bast fiber-lignins were rich in guaiacyl (G) units with S/G values inferior to 0.5. In bast fibers, the highly sensitive immunological probes allowed the detection of condensed guaiacyl-type (G) lignins in the middle lamella, cell wall junctions, and in the S1 layer of the secondary wall. In addition, lower quantities of mixed guaiacyl–syringyl (GS) lignins could be detected throughout the secondary cell wall. Chemical analyses suggested that flax bast-fiber lignin is more condensed than the corresponding xylem lignin. In addition, H units represented up to 25% of the monomers released from bast-fiber lignin as opposed to a value of 1% for the corresponding xylem tissue. Such an observation indicates that the structure of flax bast-fiber lignin is significantly different

from that of the more typical ‘woody plant lignin’, thereby suggesting that flax bast fibers represent an interesting system for studying an unusual lignification process.

Keywords Bast fiber · Cell wall · Flax · Immunolabeling · Lignin

Abbreviations AGP: Arabinogalactan protein · CWR: Cell wall residue · G: Guaiacyl unit · GRP: Glycine-rich protein · H: *p*-hydroxyphenyl unit · HPLC: High pressure liquid chromatography · S: Syringyl unit · TEM: Transmission electron microscopy

Introduction

Flax (*Linum usitatissimum* L.) is an economically important fiber crop. The cellulose fibers (bast fibers) of this plant are characterized by a number of physico-chemical and mechanical properties of importance in the textile and composite polymer industries (Baley 2002). Bast fibers are located in the stem cortex between the epidermis and the xylem core and are considered as primary sclerenchyma phloem fibers. At maturity, individual fiber cells with a thick secondary wall and extremely reduced lumen can reach 150 mm in length with a diameter of 15–25 μm .

The physical properties of flax fibers are determined not only by the chemical and structural properties of the different cell wall polymers, but also by their spatial organization within the cell wall and by the interactions between different polymers. Cellulose, the main structural component of flax fibers, is thought to play a predominant role in determining the mechanical properties of the fiber cell wall, but other components of the cell wall might also contribute to these properties (Girault et al. 1997).

Individual bast fibers (elementary fibers) are ‘glued together’ at their middle lamella with ‘a pectin-rich

A. Day · G. Neutelings · H. David · S. Hawkins
Laboratoire de Physiologie des Parois Végétales,
UPRES EA 3568-USC-INRA, UFR de Biologie,
USTL, 59655 Villeneuve d’Ascq cedex, France

A. Day · D. Crônier · B. Chabbert (✉)
INRA Parois Végétales et Matériaux Fibreux,
UMR FARE, BP 224, 51686 Reims, France
E-mail: chabbert@inra-reims.fr
Fax: +33-326-773599

K. Ruel
Centre de Recherches sur les Macromolécules Végétales,
UPR 5301 - CNRS, BP 53, 38041 Grenoble Cedex 9, France

cementing material' to form bundles orientated in parallel to the longitudinal axis of the stem. The ease with which elementary fibers can be separated depends upon the chemical nature of the 'cementing material' in the common middle lamella between cells (Girault et al. 2000). In flax fibers, it has been suggested that the presence of various 'phenolic compounds' reinforces the cementing material of the middle lamella. As a result, the middle lamella is more resistant to microbial degradation during the retting stage and fiber separation becomes more difficult (Sharma et al. 1999). While it seems likely that the presence of phenolic compounds has a negative effect on fiber bundle dissociation, the exact chemical nature of these substances has not yet been confirmed.

For example, some workers consider that the phenolic compounds in question are lignins (McDougall 1992; Akin et al. 1996; Gorshkova et al. 2000) whereas others suggest that they are composed of diverse aromatic molecules such as flavonoids, anthocyanins or cinnamic acids (Love et al. 1994; Gamble et al. 2000; Morrison et al. 2003). The uncertainty surrounding the chemical nature of flax fiber phenolics can be partly explained by the fact that they are generally present in low amounts (0.5–7.0%) as compared with the much higher amounts found in xylem tissues (Gorshkova et al. 2000; Kotelnikova et al. 2000).

Generally, there is a lack of detailed information about the nature of phenolic compounds in the bast fibers of different annual plants. Such a paucity of information is in contrast to studies on woody plants, where abundant chemical information on the lignin present in xylem tissue is available (Monties 1989). Lignin itself is a highly complex polymer composed of hydroxycinnamyl alcohols or 'monolignols' [the phenylpropane units *p*-hydroxyphenyl-propane (H), guaiacyl (G) and syringyl (S)] and displays a large diversity depending upon the plant species, cell type, and environmental factors. Monolignols are interconnected by a variety of ether (carbon–oxygen) and carbon–carbon bonds that are usually classified into condensed linkages, such as biphenyl, biphenyl ethers, and non condensed linkages including alkyl–aryl ether linkages (β -O-4 and α -O-4; Monties 1989).

Since the presence of lignin and/or phenolic compounds in flax bast fibers appears to be negatively correlated with fiber separation, the chemical characterization of such compounds represents an important first step toward the improvement of fiber quality during processing, also relevant for textile application. The aim of this study was therefore to characterize in detail the nature of the phenolic compounds associated with bast fibers, as well as providing information on their distribution within the fiber cell wall. As we also wished to know whether these phenolic compounds are characteristic of bast fibers, or of the flax plant itself, we decided to analyze the lignin and other phenolic compounds in the xylem tissue of the flax stem.

Materials and methods

Plant material

Flax plants (*Linum usitatissimum* L.); fiber cultivar hermes; were sown in March 2000 and grown under field conditions at Dunkerque (Nord, France) by professional flax producers (Institut Technique du Lin, Paris). The plants analyzed were randomly harvested at two developmental stages: (1) flowering (10-week-old plants, approximately 85 cm in height, 50% of flowers open) and (2) seed maturation (14-week-old plants, approximately 85 cm in height, yellowing capsules). These two stages were chosen, since flax plants are typically harvested after flowering, but before seed maturation. Following elimination of the first 10 cm of the stem above ground level, two further 10-cm regions were isolated for study: the 'basal region' (the next 10 cm at the basal end of the stem) and the 'apical region' (the 10 cm region under the first floral branch point). For chemical analyses, the stem samples were separated by peeling into: (1) bast fiber-rich strips ('outer tissues', also containing phloem, epidermis and cortical parenchyma accounting for approximately 20% total tissue) and (2) xylem-rich cores ('inner tissues', also containing pith tissue accounting for approximately 5% total tissue).

Histochemical analyses of flax stem samples

Fresh free-hand transverse sections were cut from the basal regions of flax stems and the presence of lignin was determined by staining with the Weisner reagent (phloroglucinol–HCl) (Clifford 1974) that gives a red coloration in the presence of lignin cinnamaldehyde groups, or the Maüle reagent (Meshitsuka and Nakano 1979), which gives a purple red coloration with syringyl lignin. Observations were made using a Nikon Eclipemicroscope (model TS 100) and Nikon Coolpix camera system (model E-950).

Transmission Electron Microscopy (TEM) procedures

Fixation and embedding

Small transverse slices of basal regions (1 mm in thickness) of flax stems, obtained by free-hand sectioning, were fixed in a freshly prepared mixture of 0.2% glutaraldehyde (v/v), 2% *para*-formaldehyde (w/v) in 0.05 M phosphate buffer (pH 7.0–7.2). After rinsing in phosphate buffer, samples were dehydrated in a graded ethanol series and embedded in LR White resin (hard mixture, TAAB) and polymerized 24 h at 50°C as previously described (Joseleau and Ruel 1997).

Potassium permanganate labeling

Free-hand transverse sections of basal regions (1 mm in thickness) of flax stems were fixed in a freshly-prepared

mixture of 5% KMnO_4 (Kerr and Goring 1975). Samples were then vacuum-incubated for 90 min at room temperature and then rinsed in water (5 min) followed by four rinses (15 min each) under vacuum. Observations were performed with a Philips CM 200 Cryoelectron microscope operating at 80 kV.

Immunocytochemical labeling

Three polyclonal antibodies raised against synthetic lignin dehydropolymers (DHPs) were prepared and used as antisera as described previously (Ruel et al. 1994; Joseleau and Ruel 1997): (1) Anti-Gzl (directed against guaiacyl (G) lignin homopolymer with condensed interunit bonds), (2) Anti-GSzl (directed against mixed guaiacyl/syringyl (GS) lignin polymer with condensed interunit bonds) and (3) Anti-GSzt (directed against mixed guaiacyl/syringyl (GS) lignin polymer with non-condensed interunits bonds).

