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Changes in pectin structure and localization during the growth of unadapted and NaCl-adapted tobacco cells

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Summary

Tobacco cells adapted to grow in high concentrations of NaCl exhibit a drastically altered growth physiology that results in cells whose fully expanded volume is only one-fifth to one-eighth those of unadapted cells. Comparison between NaCl-adapted and unadapted tobacco cells provides an opportunity to evaluate current concepts of the structural and mechanical determinants of cell wall expansion. Both biochemical studies of pectic polymers and the ultrastructural localization of pectic epitopes at three specific phases of cell culture, maximal cell division, maximal elongation, and stationary phase are reported here.

One-half of the galactosyluronic acid units in wall polymers of NaCl-adapted cells are esterified throughout the culture period, while wall polymers of unadapted cells show a rise in esterified polygalacturonic acid from 50 to 80% during elongation and then a decrease to 70% at stationary phase. Methyl esters account for only a proportion of the total esterified polygalacturonic acid at any stage in both unadapted and NaCl-adapted cell walls. Using monoclonal antibodies, we show differences in the localization of relatively methyl-esterified and unesterified pectic epitopes at different stages of growth and corroborate the chemical determinations. Fourier transform infrared (FTIR) microspectroscopy of representative walls of both NaCl-adapted and unadapted cells confirms, at the single cell wall level, that results obtained from chemical analysis of bulk samples are applicable to the entire cell population.

FTIR microspectroscopy also reveals an increase in wall protein in the walls of adapted cells. Images obtained by the fast-freeze, deep-etch, rotary-shadowed replica technique show clearly different cell wall architectures in NaCl-adapted compared with unadapted cells; walls of elongating unadapted cells contain long, thin fibres that show a net orientation with respect to the long axis of the cell, whereas walls of adapted cells have thicker, flatter bundles of fibres with no clear net orientation. Polarized FTIR microspectroscopy indicates that, in unadapted tobacco cells during elongation, pectin molecules may be oriented within the wall in a similar manner to cellulose. Possible ways in which pectin structure and conformation may affect the behaviour of the cellulose-xyloglucan network are discussed.

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Introduction

Cells in liquid culture adapted to grow under conditions of stress exhibit altered cell wall compositions. These model systems may prove useful for studies of the underlying mechanisms of cell expansion in plants (Iraki *et al.*, 1989a, 1989b, 1989c; Shedletzky *et al.*, 1990, 1992). Tobacco cells adapted to grow on nearly half molar NaCl achieve only 1/5 to 1/8 of the fully expanded volume of unadapted cells. Slower cell expansion occurs despite a threefold increase in steady-state turgor pressure and a two- to fivefold lowering of cell wall tensile strength (Iraki *et al.*, 1989a). The proportion of crystalline cellulose is only 50% and the hydroxyproline content is only 10% of that in unadapted cells, and this reduction in cellulose and hydroxyproline-rich glycoprotein is thought to account for the much lower wall tensile strength. Reduced expansion is correlated with a failure by adapted cells to secrete xyloglucans and uronic acid-rich polymers into the medium (Iraki *et al.*, 1989c). Walls of adapted cells have increased proportions of pectin and other non-cellulosic polymers and a two to threefold increase in protein (Iraki *et al.*, 1989a, 1989c). The biochemical basis of this reduced cell expansion is unclear.

Proposed mechanisms of cell expansion have focused on how the cellulose/xyloglucan network may be loosened. Because of the ability of cell walls to undergo an enzymic release of cell wall sugars *in vitro* ('autolysis'), a long-held idea is that glycanohydrolases may be secreted or activated at specific sites to cleave tension-bearing xyloglucans (for review, see Taiz, 1984). Promotion of cell extension *in vitro* by exogenous glycanases has never been demonstrated convincingly (Cosgrove, 1993), and most of the sugars released during wall autolysis are arabinose and galactose rather

