Fourier-Transform Infrared Microspectroscopy of Anatomically Different Cells of Flax (*Linum usitatissimum*) Stems during Development

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FT-IR microspectroscopy was used to study the changes in cell wall structure of four anatomically different cells of flax (*Linum usitatissimum*) stems during growth. The cell types were xylem, fiber, epidermal, and gland, a specialized epidermal cell. FT-IR spectra suggested that xylem growth from 5 to 20 days was accompanied by de-esterification of pectin and an increse in lignification. The principal feature in the spectra of the fiber cell walls was the predominance of cellulose absorbances at 7–20 days. Associated with these were acetyl absorbances which suggest the presence of acetylated glucomannan and/or xylan. The spectra of both the epidermal and gland cell walls contained suberin/cutin and protein absorbances. Epidermal development produced increased suberin/cutin deposition, while gland cell wall development was accompanied by protein accretion.

Keywords: *Flax; development; FT-IR; cell walls; cellulose; noncellulosic polysaccharides; lignin; suberin; xylem; fiber; epidermis*

INTRODUCTION

The demand for nonfood industrial plant products, such as oil and fiber, is increasing (Decanierre, 1989). The recent drive for environmentally friendly alternatives to petrochemical-derived products has led to an enhanced awareness of the value of renewable plant resources (McDougall *et al.*, 1993). The annual crop plant *Linum usitatissimum* provides an alternative source of both oil and fiber in the form of linseed oil and flax fiber. To maximize the exploitation of this crop, a more complete understanding of the biology and chemistry that underpin its development is required. Although the chemistry of the cell wall of flax fibers has been the subject of intensive research (Chesson, 1978; Morvan *et al.*, 1988, 1989; Sharma, 1989; Davis *et al.*, 1990; Rynja, 1991; McDougall, 1993), little work has been done on the chemistry of the cell walls of other types of cells during development (McDougall *et al.*, 1992). Due to the intractability of plant cell walls, analyses rely heavily on the use of harsh chemicals and derivatization, thereby altering the native cell wall structure and possibly generating artifacts. The analyses also require reasonable amounts of cell wall material (≥ milligram levels), which usually means that they are carried out on composite samples of tissues. Therefore, a study of the development and differentiation of individual cell types is extremely difficult as it requires the expert dissection of a great number of plants. However, the use of Fourier-transform infrared (FT-IR) microspectroscopy permits the study of localized changes in cell wall composition and structure of individual cells and the comparison of these with different and distant tissues.

Invented in 1949, the infrared microscope remained a curiosity until the addition of Fourier-transform techniques in 1982 [reviewed in Reffner (1990)]. Its use then became widespread throughout material (Carl and Smith, 1990; Reffner and Wihlborg, 1990; Allen, 1992; Kaito *et al.*, 1992) and forensic (Beauchaine *et al.*, 1988) science. However, the application of this technique to plant sciences has been limited and is only now coming of age (Sweeney, 1989; Homblé *et al.*, 1990; McCann *et al.*, 1992; Wells *et al.*, 1994; Stewart *et al.*, 1994).

In this paper, we report the use of FT-IR microspectroscopy to study the changes in composition and structure of four anatomically different cell types during the development of the hypocotyl of seedlings of *L. usitatissimum*.

MATERIALS AND METHODS

Flax (*L. usitatissimum* cv. Belinka; Cebecco Zaden BV Ltd., Rotterdam, The Netherlands) was grown from seed in a greenhouse. Batches of plants were harvested daily from 5 to 20 days after emergence and examined microscopically. Sections of hypocotyls at 5, 7, 11, 15, and 20 days were selected for FT-IR microspectroscopy. The basal sections were cut into small pieces (5 × 5 × 5 mm), fixed in 2.5% (v/v) glutaraldehyde in 50 mM phosphate buffer, pH 6.8, for 4–8 h, and then washed by changing into the same buffer for 3 × 30 min. The samples were preoriented in 2% (v/v) agar and then dehydrated in an ethanolic series, i.e. 2 × 30 min in 70% ethanol followed by 2 × 30 min changes into 100% ethanol dried over molecular sieves. The samples were then transferred into London resin (LR White, medium grade, Agar Scientific, Stansted, U.K.), changed into resin, and then embedded in gelatine capsules and polymerized by constant heating at 55 °C for 24 h. The blocks were trimmed and sectioned at 5 mm using a Reichert-Jung 1140 autocut microtome. For each stage of growth three sections from three different blocks were analyzed.