Immunolabeling for TEM was done on ultrathin transverse sections (50 nm) of basal regions of flax stems as previously described (Ruel et al. 2002), and observations were performed at 80 kV with a Philips CM 200 Cryoelectron microscope. In order to maintain the same experimental parameters, comparative immunolabeling experiments were carried out in parallel. The pre-immune serum for each antibody was assayed under the same conditions used for immunolabeling.

Cell wall residue preparation

Outer or inner tissues from flax stem regions were ground to a fine powder in liquid nitrogen with a mortar and pestle. Compounds that were not covalently bound were sequentially removed from the ground samples by successive extraction with 80% ethanol at room temperature. The obtained material was called Cell Wall Residue (CWR).

Lignin content determinations

CWR lignin content was estimated by a spectrophotometric procedure using acetyl bromide (Iiyama and Wallis 1990) and by the Klason procedure (Effland 1977) as modified by Monties (1984). Data were analyzed by analysis of variance (ANOVA, Minitab programme) and differences were considered as significant at the 5% level ($P \leq 0.05$).

Lignin characterization

Lignin characterization was performed using thioacidolysis and alkaline nitrobenzene oxidation that disrupt the noncondensed intermonomer linkages (alkyl-aryl ether). Nitrobenzene oxidation cuts the propane side chains giving rise to C6-C1 aldehydes, whereas,

thioacidolysis maintains the phenylpropane structure intact and gives rise to trithioether derivatives. Data were analyzed by analysis of variance (ANOVA, Minitab programme) and differences were considered as significant at the 5% level ($P \leq 0.05$).

Thioacidolysis

The thioacidolysis reaction was performed in triplicate on 10 mg of CWR at 100°C using ethanethiol/ BF_3 etherate/dioxane reagent as detailed previously (Vallet et al. 1996). After 4-h reaction, the mixture was diluted with water containing tetracosane as an internal standard and extracted with dichloromethane. Guaiacyl (G) and syringyl (S) thioethylated monomers were analyzed as their trimethylsilyl derivatives using a gas chromatograph, equipped with a fused silica capillary DB1 column (30 m \times 0.3 mm) and flame ionisation detector. The temperature gradient was 160–280°C at 2°C min (Vallet et al. 1996). Measurements were performed in triplicate.

Nitrobenzene oxidation

Nitrobenzene oxidation was performed as described by Billa et al. (1996). Sodium hydroxide (5 ml, 2 M) and nitrobenzene (0.5 ml) were added to CWR (20–30 mg) in a Teflon vial enclosed within a stainless steel autoclave and samples were heated in an oil bath (3 h, 160°C). After addition of the internal standard (3-ethoxy-4-hydroxybenzaldehyde), the cooled reaction mixture was diluted with 20 ml H_2O and extracted with 3 \times 25 ml CH_2Cl_2 , to eliminate the nitrobenzene. The aqueous residue was then adjusted to pH 1–2 with 6 M HCl, extracted with 3 \times 25 ml CH_2Cl_2 , and finally dried over anhydrous Na_2SO_4 . After removal of the solvent, the lignin oxidation products were analyzed by HPLC on a Spherisorb S5 ODS2, RP18, 4.6 \times 250 mm (WATERS) column. Gradient elution was as previously described (Beaugrand et al. 2004) using a combination of acetonitrile, methanol and 1% orthophosphoric acid in ultra-pure water. The lignin oxidation products were quantified at 280 nm using commercial standards. Measurements were performed in triplicate.

Cell wall phenolic acid characterization

For the analyses of ether- and ester-linked phenolic acids, 40 mg CWR was heated (2 h, 170°C) in the presence of 10 ml 4 M NaOH with constant stirring under nitrogen flow (Iiyama et al. 1990). Alkali filtrate was acidified to pH 1 with HCl, mixed with 3,4,5 trimethoxy-*trans*-cinnamic acid as an internal standard and extracted three times with 25 ml ether. The organic phase was dried under reduced pressure, dissolved in 1.5 ml of methanol/water (1/1, v/v) and filtered (0.45 μm) prior to HPLC as previously described for

nitrobenzene oxidation. Ether- and ester-linked phenolic acids were quantified at 302 nm using commercial standards. Measurements were performed in triplicate.

Results

Microscopic evidence for the presence of lignin in flax stems

The distribution of flax stem ‘lignin’ at the seed maturation stage (stage 2) was determined by histochemical staining of freshly prepared cross-sections using the Maüle and Wiesner reactions (Fig. 1a, b). Both stains gave an intense red coloration of the xylem, indicative of its highly lignified nature. The red-purple staining observed with the Maüle test suggests that flax xylem lignin is a G–S type lignin characteristic of dicotyledons (Iiyama and Pant 1988). In outer tissues (phloem, bast fibers, cortical parenchyma, and epidermis), a discrete red coloration with the Weisner reagent could only be detected in the cell corners and in the middle lamella of bast fibers (Fig. 1a). No red-purple coloration could be detected in the bast fibers with the Maüle test (Fig. 1b) suggesting that the ‘lignin’ associated with the fibers is poor in S units.

In addition to its role as a fixative, KMnO_4 also reacts selectively with lignin, and can therefore be used as a highly electron-sensitive stain for characterizing lignin

deposition in cell walls at the ultrastructural level. In xylem cells (Fig. 1d), the middle lamella and the primary cell wall were more heavily stained than the secondary cell wall. In addition, the S1 layer of the secondary cell wall was also more intensely stained than the S2 layer. KMnO_4 -staining of bast fibers (Fig. 1c) showed that the compound middle lamella (cell corner, middle lamella, and primary cell wall) of all bast fibers presented an intense staining, similar to that observed in xylem fibers. In contrast, the thick secondary cell wall was only weakly stained except for a narrow 1 μm -wide band at the primary/secondary cell wall junction which may correspond to the S1 layer of the secondary cell wall.

Chemical evidence for the presence of lignin in flax stems

The lignin contents of flax inner (xylem-rich) and outer (fiber-rich) stem tissues were determined by the acetyl bromide method at the flowering (stage 1) and seed maturation (stage 2) stages. Phloroglucinol–HCl staining (data not shown) confirmed that outer tissues (obtained by peeling) were not contaminated with inner (xylem) tissues that could represent a source of possible errors in subsequent chemical analyses.

The lignin content of flax stems was tissue-dependent (Fig. 2): the inner tissues were approximately 10-fold richer in acetyl bromide lignin (23.7–31.4% dry CWR) than the outer tissues in which the lignin content varied

Fig. 1 a–d Histochemical and cytochemical detection of lignin in basal regions of flax stems at the seed maturation stage. Staining by phloroglucinol–HCl (a) and by Maüle reaction (b) of free-hand cross-sections; lignin labeling by KMnO_4 -staining in bast fiber bundles (c) and xylem tissues (d) of ultrathin cross-sections. *bf* bast fibers, *cc* cell corner, *cp* cortical parenchyma, *ep* epidermis, *ml* middle lamella, *pcw* primary cell wall, *ph* phloem, *S1* S1 secondary cell wall, *S2* S2 secondary cell wall, *sx* secondary xylem, *xf* xylem fiber