than xylose and glucose (McQueen-Mason *et al.,* 1992; Terry *et al.,* 1981). The discovery of xyloglucan endo-transglycosylase activity (Fanutti *et al.,* 1993; Fry *et al.,* 1991; Nishitani and Tominaga, 1992) in plant cell walls provided some preliminary evidence for a model of wall loosening whereby the xyloglucans that span between cellulose microfibrils are cleaved, permitting movement of the microfibrils with respect to each other, and then religated to maintain wall strength. However, two proteins ca. 30 kDa isolated from cucumber hypocotyls promote wall extension *in vitro* with neither detectable hydrolase nor transglycosylase activity (McQueen-Mason *et al.,* 1992). These small proteins, tentatively called 'expansins', may displace xyloglucans from cellulose microfibrils by disrupting the hydrogen-bonding between the xyloglucan backbone and the microfibril (Cosgrove, personal communication).

Recent models of cell wall architecture (Carpita and Gibeaut, 1993; McCann and Roberts, 1991) have suggested that cell walls of dicots and non-graminaceous monocots are constructed from at least two independent but co-extensive and interactive networks, a cellulose/xyloglucan network and a pectin network, with a third interactive network of structural proteins in some cells. In tomato cells adapted to growth on 2,6-dichlorobenzonitrile, cross-bridging of pectins by Ca^{2+} constitutes the major load-bearing matrix (Shedletzky *et al.,* 1992), whereas it has been suggested that a mechanical role for the pectin network is unlikely in cells with a normal cellulose-xyloglucan network (Rayle, 1989; Virk and Cleland, 1990). Modifications in the degree of esterification of polygalacturonic acid and the size, frequency and conformation of junction zones could influence the fixed-charge density and/or porosity of the pectin gel (Carpita and Gibeaut, 1993). Loosening of the pectin network concomitant with wall expansion may involve changes in pectin gel rheology which influence metabolism of xyloglucan-cellulose by the fine control of pectin methyl esterification, calcium ion and proton concentration, and the activity of pectin methylesterase (Ricard and Noat, 1986). In grasses, increase in a non-methyl ester of galacturonic acid is associated with elongation (Kim and Carpita, 1992). Adaptation of the tobacco cells to NaCl results in marked differences in the chemical structure and organization of pectins. Although the walls contain similar proportions of total pectin, in adapted cells much of it is retained by the wall throughout the culture period, whereas in unadapted cells it is secreted into the culture medium (Iraki *et al.,* 1989b, 1989c). The rhamnosyl residues of rhamnogalacturonan from adapted cells are more highly substituted with arabinose and galactose but with shorter side-groups (Iraki *et al.,* 1989b). Extracellular polysaccharides from adapted cells include a large amount of protein and arabinogalactan but no uronic acid-rich material. Unadapted cells secrete high molecular weight uronic acid-rich pectins, arabinogalactan and low molecular weight xyloglucans into the medium (Iraki *et al* 1989c). These alterations in pectin structure may influence the cell wall metabolism responsible for decreases in wall extensibility and tensile strength.

We examined, by a variety of techniques, changes in pectin structure and localization in the cell wall at three different phases of the culture period representing maximal cell division, maximal cell expansion or cell elongation, and the stationary phase for both unadapted and NaCl-adapted cells. Biochemical determinations of total and methyl-esterified pectin, immunogold labelling on thin sections of low-temperature embedded cells with antibodies to pectic epitopes, and Fourier Transform Infrared (FTIR) microspectroscopy each document changes in the degree of esterification of the pectin in cell walls during the culture period. GLC-MS analysis and FTIR microspectroscopy indicate phenolic and novel saturated esters in adapted and unadapted tobacco cell walls. Direct visualization of the walls by the fast-freeze, deep-etch, rotary-shadowed (FDR) replica technique shows clearly the different architectures in cell walls of unadapted and NaCl-adapted tobacco cells. The application of methodologies capable of analysing single cell walls not only confirms that the chemical data are applicable to the entire cell population but also reveals novel features about pectin and protein constituents of the wall and their orientation.