FT-IR Microspectroscopy. Samples were mounted by adhering the sections to two strips of adhesive tape which had been stuck over a rectangular hole in a "slide" fashioned of cardboard. The strips of adhesive tape had a gap between them so that only the edges of the section, containing no plant tissue, were held. The mounted section was then handled easily and without fear of the loss of the section. This method has advantages over drying the plant material onto a BaF_2 disk as used by McCann *et al.* (1992); the section could be stored easily and, if necessary, scanned at a later date. All

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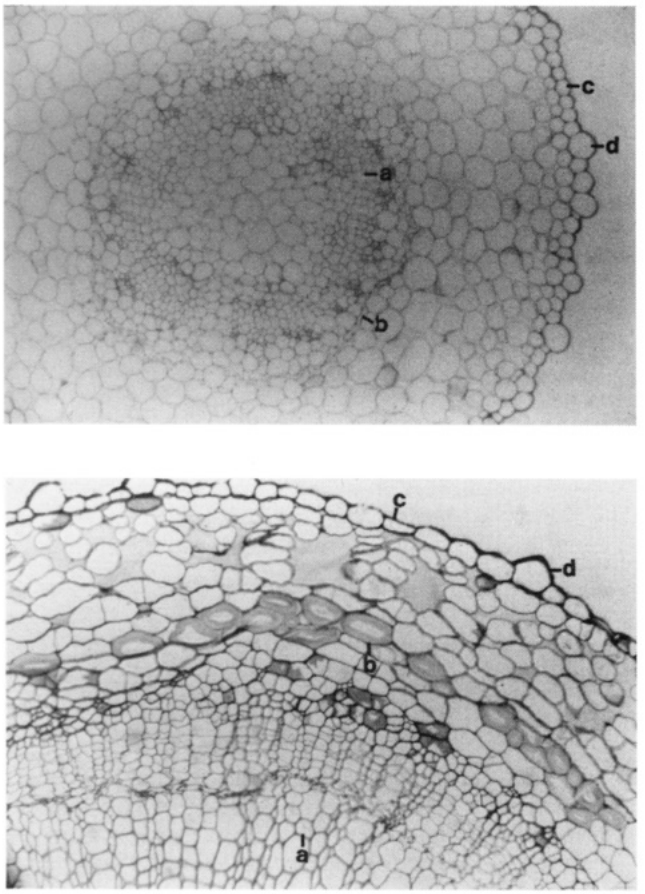

Figure 1. Photomicrographs of transverse sections of flax stems at 5 days (top) and 17 days (bottom). Highlighted are the xylem (a), fiber bundles (b), epidermis (c), and gland cell (d).

spectra were acquired using a Bruker IFS 66 FT-IR spectrometer with microscope attachment. The incident IR laser beam was reduced to *ca.* 8 mm by redundant aperturing. Spectra were routinely acquired from 1000 interferograms, at a resolution of 4 cm^{-1}. Background spectra were acquired on the air gap between the strips of Sellotape and the resin for each section. The spectra of the plant tissues were, therefore, both background- and resin-subtracted. The region from 4000 to 2500 cm^{-1} contained only unresolved C−H, O−H absorbances, and the spectra presented are only shown over the region 1900−700 cm^{-1}. Photomicrographs of equivalent sections stained with toluidine blue (O'Brien and McCully, 1981) were taken using a Zeiss Universal research microscope fitted with an Olympus camera.

RESULTS AND DISCUSSION

Photomicrographs of the tissues over the period of secondary thickening of the flax hypocotyls are shown in Figure 1. Representative examples of the xylem (a), fiber bundles (b), epidermis (c), and gland cells (d) are also shown.