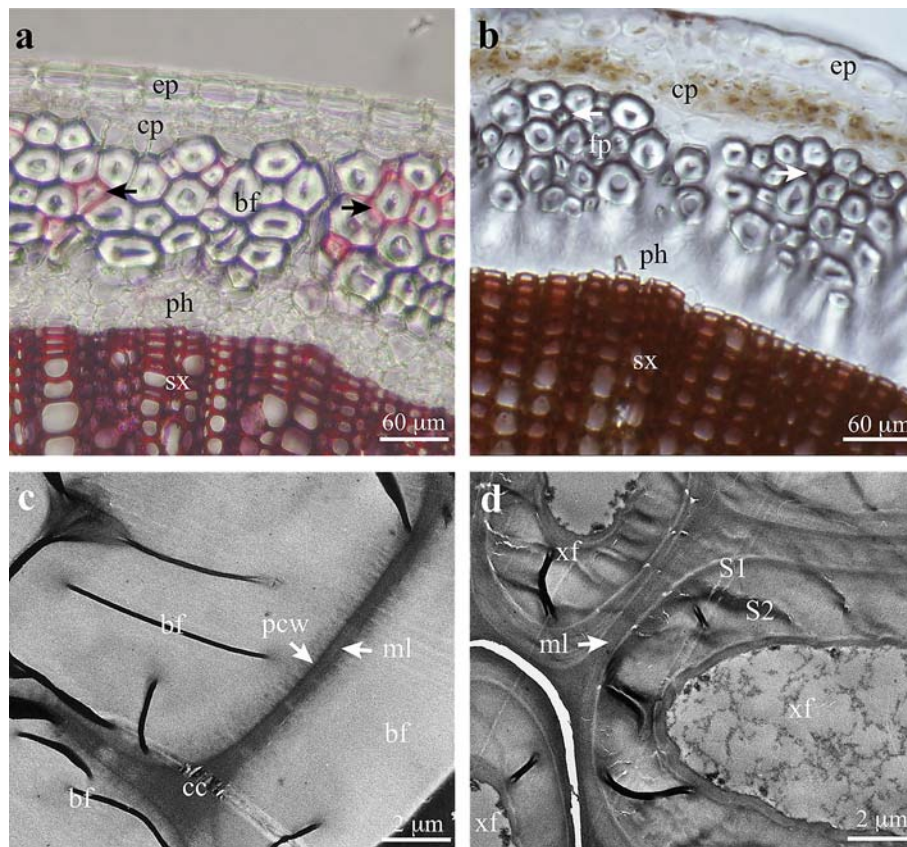


Fig. 2 a, b Acetyl-bromide lignin content in outer- (a) and inner (b) tissues of flax stems. Stage 1: flowering stage, stage 2: seed maturation stage. Each value is the mean \pm standard deviation of three separate assays

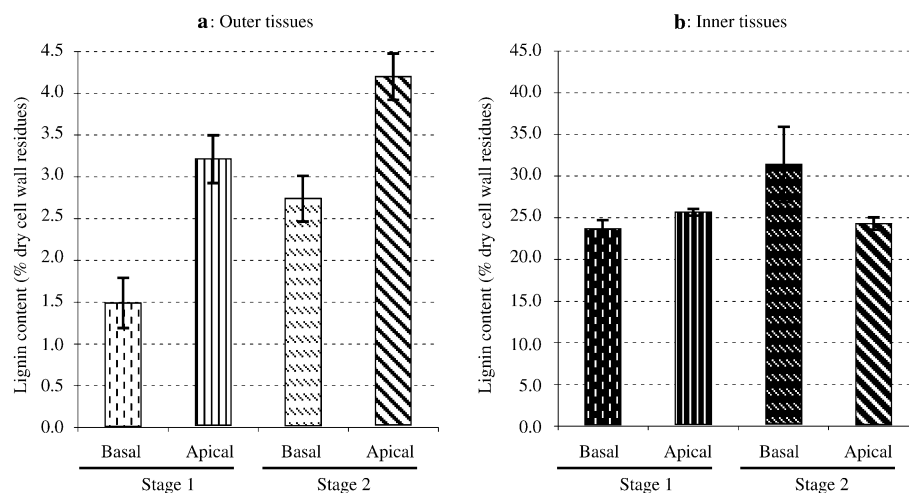


Table 1 Total phenolic acid content (*p*-coumaric + ferulic + sinapic acids) in inner- and outer flax stem tissues as determined by alkaline hydrolysis. Each value is expressed in $\mu\text{mol/g}$ dry CWR and is the mean \pm SD (in brackets) of three separate assays and the variation was estimated to be 5%

	Inner tissues	Outer tissues
Basal region stage 1	< 0.01	0.22 (0.01)
Apical region stage 1	< 0.01	0.31 (0.02)
Basal region stage 2	< 0.01	0.51 (0.07)
Apical region stage 2	< 0.01	0.59 (< 0.01)

from 1.5% (basal region, stage 1) to 4.2% (apical region, stage 2) of dry CWR. In the outer tissues, the lignin content was always higher ($\geq 1.5\times$) in apical regions as compared to basal regions, whereas no significant differences were detected between apical and basal regions in the inner tissues. The lignin content increased in the outer tissues by 83% (basal regions) and 31% (apical regions) between the two developmental stages. In contrast, in the inner tissues a significant increase (32%) in lignin content was only observed in basal regions.

Table 2 Immunolabeling pattern in xylem- and bast-fibers from basal regions of flax stems using three different polyclonal antibodies raised against synthetic lignins (DHPs). Anti-Gzl directed against G lignin homopolymer with condensed interunit bonds,

Cell type	Antisera	Epitope	Cell wall region					
			cc	ml	pI	S1	S2	S3
Xylem fibers	Anti-Gzl	G, condensed	+	-	-	++	++	++
	Anti-GSzl	G-S, condensed	-	+	+	++	++	+
	Anti-GSzt	G-S, noncondensed	-	-	-	+	++	+
Bast fibers	Anti-Gzl	G, condensed	+	+	ND	+	+/-	-
	Anti-GSzl	G-S, condensed	-	+/-	ND	+	+	-
	Anti-GSzt	G-S, noncondensed	-	-	ND	+	+/-	-

cc cell corner, ml middle lamella, pI primary cell wall, ND not determined (individual elementary bast fibers become separated in the primary cell wall region during thin-section preparation and it is therefore difficult to estimate the labeling intensity)

S1 = S1 secondary cell wall layer, S2 = S2 secondary cell wall layer, S3 = S3 secondary cell wall layer

++ = intense labeling, + = medium labeling, +/- = weak labeling, - = no labeling

In order to verify the possibility of an overestimation of lignin levels due to the presence of phenolic acids, we estimated the yield of wall-linked phenolic acids released by alkali treatment (Table 1). Inner tissues contained phenolic acids in trace amounts ($< 0.01 \mu\text{mol/g}$ CWR), whereas outer fiber-rich tissues had quantifiable *p*-coumaric acid, ferulic acid, and at stage 2, sinapic acid. However, even when the three acids are taken into account, they represent only $0.60 \mu\text{mol/g}$ CWR or 0.012% (apical region, stage 2) of dry CWR, indicating that phenolic acids do not make a significant contribution to the lignin estimation.

To confirm these results, we used a gravimetric procedure (Klason method) for lignin determination. The use of this technique gave lignin values similar to those obtained with acetyl bromide for the inner tissues (data not shown). However, Klason lignin values obtained with the outer tissues were highly variable and irreproducible. These observations suggest that, as previously observed for several other annual plants and/or young woody plants (Morrison 1976; Dence 1992), the Klason method is inappropriate for quantifying lignin in weakly lignified tissues.

Anti-GSzl directed against mixed GS lignin polymer with condensed interunit bonds, Anti-GSzt directed against mixed GS lignin polymer with noncondensed interunit bonds