Results

FTIR microspectroscopy

We have recently shown that FTIR microspectroscopy can be usefully applied to the study of cell walls (McCann *et al.,* 1992b, 1993). In the mid-IR spectrum, saturated esters absorb at 1740 cm^{-1} (Morikawa *et al.,* 1978), amide-stretching bands of protein occur at 1650 and 1550 cm^{-1} (Sutherland, 1952), carboxylic acid groups absorb at 1600 and 1414 cm^{-1} (Morikawa *et al.,* 1978), phenolics at 1620 and 1515 cm^{-1} (Williams and Fleming, 1980), and carbohydrates absorb between 1200 and 900 cm^{-1} (Williams and Fleming, 1980). Below 1500 cm^{-1}, in the so-called fingerprint region, peaks cannot be readily assigned to particular vibrations because many complex vibration modes overlap in this region. Highly reproducible FTIR spectra show a marked change in the proportions of esterified and unesterified pectins in unadapted cells at different stages of growth (Figure 1). At 9 days, the ester peak at 1740 cm^{-1} has increased whilst free acid stretches at 1600 and 1414 cm^{-1} have decreased proportionally. There is a decrease in the

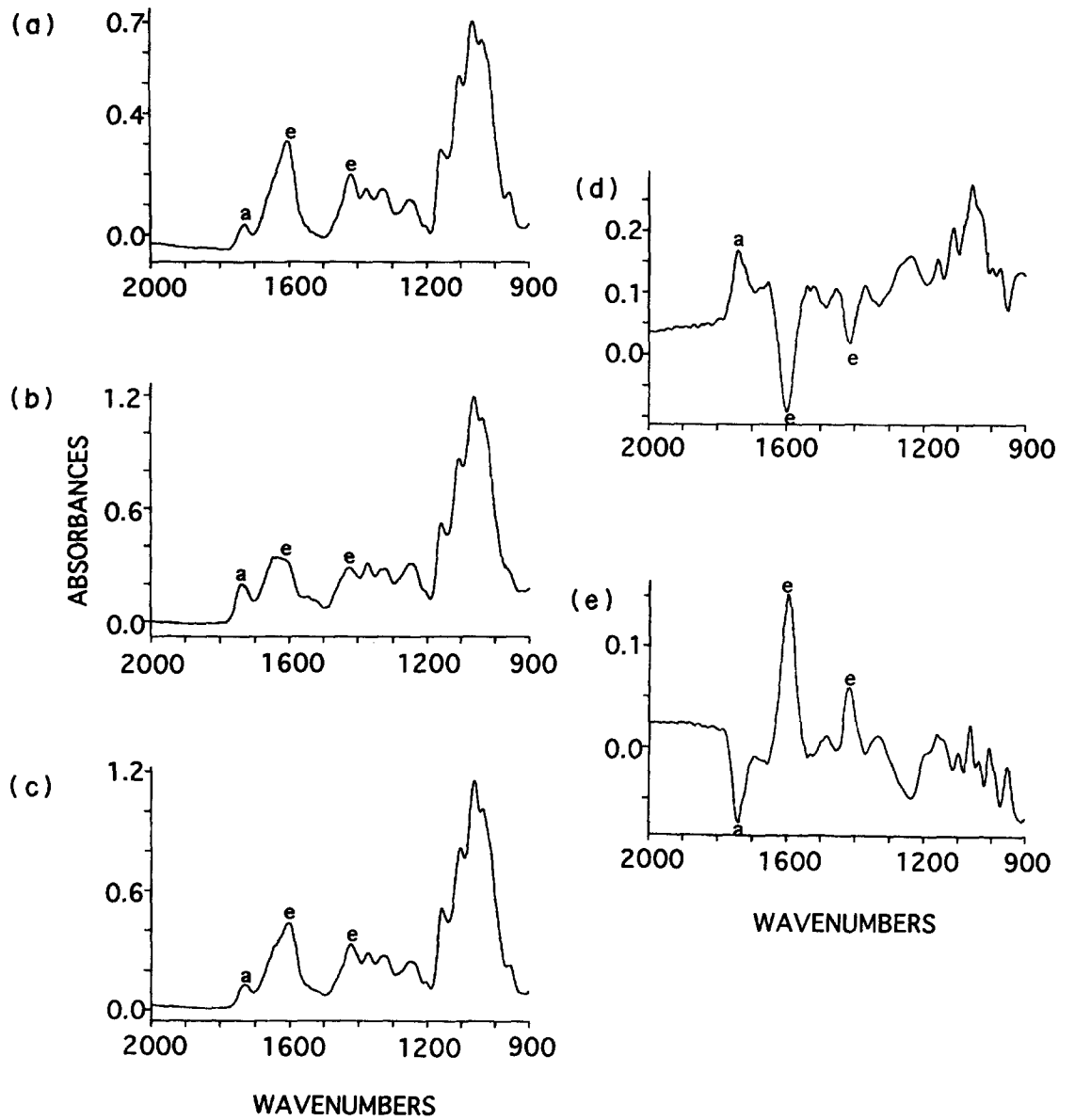

Figure 1. FTIR spectra obtained from single wall fragments of unadapted tobacco cells at different growth stages show that an increase in esterification of the wall occurs between 5 and 9 days during cell elongation and a decrease in esterification of the wall occurs between 9 and 16 days.
(a) Spectrum of walls of 5-day old tobacco cells.
(b) Spectrum of walls of 9-day old tobacco cells. There is a change in the relative intensities of the ester band at 1740 cm^{-1} (labelled a) and the carboxylic acid stretches at 1600 and 1414 cm^{-1} (labelled e).
(c) Spectrum of walls of 16-day old tobacco cells.
(d) Difference spectrum generated by digital subtraction of spectrum (a) from spectrum (b) shows the increase in the ester band at 1740 cm^{-1} (labelled a) and the decrease in the carboxylic acid stretches at 1600 and 1414 cm^{-1} (labelled e).
(e) Difference spectrum generated by digital subtraction of spectrum (b) from spectrum (c) shows the increase in free acid stretches at 1600 and 1414 cm^{-1} (labelled e) and the decrease in ester at 1740 cm^{-1} (labelled a). Some of the peaks in the carbohydrate region in spectrum (d) show inversion in spectrum (e) and are therefore possibly assignable to the ester group.

Key to principal absorbances for Figures 1, 2, 4 and 7 and references for peak assignments

Wavenumbers	Label	Assignment	Reference
1740	a	Carboxylic ester	Morikawa *et al.* (1978)