At 5 days, the flax hypocotyl was still extending and the tissues are simple and undifferentiated [Figure 1 (top)]. Although xylem and epidermal cells could easily be discerned, fiber cells cannot be readily identified in cross sections because their cell walls have not yet begun to thicken. Fiber cells are known to cause crushing and obliteration of cells in the phloem as they elongate and develop (Esau, 1943), so they were putatively identified as those to the centripetal side of crushed cells in the phloem [Figure 1 (top) b]. To ensure that the correct cells were targeted for FT-IR analysis, the relative position of the long fiber cells

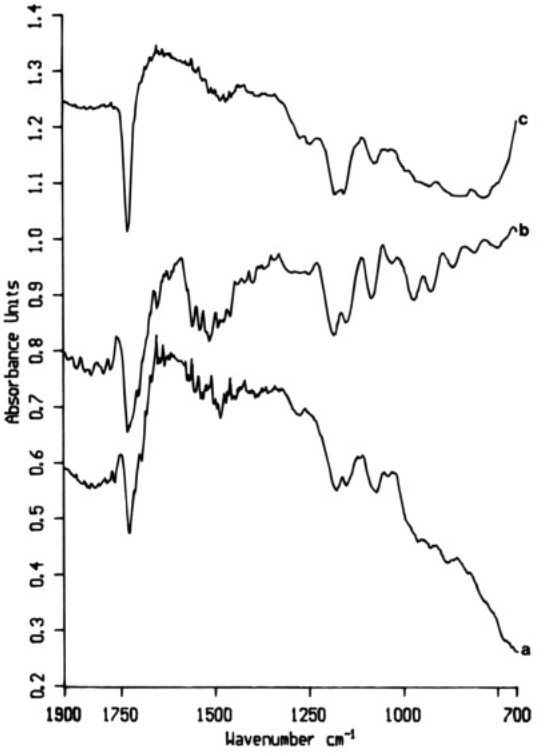

Figure 2. FT-IR spectra of xylem cell walls after (a) 5, (b) 11, and (c) 20 days of growth.

was noted in longitudinal sections of 5-day hypocotyls. This positively identified the cells selected by the method above.

At 7 days the fiber cells had begun to produce thickened cell walls, and by 10 days the cells could be easily discerned (not shown). At 15 days the secondary walls of the fiber were well developed, and at 20 days [Figure 1 (bottom) b] the fibers almost form a complete ring of cells.

Over this period, the xylem undergoes a marked expansion from a number of primary bundles at 5 days to a near complete ring of xylem, seven to eight cells deep, at 20 days [Figure 1 (bottom) a].

Xylem Cells. The FT-IR spectra of the xylem cell walls at 5, 11, and 20 days are shown in Figure 2. There are distinct differences between the spectra. The absorbance at 1740 cm^{-1} decreases in intensity from 11 to 20 days and can be assigned to the carbonyl C=O stretch of ester groups (Kemp, 1991; Stewart and Morrison, 1992). However, the intensity of the signal at 1260 cm^{-1}, which can be assigned to the corresponding ester C−O stretch, remains similar. This absorbance is undoubtedly linked to the broad absorbance at 1680−1600 cm^{-1}, which has principally been associated with the C=O stretch of carboxylic acids (1680 cm^{-1}) and anions (1600 cm^{-1}). Also evident is the increase and then decrease in intensity of the carboxylate anion absorbance at 1600 cm^{-1} from 7−11 days and 11−20 days, respectively. These changes in the relative intensities of the ester and carboxylic acid/anion absorbances, with no great change in the corresponding C−O stretch at 1260 cm^{-1}, suggest that the degree of esterification, rather than the total amount of ester/acid/anion groups, changes during development of the tissue. Pectins present in the cell walls of flax are reported to be 10−20% methyl esterified (Morvan *et al.*, 1988, 1989; Davis *et al.*, 1990). These changes in carbonyl absorbance suggest that the galacturonic acid residues of pectin are predominantly ionized at 7−11 days, but the

process of secondary thickening of the hypocotyl from 11 to 20 days is accompanied by a reduction in both the esterified and ionized pectin, which may be indicative of increased pectin methylesterase activity. The reduction in resolution of the spectra of xylem tissue after 11 days of growth is probably due to the increase in overlapping signals from the deposition of noncellulosic polysaccharides (NCPs).