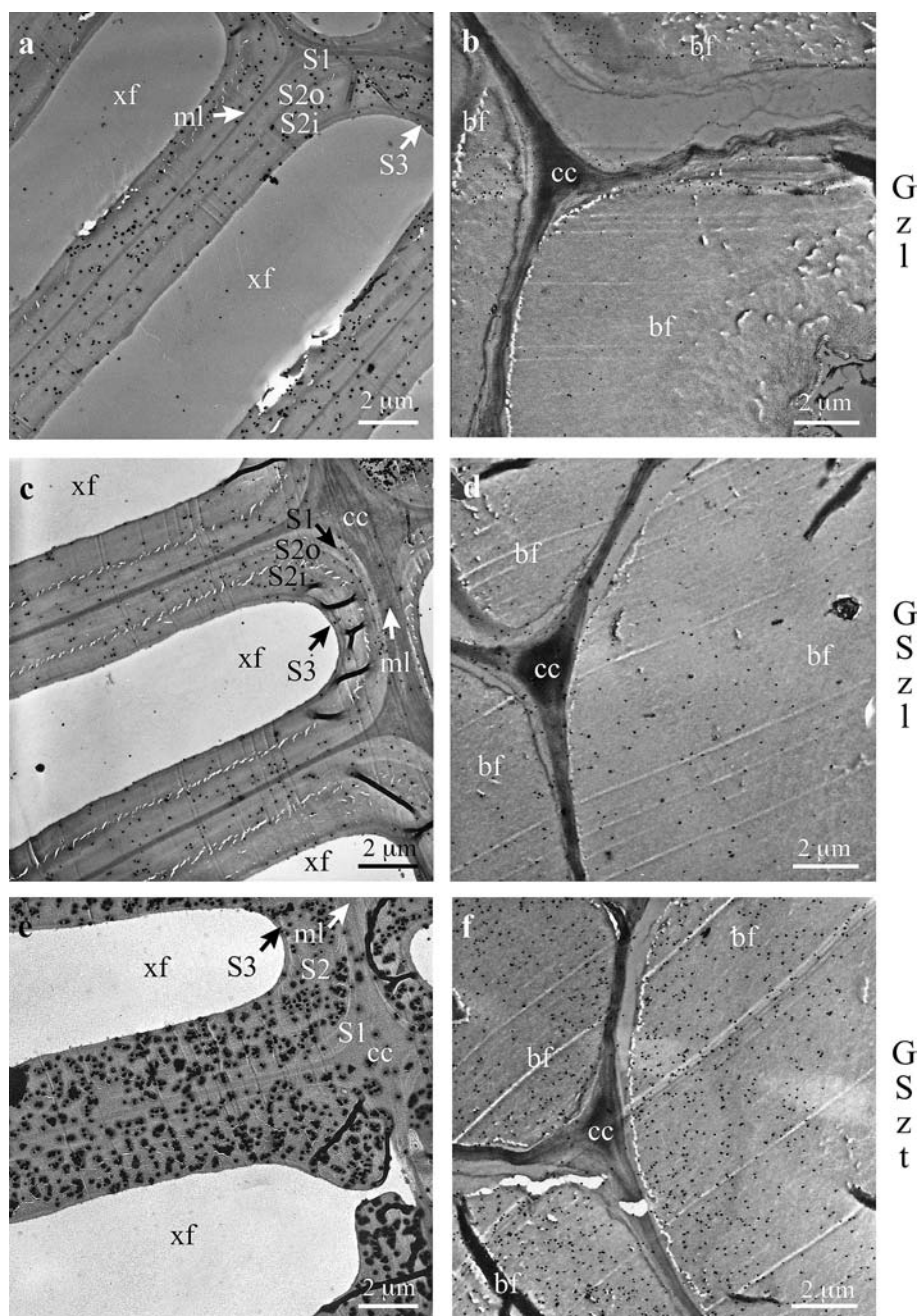
Lignin characterization and distribution in flax inner- and outer tissues

In order to obtain data on lignin distribution at the ultrastructural level in basal stem regions, we utilized three different polyclonal antibodies, anti-Gzl, anti-GSzl, and anti-GSzt raised against synthetic lignins (DHPs; Ruel et al. 1994; Joseleau and Ruel 1997). The results are summarized in Table 2. The labeling pattern (Fig. 3a, c, e) in flax xylem cell walls at stage 2 is characteristic of that previously observed in dicotyledonous wood (Ruel et al. 1999). Although such observations were not confirmed by statistical analyses of the labeling pattern, and the size of silver grains may vary from one preparation

to the other as a result of the labeling process (temperature and enhancement time), the labeling density did not appear to change dramatically between stage 1 and stage 2.

The use of the antiserum anti-Gzl (condensed guaiacyl epitopes, prevalence of C–C interunit linkages) gave rise to a significant and homogenous labeling of the whole secondary cell wall and the cell corners of xylem fibers; no labeling was detected in the middle lamella and the primary cell wall (Fig. 3a). The GSzl antibodies (condensed G–S epitopes) gave rise to a dense labeling in the S1 and outer part of the S2 secondary cell wall layers, but only a weak labeling in the middle lamella and the primary wall (Fig. 3c). GSzt antibodies

Fig. 3 a–f Identification and topochemistry of lignin subunits in xylem fibers (**a, c, e**) and bast fibers (**b, d, f**) Immunocytochemical localization of condensed G lignin subunits (**a, b**), condensed mixed GS lignin subunits (**c, d**), and noncondensed mixed GS lignin subunits (**e, f**). *bf* bast fibers, *cc* cell corner, *ml* middle lamella, *pcw* primary cell wall, *ph* phloem, *S1* S1 secondary cell wall, *S2i* inner region of S2 secondary cell wall, *S2o* outer region of S2 secondary cell wall, *S3* S3 secondary cell wall, *xf* xylem fiber



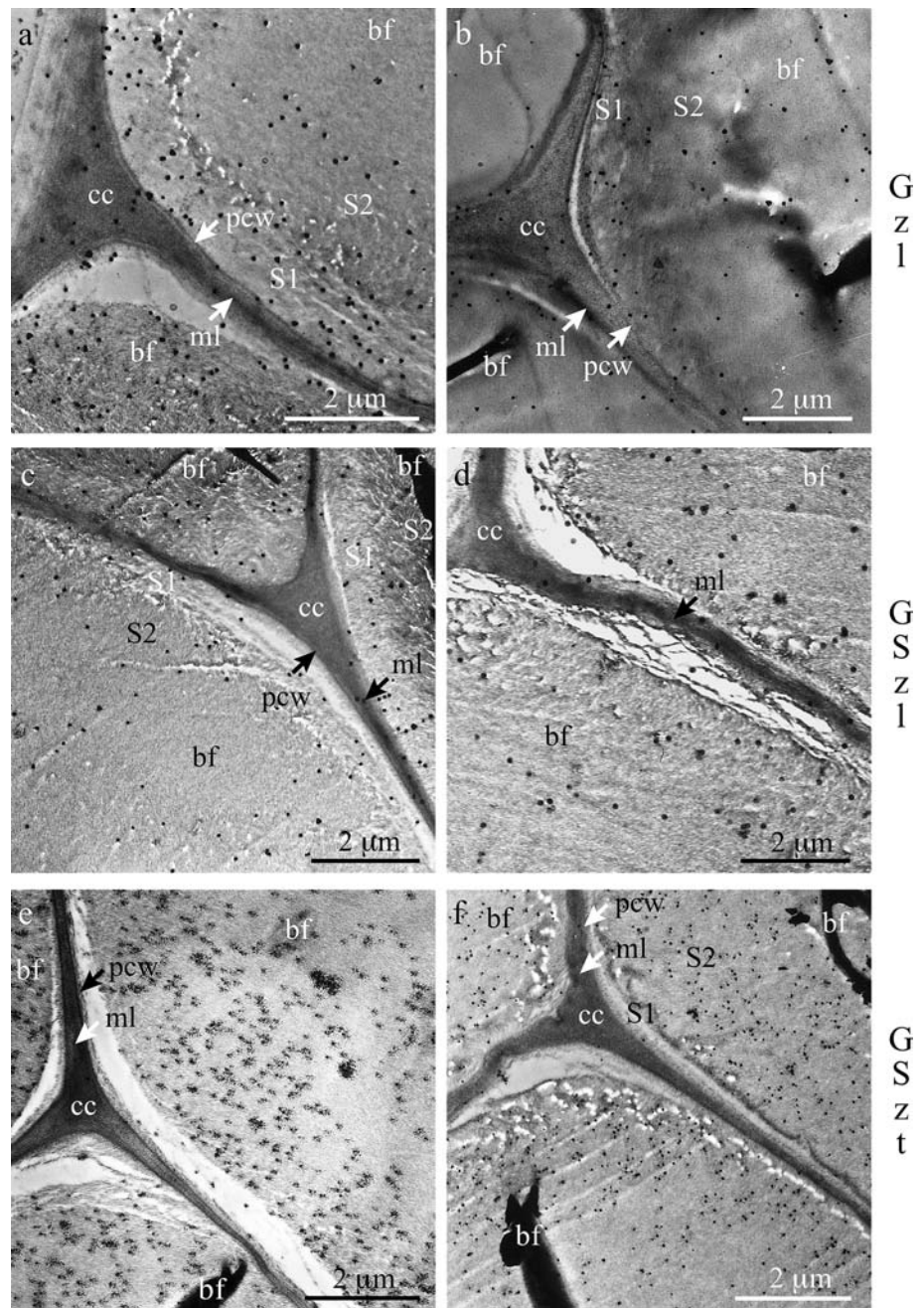
(noncondensed G–S epitopes) gave a strong labeling pattern uniquely in the secondary cell wall, particularly in the S2 layer (Fig. 3e).