1650,1550	b	Amide stretches	Sutherland (1952)
1620, 1515	c	Phenolic stretches	Williams and Fleming (1980)
1300 to 900	d	Carbohydrate region	Williams and Fleming (1980)
1600, 1414	e	Carboxylic acid stretches	Morikawa *et al.* (1978)

proportion of unesterified pectin between 5 (maximal cell division) and 9 days (maximal cell elongation). At 16 days, stationary phase, the ester peak decreases relative to the free acid stretches which increase, indicating a rise in the proportion of relatively unesterified pectin between 9 and 16 days. In contrast, the NaCl-adapted cell walls show no change at equivalent growth stages (10, 19 and 26 days) apart from a substantial increase in protein and phenolic content by 26 days (stationary phase) (Figure 2).

Polymer orientations

Samples of walls from both NaCl-adapted and unadapted cells were imaged at high resolution in the electron microscope using the fast-freeze, deep-etch, rotary-shadowed replica technique (Heuser, 1981; McCann *et al.*, 1990). Many long thin fibres are apparent in the walls of unadapted cells, whereas many thicker bundles are present in the walls of adapted cells (Figure 3). Some images of unadapted tobacco cell walls from elongation and stationary phases show a predominant net orientation of fibrils. Adapted cell walls show no net orientation of fibrils at any time during the culture period. The adapted cells slowly expand isodiametrically but do not elongate. With a microscope accessory on an FTIR spectrometer, polarizers can be inserted in the path of the IR beam to determine if band frequencies of specific functional groups are oriented either transversely or longitudinally with respect to the long axis of the cell (McCann *et al.*, 1993). Walls of round unadapted cells and all NaCl-adapted cells show no polarization of their wall components; digital subtraction of polarized spectra taken at right angles to each other generates a null spectrum regardless of the pair of angles chosen. However, all elongated tobacco cells show strong polarization of carbohydrate bands transverse to the long axis of the cell (Figure 4). This may be attributed to the net transverse orientation of cellulosic microfibrils. Walls of elongated unadapted cells are strongly birefringent by polarized light microscopy but walls of round unadapted cells and NaCl-adapted cells are not (data not shown). The presence of a free acid stretch at 1600 cm^{-1} suggests that unesterified pectin may also be preferentially oriented.