The absorbances at 1130, 1098, 1050, and 900 cm^{-1} seen in the spectra of the xylem tissue are associated with cellulose (Stewart and Morrison, 1992; Gilbert *et al.*, 1993; Hulleman *et al.*, 1994). There are conflicting reports concerning the relationship of the absorbance at 900 cm^{-1} and cellulose crystallinity. Hulleman *et al.* (1994) suggested that the absorbance at 900 cm^{-1} is representative of changes in the molecular environment of cellulose but not related to crystallinity. However, Michell (1990) presented evidence from cellulose mercerization and ball-milling studies which suggested that a direct relationship exists between this absorbance and amorphous cellulose. Since the origin of this absorbance is still debatable, it can only be used as a marker for the presence of cellulose.

The lignin-related absorbances at 1595 and 1510 cm^{-1} (Stewart and Morrison, 1992) are weak and poorly resolved in the 5 and 11 day tissues. This seems odd as mature flax xylem (*i.e.* flax shive) has a lignin content of approximately 15% (w/w) (results not shown). However, it may be that lignin is present but its absorbance is masked by other components.

All xylem cells examined have lignified cell walls, but the xylem cells at 5 days are not part of a complete ring and may be surrounded by primary-walled cells, whereas those at 11 and 20 days are part of a more complete xylem ring after initiation of a vascular cambium. Therefore, at 5 days, it is possible that the xylem cell walls selected for FT-IR analysis were adjacent to a cell with a primary cell wall and will, therefore, include signals from other components of primary cell walls such as pectin. Indeed, the spectra of the 5 day and, to a lesser extent, 11 day tissues have prominent absorbances at 1740, 1680–1600, and 1260 cm^{-1}, the pectin absorbances. At 20 days, the reduced absorbance at 1260 cm^{-1} suggests that the relative amount of pectin present has decreased. However, the lignin absorbances at 1595 and 1510 cm^{-1} have increased in intensity, which reflects the progressive lignification that accompanies secondary wall formation.

Fibers. The fiber bundles form a ring in the cortex centripetal to the xylem which extends throughout the length of the stem in the mature plant [Figure 1 (bottom) b].

The FT-IR spectra of the fiber bundles, at five stages of growth, are shown in Figure 3. The most obvious feature is the similarity of the spectra at 7–20 days of growth and the dissimilarity of these to the spectrum of the fiber bundle at 5 days. The spectrum of the youngest tissue has an intense pectin-related absorbance (1680–1600 cm^{-1}) and the corresponding general carbonyl absorbance at 1260 cm^{-1}, which are lacking in the other spectra. Additional evidence for the presence of pectin comes from the absorbances at 1440 and 955 cm^{-1} (Marchessault, 1962; Kemp, 1991). These absorbances can be assigned to methyl C–H wagging vibrations (pectin methyl ester) and carboxylic acid O–H deformations, respectively.

The cellulose absorbances (1130, 1098, and 1050 cm^{-1}) in the spectrum of the 5-day-old fiber bundle are poorly

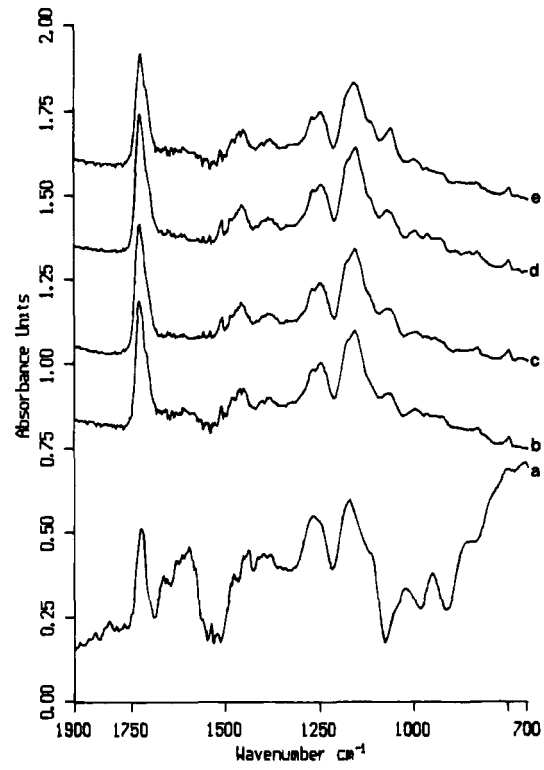

Figure 3. FT-IR spectra of fiber bundles walls after (a) 5, (b) 7, (c) 11, (d) 15, and (e) 20 days of growth.

resolved in comparison with those of the older tissues. In fact, the absorbances in the older tissues are remarkably similar in resolution, maxima, and intensity. This pattern correlates with deposition of the first layer of the secondary wall in the fiber cells, which begins around 7 days (Figure 1).