The immunocytochemical labeling pattern observed in all the basal stem regions of stage 2 bast fibers was generally much less dense than in xylem fiber cells, suggesting a limited lignification as indicated by acetyl bromide analyses. Nevertheless, labeling with Gz1 antibodies could be observed in the cell corner and middle lamella and in the secondary cell wall, the labeling intensity decreased from the S1 to the S2 layers, the S3 layer was not labeled (Fig. 3b). Condensed GS lignin subunits (GSz1 antibodies) were detected throughout the S1 and the S2 layers of the bast fiber secondary cell wall,

while the S3 layer was not labeled (Fig. 3d). Such a distribution is similar to that observed in xylem fibers. The GSz1 antibodies also recognized some epitopes in the middle lamella but not in the cell corners (Fig. 3d). As with xylem fibers, the GSzt antibodies labeled both the S1 and S2 layers of the secondary cell wall, however, in contrast to xylem fibers, it was the S1 layer that was the most intensely labeled in bast fiber cells (Fig. 3f).

Immunocytochemical examination of the lignin distribution in bast fiber- (Fig. 4) and xylem fiber (data not shown) cell walls at stage 1, showed a very similar pattern to that observed at stage 2, suggesting that the extent of the lignification does not change significantly between these two developmental stages. Although such

Fig. 4 a–f Immunolabeling of condensed G lignin subunits (**a, b**), condensed mixed GS lignin subunits (**c, d**), and noncondensed mixed GS lignin subunits (**e, f**) in flax bast fibers at flowering (**a, c, e**) and seed maturation stages (**b, d, f**). *bf* bast fibers, *cc* cell corner, *ml* middle lamella, *pcw* primary cell wall, *S1* S1 secondary cell wall, *S2* S2 secondary cell wall



observations were not confirmed by statistical analysis of the labeling pattern, these observations suggest that lignification of flax bast fibers is already well-advanced by the flowering stage.

Chemical characterization of flax inner- and outer stem tissue lignin

The immunocytochemical characterization of flax stem lignin was completed by chemical analyses (thioacidolysis (Table 3) and nitrobenzene oxidation (Table 4)). Such powerful degradation techniques provide estimates of the relative proportions of monolignols involved in labile ether linkages (β -O-4 and α -O-4).

Table 3 shows the recovery yields of thioacidolysis products from flax basal and apical stem regions in both outer- and inner tissues. Only mono- (G) and di-methoxylated (S) monomers were detected indicating that flax stem lignin can be classified as an angiosperm GS lignin. In inner stem tissues, the total (S + G) values ranged from 285.5 μ mol/g to 324.9 μ mol/g dry CWR, as compared to values of 4.8–12.8 μ mol/g dry CWR in outer stem tissues. For the inner stem tissues, the G and S values did not vary significantly, neither as a function of the developmental stage nor of the stem region analyzed (basal versus apical region). These results gave rise to an S/G value varying between 0.14 and 0.21 suggesting that flax stem secondary xylem is rich in G units.

With regard to the outer stem tissues, G and S values varied as a function of the stem regions analyzed (basal versus apical) but did not appear to vary significantly as a function of the developmental stage. The S/G values (0.31–0.45) of outer stem tissues were higher than those observed in inner tissues but remained inferior to 1, thereby indicating that the lignin associated with flax bast fibers is also rich in G units.

Assuming that (1) acetyl bromide determination gives a correct estimation of the lignin content in flax stems and (2) thioacidolysis and nitrobenzene oxidation result in 100% cleavage of the noncondensed lignin structures,

the proportion of noncondensed lignin can be determined. Calculations of the quantity of lignin monomers (S + G) released per g lignin (acetyl bromide) indicated that the quantities released from outer stem tissues (2.3–17.2%) were lower (except for the stage 1 basal stem regions with a value of 17.2%), and more variable, than from inner stem tissues (20.7–25.2%). These observations suggest that flax outer tissue lignin is more condensed than inner tissue lignin.

After nitrobenzene oxidation (Table 4), three lignin degradation products: 4-hydroxybenzaldehyde, vanillin, and syringaldehyde corresponding to H, G, and S lignin subunits, respectively, could be detected in all samples. Trace amounts of phenolic acids were also detected (data not shown). In inner stem tissues, the total (H + V + S) values were between 15- and 35-fold higher (for similar stages and stem regions) than in outer tissues. This difference is of the same order of magnitude to that observed with the thioacidolysis results (22 \times –64 \times). In contrast to the inner stem tissues, where H units represent only 1% of total degradation products, in the outer stem tissues this value rises to approximately 25% (basal region, stage 1). The observation of H units with nitrobenzene oxidation, but not with thioacidolysis is potentially related to the increased monomer yield obtained with the first method as compared to the second. In inner tissues, nitrobenzene oxidation gave rise to S/V ratios that varied from 0.14 (basal region, stage 2) to 0.21 (apical region, stage 1), values that are almost identical to the S/G value obtained with thioacidolysis. In contrast, in outer stem tissues, the S/V ratio varied from 0.22 (basal region, stage 1) to 0.43 (apical region, stage 2), suggesting that the developmental stage and, in the case of the stage 2 samples, the sample type (basal versus apical region) has an influence on the S/V ratio.

Calculation of the quantity of lignin monomers (H + V + S) released per g lignin (acetyl bromide) indicated that, as observed with the thioacidolysis results, the quantities released by outer stem tissues were, in general, lower (5.5–17.1%) and more variable than with inner stem tissues (16.6–30.1%). The stage 1 samples (outer

Table 3 Monomeric composition of lignin determined by thioacidolysis of inner- and outer flax stem tissues

	Inner tissues				Outer tissues			
	Stage 1		Stage 2		Stage 1		Stage 2	
	Basal	Apical	Basal	Apical	Basal	Apical	Basal	Apical
H	0	0	0	0	0	0	0	0
G	241.01 (13.22)	238.44 (29.14)	284.02 (27.53)	253.20 (11.84)	9.73 (1.78)	3.99 (0.04)	8.04 (0.69)	3.50 (0.05)
S	44.45 (5.07)	51.14 (8.34)	40.87 (4.73)	52.92 (2.43)	3.09 (1.70)	1.69 (0.50)	3.69 (1.58)	1.32 (0.13)
S/G	0.18 (0.01)	0.21 (0.01)	0.14 (<0.01)	0.21 (<0.01)	0.31 (0.12)	0.42 (0.12)	0.45 (0.16)	0.38 (0.04)
S+G	285.5 (18.2)	289.6 (37.3)	324.9 (32.3)	306.1 (14.3)	12.8 (3.5)	5.7 (0.5)	11.7 (2.3)	4.8 (0.1)
S+G/lignin	1204.5 (76.8)	1129.0 (145.2)	1035.7 (102.8)	1260.3 (58.8)	860.5 (233.4)	177.0 (16.9)	428.0 (82.7)	114.8 (1.9)

H, G, S = yields of the thioethylated products of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin units expressed as μ moles per gram of CWR; S/G = ratio of G to S lignin units; S+G = total S plus G lignin units; S+G/lignin: yields of total (S+G) expressed as μ moles per gram of lignin

Each value is the mean \pm SD (in brackets) of three separate assays. The analytical data varied by nearly 5% and 10% in inner- and outer tissues respectively

Table 4 Monomeric composition of lignin determined by alkaline nitrobenzene oxidation of inner- and outer flax stem tissues

	Inner tissues				Outer tissues			
	Stage 1		Stage 2		Stage 1		Stage 2	
	Basal	Apical	Basal	Apical	Basal	Apical	Basal	Apical
H	3.33 (0.49)	3.42 (0.27)	2.48 (0.31)	2.82 (0.22)	2.80 (0.25)	5.92 (0.57)	1.80 (0.07)	1.95 (0.36)
V	296.49 (2.52)	297.64 (7.27)	226.36 (3.72)	247.08 (1.73)	8.16 (0.15)	14.08 (0.66)	4.36 (0.58)	8.76 (1.00)
S	56.83 (1.61)	61.36 (3.37)	31.16 (1.26)	48.05 (0.45)	1.79 (0.06)	3.43 (0.09)	1.31 (0.17)	3.76 (0.38)
S/V	0.19 (<0.01)	0.21 (<0.01)	0.14 (<0.01)	0.19 (<0.01)	0.22 (<0.01)	0.24 (0.02)	0.30 (0.02)	0.43 (0.02)
H + V + S	356.7 (2.3)	362.4 (9.8)	260.0 (4.7)	298.0 (1.9)	12.8 (0.4)	23.4 (0.8)	7.5 (0.7)	14.5 (1.6)
H + V + S/lignin	1504.9 (9.6)	1412.9 (37.9)	828.8 (15.0)	1226.6 (7.9)	856.1 (25.18)	730.2 (23.78)	272.8 (25.6)	344.4 (37.7)