Ester determination

Using a chemical reduction technique whereby the esterified uronic acids are selectively reduced to their 6,6-dideuteriosugars (Kim and Carpita, 1992) and assayed by GLC-MS, the proportion of total esters in walls of unadapted cells is initially about 50% in dividing cells, rises to 78% during elongation and then drops to 68% at stationary phase (Table 1). In contrast, the total ester in walls of NaCl-adapted cells remains just above 50% throughout the culture period. As the selective reduction technique revealed in developing maize coleoptiles (Kim and Carpita, 1992), methyl esters account for only a portion of the total esterified GalA units (Table 1). Methyl esters account for only one half of total ester in tobacco cells during the cell division phase. The proportion of methyl esters rises to about two-thirds during elongation and the increase in esterification is accounted for entirely by methyl esters. However, the proportion of methyl ester does not decline at stationary phase, so the decrease in

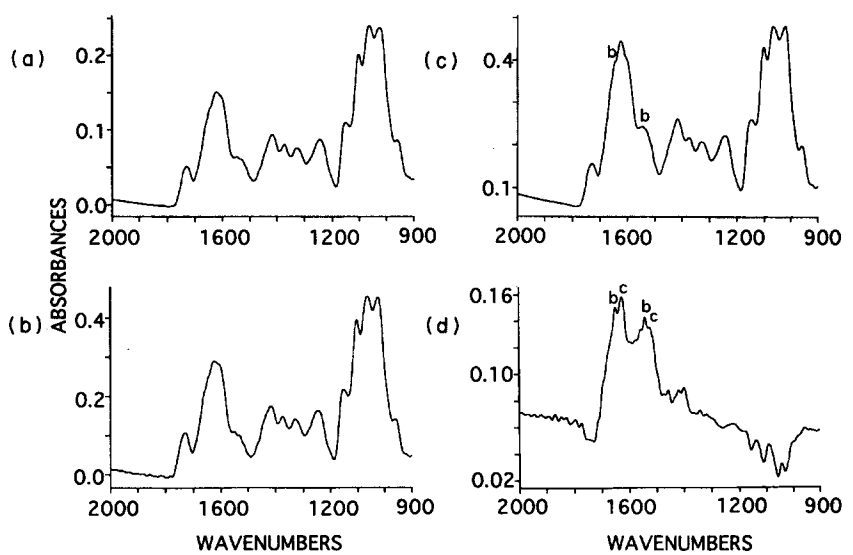

Figure 2. FTIR spectra obtained from single wall fragments of NaCl-adapted tobacco cells at different growth stages show no change occurring between 10 and 19 days during cell expansion but a large increase in protein and phenolic material in the wall between 19 and 26 days.
(a) Spectrum of walls of 10-day-old NaCl-adapted tobacco cells.
(b) Spectrum of walls of 19-day-old NaCl-adapted tobacco cells. The difference spectrum generated by digital subtraction of spectrum (a) from spectrum (b) is a null spectrum.
(c) Spectrum of walls of 26-day-old NaCl-adapted tobacco cells shows an increase in the amide stretches at 1650 and 1550 cm^{-1} (labelled b).
(d) The difference spectrum generated by digital subtraction of spectrum (b) from spectrum (c) shows the increase in protein (amide stretches at 1650 and 1550 cm^{-1}) (labelled b) and phenolics (1620 and 1515 cm^{-1}) (labelled c).

total ester must be due to loss of a different ester. The adapted cells show a continuous increase in methyl ester content from two to four-fifths of total esters in the adapted cells but without an increase of the total ester from about 50%.