The spectra of the fiber bundles from 7–20 days (Figure 3b–e) exhibit ester carbonyl absorbances at 1730 cm^{-1} and a small methyl C–H absorbance (1440 cm^{-1}). The intensity of these absorbances in conjunction with the virtual absence of the pectin absorbances at 1680 and 1600 cm^{-1} suggests that acetylated groups are present and, due to the absence of pectin, that the pectin methyl ester carbonyl absorbs at a higher frequency (1740 cm^{-1}). These groups are likely to be attached to noncellulosic polysaccharides, as van Hazendonk *et al.* (1994) found acetylated glucomannan and xylan were intimately associated with the cellulose microfibrils of flax fibers. Similarly, Rättö *et al.* (1993) found that acetylated glucomannans were associated with the cellulose microfibrils of softwoods.

The lignin absorbances at 1595 and 1510 cm^{-1} are poorly represented in all of the fiber bundle spectra, reflecting the reported low level of lignification of this tissue (McDougall, 1992). Indeed, at this stage of development, fiber bundles in the hypocotyl do not show any signs of lignin by acid phloroglucinol staining or by autofluorescence (results not shown). The deposition of lignin in the middle lamella of flax fibers (Langer and Hill, 1982) occurs much later during stem maturation. These absorbances may not be due to lignin since a solid-state nuclear magnetic resonance study of industrial grade flax fiber by Love *et al.* (1994) suggested that aromatic residues associated with the fibers had structures more consistent with that of anthocyanins.

Epidermal Cells. At the outer edge of the transverse section is the epidermis (Figure 1d). At 5 days, the cell walls of the epidermis exhibit signals that can

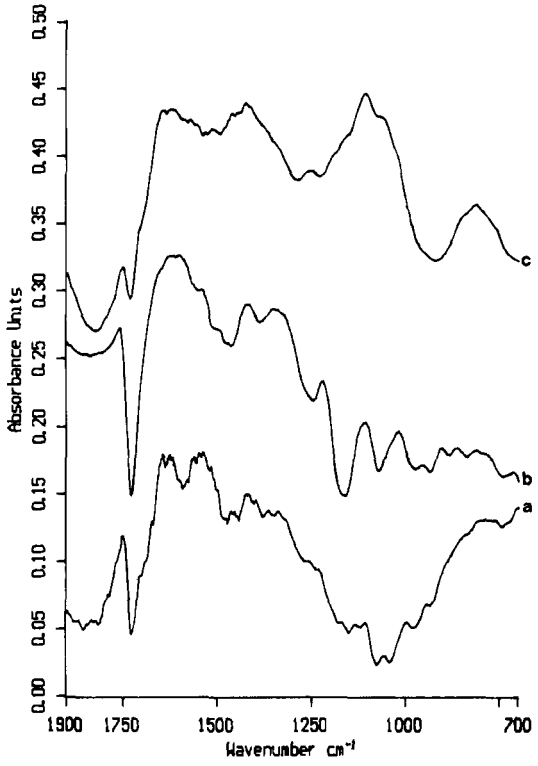

Figure 4. FT-IR spectra of epidermal cell walls after (a) 5, (b) 11, and (c) 20 days of growth.