H, V, S = yields of *p*-hydroxybenzaldehyde (H), vanillin (V), and syringaldehyde (S) units expressed as μ moles per gram of CWR; S/V = ratio of V to S lignin units; H + V + S = total H plus V plus S;

H + V + S/lignin: yields of total (H + V + S) expressed as μ moles per gram of lignin. Each value is the mean \pm SD (in brackets) of three separate assays and the variation was estimated to be 6% in inner tissues and 4% in outer tissues

tissue) and stage 2 samples (inner tissue) from basal regions were, however, exceptions to this general trend. These results suggest that flax outer tissue lignin is more condensed than inner tissue lignin. In addition, and only for the outer stem tissues, more lignin monomers were released at stage 1 as compared to stage 2. Such an observation suggests that bast fiber maturation is associated with the formation of a more condensed lignin.

Discussion

Flax fibers contain lignin

The results of our microscopic and chemical analyses of flax stems provide evidence that the ‘phenolic compounds’ associated with bast fibers correspond to lignin. While a phloroglucinol–HCl-positive response in flax bast fibers has consistently been reported in other studies, the intensity and the localization of the red coloration may differ according to the study. For example, Gorshkova et al. (2000) observed a discrete staining in the middle lamella of some bast fibers, whereas Akin et al. (1996) detected an intense, but sporadic red coloration in the middle lamella and cell corners. However, the Weisner reaction is not specific to lignin and other cell wall-associated phenolic monomers such as cinnamyl alcohols, and/or cinnamaldehydes also give a positive reaction with this reagent (Clifford 1974). KMnO_4 -staining and concomitant immunolocalization of lignin epitopes in our study indicated that the cell wall regions that stained red with the Wiesner reaction also gave a positive labeling with both KMnO_4 -staining and anti-lignin immunosera. The use of such high-resolution techniques enabled us to detect lignin in both the compound middle lamella and (to a lesser degree) the secondary cell wall, notably in the S1 layer.

Consistent with these microscopic studies, chemical analyses of flax outer stem tissues (principally composed of bast fibers) confirmed the presence of low quantities of lignin, which ranged between 1.5% and 4.2% dry cell wall. Such a value is higher than other published values

(Gorshkova et al. 2000; Morrison and Akin 2001; Morrison et al. 2003) and only one FTIR study (Kotelnikova et al. 2000) indicated a higher value (7%). Microscopic control of the outer tissue samples used for chemical analyses indicated that (1) such samples were not contaminated by xylem tissue and (2) other potentially contaminating tissues such as the cuticle did not give a positive reaction with phloroglucinol–HCl. While the acetyl bromide method is an appropriate and sensitive technique for lignin determination (Yokoyama et al. 2002), one drawback is that potential artifacts due to UV absorption by other non lignin wall components such as aromatic amino-acids, phenolic acids, and/or hemicelluloses must be taken into account. Our results indicate that the low levels of *p*-coumaric, ferulic, and sinapic acids present in outer tissues do not significantly contribute to the overall acetyl bromide estimation of lignin. While cell wall proteins can constitute up to 6% of the cell wall in flax stems (data not shown), they are generally composed of AGP- and GRP-types that are poor in aromatic amino acids (Girault et al. 2000). In addition, UV absorption of aromatic amino-acids and phenolic acids are strongly reduced after cell wall solubilization by acetyl bromide (Iiyama and Wallis 1990), thereby limiting their potential contribution to an overestimation of lignin content. With regard to non-cellulose polymers, the use of perchloric acid, which facilitates cell wall solubilization, can also partially degrade hemicelluloses, thereby giving rise to UV-absorbing furfural derivatives (Hatfield et al. 1999). Nevertheless, in the case of both annual and woody plants, the contamination by such furfural derivatives is slight (Iiyama and Wallis 1990) and the addition of perchloric acid generally results in a better estimation of lignin content. Our results (data not shown) indicated that furfural derivatives did not contribute significantly to the overall estimation of lignin in flax fibers. In conclusion, the results of our chemical analyses strongly suggest that the lignin content in bast fibers is not due to contamination.

The acetyl bromide analyses indicated that the lignin content of outer tissues increased between the flowering

and seed maturation stages, as previously reported (Meijer et al. 1995). The observation that lignin can already be detected at the flowering stage could therefore suggest that the lignification of flax bast fibers is actually initiated prior to this stage. Such an interpretation is in agreement with the detection of cell wall peroxidases in immature flax bast fibers (McDougall 1992).

Spatial heterogeneity of lignin in flax stems

High-resolution TEM associated with immunolabeling provided information on the spatial distribution of lignin in bast and xylem fibers. Immunolabeling of flax xylem fibers shows that the compound middle lamella (cell corner, middle lamella, and primary cell wall) is rich in condensed lignin (only antibodies directed against condensed subunits reacted). The secondary cell wall contains GS lignin, and the degree of lignin condensation decreases from the S1 towards the S3 layer. The labeling pattern obtained in flax xylem fiber cell walls is thus typical of that observed in dicotyledon wood (Grünwald et al. 2002).

In bast fibers, according to the labeling pattern, condensed G-type lignins are deposited in cell wall corners and GS lignins are present in the secondary walls. However, the intense KMnO_4 -staining and relatively dense anti-Gzl labeling pattern observed in the S1 layer of the secondary cell wall also suggest the presence of relatively important quantities of condensed G lignin.

In conclusion, our results indicate that bast fiber lignins consist mainly of G-condensed epitopes, located in the compound middle lamella, together with the regularly distributed GS lignin epitopes present in the entire secondary cell wall. Such an observation is in disagreement with the negative reaction of the secondary cell wall with classical histochemical reagents; however, the low amounts of lignin in the secondary cell wall (detected by the immunocytochemical approach) might be responsible for the failure of the histochemical tests. The combination of different microscopy approaches utilized in this paper provides, for the first time to our knowledge, a detailed picture of lignin deposition in the cell walls of flax bast fibers.

Flax fiber lignin is G-rich and condensed

Chemical analyses (thioacidolysis and nitrobenzene oxidation) indicated the presence of a G-rich lignin (S/G and S/V ratios ≤ 0.5) in both outer- and inner stem tissues. Based on the amount of lignin monomers released by these two techniques, only 2.3–17.2% of outer tissue lignin would be non-condensed. Since nitrobenzene oxidation and thioacidolysis are highly sensitive and efficient techniques as compared to other reactions (Monties 1989; Lapiere 1993), these low values likely make bast fiber lignins particularly condensed. However, preliminary analyses did not reveal the presence of any

unusual features in the dimeric structures as compared to other dicotyledon lignin (C. Lapiere, Institut National Agronomique Paris-Grignon France, personal communication).

Nitrobenzene oxidation analyses of outer stem tissues revealed a major difference between bast fiber- and xylem lignin. The relative proportions of H subunits were much higher ($\leq 25\%$) in outer tissues as compared to inner tissues ($\leq 1\%$). In contrast, no H units were detected by thioacidolysis. In order to exclude the possibility that the H units were derived from non-lignin molecules, we quantified potentially contaminating molecules such as phenolic acids (Monties 1989) and aromatic amino-acids (Lam et al. 1990). Our results indicated that such compounds did not contribute significantly to the overall determination of lignin content.

One interpretation of these results would be that H units are preferentially implicated in condensed structures such as diarylpropane and/or resinol bonds that are sensitive to nitrobenzene oxidation (Chang and Allan 1971). Such an interpretation is also supported by our immunocytochemical data indicating that bast fibers contain condensed lignins since the presence of H units is often related to the deposition of highly condensed lignins (Monties 1989).