Immunogold labelling

Immunogold-labelled sections of low-temperature embedded cell walls from unadapted and NaCl-adapted tobacco cells show differences in the localization of relatively methyl esterified and unesterified pectic epitopes at different stages of growth (Figures 5 and 6) and corroborate the chemical determinations. JIM 5, an antibody that recognizes a relatively unesterified pectic epitope (Knox *et al.*, 1990), strongly labels cell walls of unadapted cells in a population at cell division, labels the walls weakly during maximum elongation, and labels them strongly once again at stationary phase (Figure 5). The JIM 5-reactive epitope appears to be secreted into the medium during elongation. JIM 7, an antibody that recognizes a relatively methyl-esterified pectic epitope (Knox *et al.*, 1990), shows no labelling in the 5-day-old population but weak labelling in the 9-day-old and 16-day-old population (Figure 6). The adapted cells showed no changes in labelling density at different stages of growth. JIM 5 strongly labelled the walls of adapted cells at the equivalent growth stages of 10, 19 and 26 days (Figure 5), and JIM 7 labelled all walls weakly (Figure 6). A subpopulation of the walls of both adapted and unadapted cells labelled with JIM 13, an antibody that recognizes a carbohydrate epitope on arabinogalactan protein (Knox *et al.*, 1991), at all stages of growth (data not shown). From measurements of thin sections, the thickness of the purified cell walls of both unadapted and NaCl-adapted cells remains constant at about 200 nm.