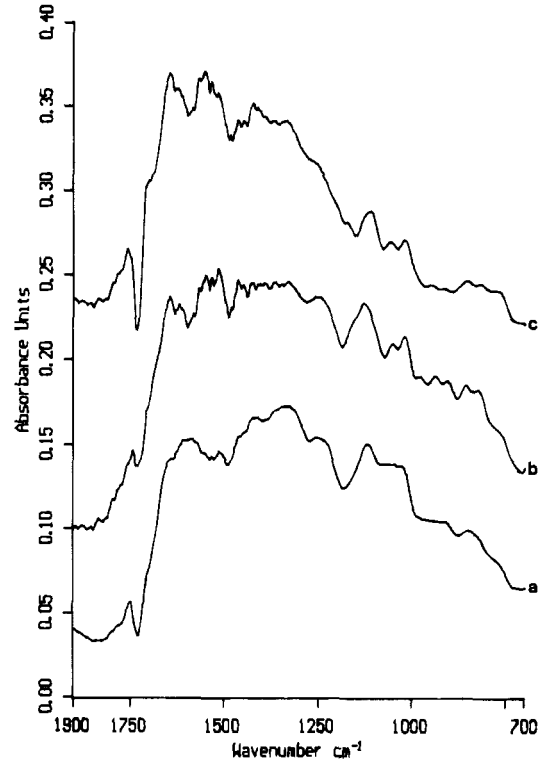

Figure 5. FT-IR spectra of gland cell walls after (a) 7, (b) 11, and (c) 20 days of growth.

be assigned to the presence of ester (1740 and 1260 cm^{-1}), pectin (1680–1600 and 955 cm^{-1}), and lignin (1595 and 1510 cm^{-1}) groups (Figure 4a). However, the major absorbances are centered at 1640 and 1550 cm^{-1}. When seen together, these absorbances suggest the presence of protein and can be assigned to the C=O stretch (1660 cm^{-1}) and N–H (1550 cm^{-1}) of amide bonds (Kemp, 1991).

At 11 days, the protein peaks have collapsed into a poorly resolved broad absorbance centered at 1600–1595 cm^{-1} (Figure 4b). This loss of resolution could be due to signals from increased pectin content, but this is unlikely since the intensity of absorbance at 1260 cm^{-1} has remained relatively unchanged. However, these signals may arise from the deposition of lignin. At 20 days, the absorbance at 1600–1595 cm^{-1} is still broad but is accompanied by more intense absorbances at 1510 and 1460–1430 cm^{-1}. Signals in this latter region can be assigned to hydrocarbon ($-CH_2-$) C–H deformation (Sarkanen *et al.*, 1967) and suggest that the deposition of suberin/cutin, rather than lignin, has occurred (Kolattukudy *et al.*, 1981). This is consistent with the observed distribution of suberin/cutin in other plants where the suberin/cutin acts a first line of defense against parasites and as a water-impermeable barrier (Kolattukudy *et al.*, 1981).

The spectrum of the 20-day-old epidermis (Figure 4c) contains an intense absorbance at 816 cm^{-1}. In classical infrared spectroscopy, this absorbance is representative of the presence of 1,4-substituted aromatic rings (Williams and Fleming, 1976; Kemp, 1991) and could be used to suggest that *p*-coumaric acid is a constituent of the epidermal suberin/cutin. A recent study by Borg-Olivier and Monties (1993) found that tyramine [4-(2-ethylamino)phenol] was associated with the periderm of potato in both natural and wounded states. Tyramine is 1,4-substituted and would also produce an absorbance at 816 cm^{-1}. It is not possible to tell by FT-IR spectroscopy alone if this compound is present in flax epidermal cells since the corresponding amine N–H and phenol O–H stretches would be masked by the carbohydrate and lignin/suberin O–H vibrations. The definitive assignment of this peak would require a detailed chemical study of the flax epidermis.

The epidermal cell walls, like those of the fibers, appear to show increased cellulose deposition with increasing age shown by an increase in the absorbances at 1130, 1098, and 1050 cm^{-1} (Figure 4).

Gland Cell. The final cell type studied was what we have called a gland cell. This specialized epidermal cell appears at fairly regular intervals around the stem (Figure 1).

At 7 days, the gland cell wall shows definite absorbances representative of lignin (1595 and 1510 cm^{-1}), cellulose (1130, 1098, and 1050 cm^{-1}), and esterified pectin/NCP (1740, 1680, 1600, and 1260 cm^{-1}) (Figure 5). The spectrum also contains broad absorbances centered at 1320 cm^{-1}, the region of phenolic O–H bending (Sarkanen *et al.*, 1967), and 1460–1430 cm^{-1}, the region of the hydrocarbon ($-CH_2-$) C–H deformation (Kemp, 1991), which can be assigned to the presence of suberin/cutin. However, these absorbances decrease in intensity with age. Cellulose absorbances are present (1130, 1098, and 1050 cm^{-1}), and these also decrease in intensity with age.