Lignin condensation is related to bast fiber maturation

In contrast to xylem lignin, the S/G ratio of the outer tissues increased slightly (but significantly) between the flowering and the seed maturation stages. Such an evolution is similar to the typical lignification process in maturing dicotyledon wood, where intense S subunit polymerization occurs in the secondary walls during the late stages of lignification (Terashima et al. 1993). However, this classic scheme cannot fully account for the lignification pattern observed in flax bast fibers. Our chemical data suggested an increase in the proportion of the condensed structures between the two developmental stages analyzed, which is consistent with the previous data using cupric oxidation and alkaline hydrolysis (Akin et al. 1996; Gorshkova et al. 2000). This increase in condensation could be mediated via the differential supply of monolignols. Alternatively, the presence of high amounts of crystalline cellulose may affect the polymerization process. *In vitro* and computer-modeling studies (Jurasek 1996; Touzel et al. 2003) have previously shown that preformed cellulose and non-cellulose templates affect lignin polymerization. In addition, the presence of a thick cellulose secondary cell wall could hinder monolignol transport, thereby leading to an atypically high peroxidase/monolignol ratio—conditions that are known to favor *in vitro* polymerization of a highly condensed lignin (Cathala et al. 2001). Histochemical evidence has shown that peroxidase activity can be detected at the cell corners of relatively mature bast fibers (data not shown).

Flax bast fiber lignin—a special case?

On the whole, flax xylem lignin is similar to that observed in the other annual plants with an S/G value (0.2) comparable to that observed in *Arabidopsis* (0.3, Goujon et al. 2003), lucerne (0.2–0.6, Vallet et al. 1996), hemp (0.7, data not shown) and tobacco (1.0, Atanassova et al. 1995).

In contrast, the flax bast fiber lignin presents a number of differences, not only with regard to ‘xylem lignin’ in general, but also with regard to ‘bast fiber lignin’ from other annual plants.

As compared to xylem lignin, flax bast fiber lignin is (1) present in much lower amounts (2) more highly condensed (273 $\mu\text{mol/g}$ lignin degradation products) as compared to the value of 2,400 $\mu\text{mol/g}$ lignin in poplar xylem lignin (Lapierre 1993) and (3) contains a greater proportion of H units ($\leq 25\%$) as compared to the negligible amounts typically detected in dicotyledon xylem lignin (Terashima et al. 1998).

With regard to other annual plants, flax bast fiber lignin also presents a number of important differences. Firstly, with regard to lignin content, only ramie fibers contain less lignin (1.5%) than flax fibers. In other species, the fiber lignin content is higher [hemp 4% (Vignon et al. 1995); lucerne 8% (Vallet et al. 1998); kenaf 8% (Neto et al. 1996) and jute 12% (Islam and Sarkanen 1993)]. However, some care should be taken in comparing these values, since, as our results show, the lignin content varies as a function of the stem region analyzed, the developmental stage, and the environmental conditions. Secondly, flax bast fiber lignin also appears to have the lowest S/G ratio (0.2–0.4) as compared to lucerne (0.4, Vallet et al. 1998), hemp (0.9, data not shown), jute (2.1, Islam and Sarkanen 1993) and kenaf (3.9, Morrison et al. 1999). Thirdly, flax bast fiber lignin also appears to be the richest in condensed lignins with the lowest degradation product yields (flax: 273 $\mu\text{mol/g}$ lignin, this study; kenaf: 450 $\mu\text{mol/g}$ lignin, Morrison et al. 1999; lucerne: 645 $\mu\text{mol/g}$ lignin, Vallet et al. 1998; hemp: 750 $\mu\text{mol/g}$ lignin, data not shown; jute: 1,350 $\mu\text{mol/g}$ lignin, Islam and Sarkanen 1993). Finally, degradation of flax bast fiber lignin also gives rise to the highest proportion ($\leq 25\%$) of liberated H units as compared to values of 6% in lucerne (Jung et al. 1992) and 10% in kenaf (Neto et al. 1996).

From an evolutionary point of view (Faix 1991), S-rich lignins are considered to be less primitive than G-rich lignins. An overall comparison of bast fiber lignins from different annual species suggests the existence of two groups, the first including flax, lucerne, and hemp, and the second including kenaf and jute. The first group corresponds to the Rosidae I super-order which is considered to be more primitive than the second, corresponding to the Rosidae II super-order (Chase and The Angiosperm Phylogeny Group 1998).

In conclusion, our results indicate that low quantities of an ‘atypical lignin’ are present in flax bast fibers. Since it is known that ‘phenolic compounds’ have a negative impact on the flax fiber quality, evidence for the

deposition of lignin is important. In addition, our results demonstrate the particular nature of flax fiber lignins, as compared to xylem lignins of woody species and *Arabidopsis*. This special lignin structure could be due to the physical/chemical constraints imposed on the lignification process by the presence of high amounts of structural polysaccharides. As such, and in a more fundamental context, the flax bast fiber may represent an interesting system, not only for studying an atypical lignification process, but also as a model for weakly lignified, cellulose-rich secondary cell walls. Our laboratory has already initiated such studies with the characterization of a flax CCoAOMT (Day et al. 2001) and, more recently, expression analyses of flax ESTs in outer stem tissues (Day et al. 2005). A better understanding of the flax bast fiber lignification and secondary cell wall formation should therefore be complementary to other programs concerned with xylem in woody plants and *Arabidopsis*.

Acknowledgements The authors would like to thank M. François Bert (Institut Technique de Lin, Paris) for his professional advice on Flax plants. A. Day thanks the Regions Champagne-Ardenne and Nord-Pas de Calais for financial support. This work was carried out in the context of the Regional Project ‘Etude de la modulation de la cohésion intercellulaire chez le lin’.

References

- Akin DE, Gamble GR, Morrison WH, Rigsby LL, Dodd RB (1996) Chemical and structural analysis of fibre and core tissues from flax. *J Sci Food Agr* 72:155–165
- Atanassova R, Favet N, Martz F, Chabbert B, Tollier MT, Monties B, Fritig B, Legrand M (1995) Altered lignin composition in transgenic tobacco expressing O-methyltransferase sequences in sense and antisense orientation. *Plant J* 8:465–477
- Baley C (2002) Analysis of the flax fibres tensile behaviour and analysis of the tensile stiffness increase. *Compos Part A-Appl Sci Manuf* 33:939–948
- Beaugrand J, Crônier D, Debeire P, Chabbert B (2004) Arabinoxylan and hydroxycinnamate content of wheat bran in relation to endoxylanase susceptibility. *J Cereal Sci* 40:223–230
- Billa E, Tollier MT, Monties B (1996) Characterisation of the monomeric composition of in situ wheat straw lignins by alkaline nitrobenzene oxidation: effect of temperature and reaction time. *J Sci Food Agr* 72: 250–256
- Cathala B, Chabbert B, Joly C, Dole P, Monties B (2001) Synthesis, characterisation and water sorption properties of pectin-dehydrogenation polymer (lignin model compound) complex. *Phytochemistry* 56:195–202
- Chang HM, Allan GG (1971) Oxidation. In: Sarkanen KV, Ludwig CH (eds) *Lignins: occurrence, formation, structure and reactions*. Wiley, New York, pp 433–485
- Chase M, The Angiosperm Phylogeny Group (1998) An ordinal classification for the families of flowering plants. *Ann Missou Botanical Garden* 85:531–553
- Clifford MN (1974) Specificity of acidic phloroglucinol reagents. *J Chromatogr* 94:321–324
- Day A, Dehorter B, Neutelings G, Czeszak X, Chabbert B, Bellingheri L, David H (2001) Caffeoyl-coenzyme A 3-O-methyltransferase enzyme activity, protein and transcript accumulation in flax (*Linum usitatissimum*) stem during development. *Physiol Plant* 113:275–284
- Day A, Addi M, Kim W, David H, Bert F, Mesnage P, Rolando C, Chabbert B, Neuteulings G, Hawkins S (2005) ESTs from the fibre-bearing stem tissues of flax (*Linum usitatissimum* L): expression analyses of sequences related to cell wall development. *Plant Biol* 7:23–32