As the gland cells age, they begin to accumulate protein. This is seen as an increase in absorbance at 1640 and 1550 cm^{-1} (Figure 5). In fact, the changes in the gross chemical composition of the gland cell walls appear to be the reverse of those seen in the spectra of the epidermal cells (Figure 4) as the gland cells initially show pronounced suberin (possibly lignin/anthocyanin) deposition followed by increased protein accretion. This may indicate a role for these cells possibly in defense mechanisms (proteases) or as storage cells.

Comparison of Spectra of Cell Walls at 20 Days. The differences between the types of mature (20 days

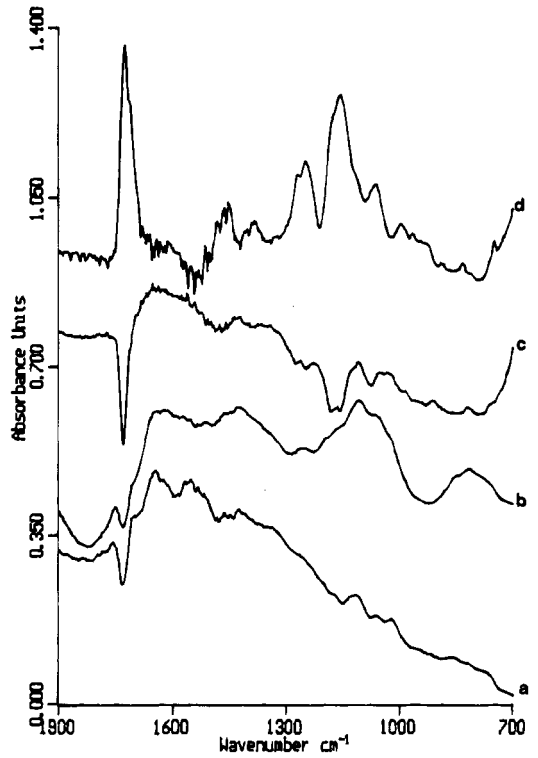

Figure 6. FT-IR spectra of (a) xylem, (b) fiber, (c) epidermal, and (d) gland cell walls after 20 days of growth.

old) cell walls are highlighted in their corresponding spectra (Figure 6). All of the spectra contain distinct differences, with the spectrum of the 20-day-old fiber cell wall (Figure 6d) being particularly dissimilar. The dominant features of this spectrum are the cellulose (1130 and 1150 cm^{-1}) absorbances and acetyl carbonyl (1730 and 1250 cm^{-1}) and methyl absorbances (1440 cm^{-1}), all of which are corroborative evidence for the report of glucomannans and xylans intimately associated with the cellulose microfibril (van Hazendonk *et al.*, 1994). Significantly, the lignin absorbances (1595 and 1510 cm^{-1}) are very small.

The spectra of the remaining three cell types have broadly similar line shapes but contain differences. For example, the spectrum of the xylem cell wall (Figure 6c) contains broad pectin and lignin absorbances as well as a small cellulose absorbance. The epidermal cell wall spectrum (Figure 6b), in comparison, has increased "lignin" and hydrocarbon absorbances, which suggest the presence of suberin and/or cutin. The cellulose absorbances are also increased.

The most obvious features in the spectrum of the gland cell wall (Figure 6a) are the absorbances at 1650 and 1540 cm^{-1} which, due to their concomitant increase in intensity with age, suggest protein accretion. The remainder of the spectrum is, in general, poorly resolved, with only small cellulose absorbances as a distinguishing feature.

Conclusion. FT-IR microspectroscopy allowed us to follow, by inference from characteristic absorbances, changes in the deposition of cellulose, lignin, and pectins that occurred during the development of four anatomically different cells of flax hypocotyls. The changes in wall structure of flax fibers inferred with this method between 5 day flax hypocotyls and the later samples correlated with the deposition of the secondary wall of flax fibers. Changes in NCPs that were inferred from associated acetyl carbonyl and methyl absorbances gave supportive evidence for the association of acetylated glucomannan with cellulose deposition. FT-IR microspectroscopy also highlighted differences between the epidermal and gland cells. These seemed to be suberized/cutinized epidermal cells with putative roles in desiccation and parasite resistance, but the gland cell accumulates protein during development for an, as yet, unknown reason. Therefore, we conclude that FT-IR microspectroscopy may be a valuable tool for following the changes in chemical structure and composition in developing plant tissues.

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