- Dence CW (1992) The determination of lignin. In: Lin SY, Dence CW (eds) *Methods in lignin chemistry*. Springer, Berlin, Heidelberg, New York, pp 33–61
- Effland MJ (1977) Modified procedure to determine acid-insoluble lignin in wood and pulp. *Tappi J* 60:143–144
- Faix O (1991) Classification of lignins from different botanical origins by FT-IR spectroscopy. *Holzforschung* 45:21–27
- Gamble GR, Snook ME, Henriksson G, Akin DE (2000) Phenolic constituents in flax bast tissue and inhibition of cellulase and pectinase. *Biotechnol Lett* 22:741–746
- Girault R, Bert F, Rihouey C, Jauneau A, Morvan C, Jarvis M (1997) Galactans and cellulose in flax fibres: putative contributions to the tensile strength. *Int J Biol Macromol* 21:179–88
- Girault R, His I, AndemeOnzighi C, Driouich A, Morvan C (2000) Identification and partial characterization of proteins and proteoglycans encrusting the secondary cell walls of flax fibres. *Planta* 211:256–264
- Gorshkova TA, Salnikov VV, Pogodina NM, Chemikosova SB, Yablokova EV, Ulanov AV, Ageeva MV, VanDam JEG, Lozovaya VV (2000) Composition and distribution of cell wall phenolic compounds in flax (*Linum usitatissimum* L) stem tissues. *Ann Bot* 85:477–486
- Goujon T, Ferret V, Mila I, Pollet B, Ruel K, Burlat V, Joseleau JP, Barriere Y, Lapiere C, Jouanin L (2003) Down-regulation of the AtCCR1 gene in *Arabidopsis thaliana*: effects on phenotype, lignins and cell wall degradability. *Planta* 217:218–28
- Grünwald C, Ruel K, Kim YS, Schmitt U (2002) On the cytochemistry of cell wall formation in poplar trees. *Plant Biol* 4:13–21
- Hatfield RD, Grabber J, Ralph J, Brei K (1999) Using the acetyl bromide assay to determine lignin concentrations in herbaceous plants: some cautionary notes. *J Agric Food Chem* 47:628–32
- Iiyama K, Pant R (1988) The mechanism of the Maüle color reaction. Introduction of methylated syringyl nuclei into softwood lignin. *Wood Sci Technol* 22:167–175
- Iiyama K, Wallis AFA (1990) Determination of lignin in herbaceous plants by an improved acetyl bromide procedure. *J Sci Food Agr* 51:145–161
- Iiyama K, Lam TBT, Stone BA (1990) Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochem* 29:733–737
- Islam A, Sarkanen K (1993) The isolation and characterization of lignins of jute (*Corchorus capsularis*). *Holzforschung* 47:123–132
- Joseleau JP, Ruel K (1997) Study of lignification by noninvasive techniques in growing maize internodes. An investigation by Fourier transform infrared cross-polarization-magic angle spinning ¹³C-nuclear magnetic resonance spectroscopy and immunocytochemical transmission electron microscopy. *Plant Physiol* 114:1123–33
- Jung H, Valdez F, Hatfield R, Blanchette R (1992) Cell wall composition and degradability of forage stems following chemical and biological delignification. *J Sci Food Agric* 58:347–355
- Jurasek L (1996) Morphology of computer-modeled lignin structure: fractal dimensions, orientation and porosity. *J Pulp Pap Sci* 22: 376–380
- Kerr AI, Goring DAI (1975) The ultrastructural arrangement of wood cell wall. *Cell Chem Technol* 9:563–573
- Kotelnikova NE, Panarin EF, Serimaa R, Paakkari T, Sukhanova TE, Gribanov AV (2000) Study of flax fibre structure by Waxes, IR and ¹³C NMR spectroscopy, and SEM. In: Kennedy J, Phillips GO, Williams PA (eds) *Cellulosic pulps, fibres and materials*. Woodhead publishing Ltd Abington Hall, Cambridge, pp 169–179
- Lam TBT, Iiyama K, Stone BA (1990) Lignin in wheat internodes. Part 2: Alkaline nitrobenzene oxidation by wheat straw lignin and its fractions. *J Sci Food Agric* 51:493–506
- Lapiere C (1993) Application of new methods for the investigation of lignin structure. In: Jung HG, Buxton DR, Hatfield RD, Ralph J (eds) *Forage cell wall structure and digestibility*. ASA CSSA SSSA, Madison, pp 133–166
- Love GD, Snape CE, Jarvis MC, Morrison IM (1994) Determination of phenolic structures in flax fibre by solid-state ¹³C NMR. *Phytochem* 35:489–491
- McDougall GJ (1992) Changes in cell wall-associated peroxidases during the lignification of flax fibres. *Phytochem* 31:3385–3389
- Meijer WJM, Vertregt N, Rutgers B, Vandewaart M (1995) The pectin content as a measure of the retting and retrainability of flax. *Ind Crops Products* 4:273–284
- Meshitsuka G, Nakano J (1979) Studies on the mechanism of lignin color reaction (XIII): Maüle color reaction (9). *Mokuzai Gakkaishi* 25: 588–594
- Monties B (1984) Dosage de la lignine insoluble en milieu acide: influence du prétraitement par hydrolyse acide sur la lignine Klason de bois et de paille. *Agronomie* 4:387–392
- Monties B (1989) Lignins. In: Dey PM, Harborne JB (eds) *Methods in plant biochemistry*. Academic, New York, pp 113–157
- Morrison IM (1976) New laboratory methods for predicting the nutritive value of forage crops. *World Review of Animal Production* XII:75–82
- Morrison WH, Akin DE (2001) Chemical composition of components comprising bast tissue in flax. *J Agr Food Chem* 49:2333–2338
- Morrison WH, Akin DE, Archibald DD, Dodd RB, Raymer PL (1999) Chemical and instrumental characterization of maturing kenaf core and bast. *Ind Crops Products* 10:21–34
- Morrison WH, Himmelsbach DS, Akin DE, Evans JD (2003) Chemical and spectroscopic analysis of lignin in isolated flax fibers. *J Agri Food Chem* 51:2565–2568
- Neto CP, Seca A, Fradinho D, Coimbra MA, Domingues F, Evtuguin D, Silvestre A, Cavaleiro JAS (1996) Chemical composition and structural features of the macromolecular components of *Hibiscus cannabinus* grown in Portugal. *Ind Crops Products* 5:189–196
- Ruel K, Faix O, Joseleau J (1994) New immunogold probes for studying the distribution of the different lignin types during plant cell wall biogenesis. *J Trace Microprobe Tech* 12:247–265
- Ruel K, Burlat V, Joseleau JP (1999) Relationship between ultrastructural topochemistry of lignin and wood properties. *Iawa J* 20:203–211
- Ruel K, Montiel MD, Goujon T, Jouanin L, Burlat V, Joseleau JP (2002) Interrelation between lignin deposition and polysaccharide matrices during the assembly of plant cell walls. *Plant Biol* 4:2–8
- Sharma HSS, Faughey G, Lyons G (1999) Comparison of physical, chemical, and thermal characteristics of water-, dew-, and enzyme-retted flax fibers. *J Appl Polym Sci* 74:139–143
- Terashima N, Fukushima K, He L-F, Takabe K (1993) Comprehensive model of the lignified plant cell wall. In: Jung HG, Buxton DR, Hatfield RD, Ralph J (eds) *Forage cell wall structure and digestibility*. ASA-CSSA-SSSA, Madison, pp 247–270
- Terashima N, Nakashima J, Takabe K (1998) Proposed structure for protolignin in plant cell walls. In: Lewis NG, Sarkanen S (eds) *Lignin and lignan biosynthesis*. American Chemical Society, Washington DC, pp 180–193
- Touzel JP, Chabbert B, Monties B, Debeire P, Cathala B (2003) Synthesis and characterization of dehydrogenation polymers in *glucunoacetobacter xylinus* cellulose and cellulose/pectine composite. *J Agr Food Chem* 51:981–986
- Vallet C, Chabbert B, Czaninski Y, Monties B (1996) Histochemistry of lignin deposition during sclerenchyma differentiation in alfalfa stems. *Ann Bot* 78:625–632
- Vallet C, Lemaire G, Monties B, Chabbert B (1998) Cell wall fractionation of alfalfa stem in relation to internode development: biochemistry aspect. *J Agr Food Chem* 46:3458–3467
- Vignon M, Dupeyre D, Garciajaldon C (1995) Steam explosion of woody hemp chènevotte. *Int J Biol Macromol* 17:395–404
- Yokoyama T, Kadla JF, Chang HM (2002) Microanalytical method for the characterization of fiber components and morphology of woody plants. *J Agr Food Chem* 50:1040–1044