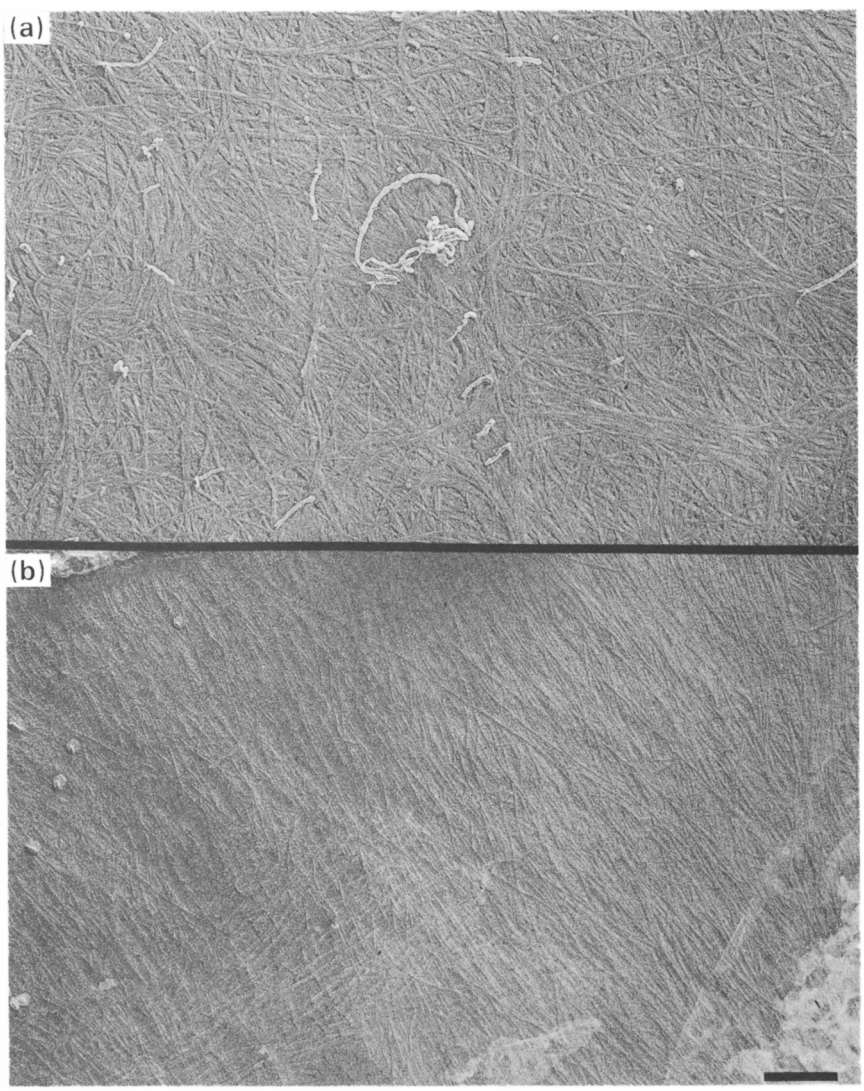

Figure 3. Micrographs of platinum/carbon replicas prepared by the fast-freeze deep-etch rotary-shadow (FDR) replica technique printed in reverse contrast.
(a) Cell walls from expanding NaCl-adapted cells have aggregated bundles of fibres of random orientation.
(b) Some images of cell walls of elongating unadapted tobacco cells show a high degree of orientation of the many thin discrete fibres.
Scale bar represents 200 nm.

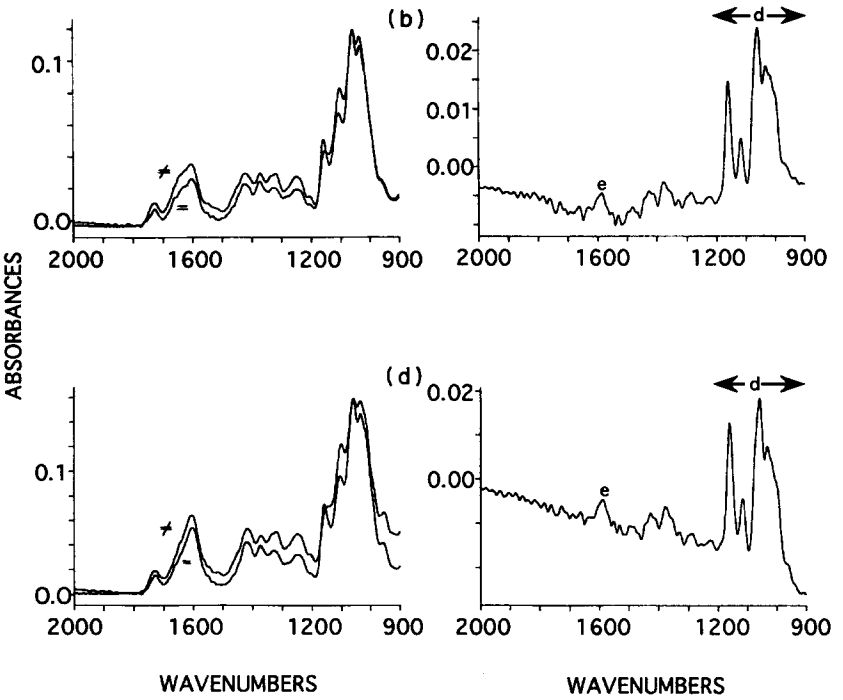

Figure 4. Polarized FTIR spectra and difference spectra from single cell wall fragments of unadapted tobacco cells at (a) 9 days old and (c) 16 days old show that cell walls from elongated cells have carbohydrate components oriented perpendicularly to the long axis of the cell. In each case, the spectrum labelled ≠ is obtained with the polarizer perpendicular to the long axis of the cell, whilst the spectrum labelled = is obtained with the polarizer parallel to the long axis of the cell. Difference spectra generated by digital subtraction of spectrum (≠) from spectrum (=) for (b) 9-day-old cell walls and (d) 16-day-old cell walls, show peaks in the carbohydrate fingerprint region of the spectrum (labelled d) which may be attributable to cellulose, but also a peak at 1600 cm^{-1} (labelled e) which may indicate some polarization of pectin. Digital subtraction of polarized spectra from either round tobacco cells (5 days old) or NaCl-adapted tobacco cells at all stages generates a null spectrum (not shown).

Table 1. Changes in uronic acid content and their esters in walls of unadapted and NaCl-adapted cells

		Mol%			
Source of cell walls	Days	Uronic acid[a]	GalA total ester[b]	GalA methyl ester[c]	GalA non-methyl ester
Unadapted cells	5	26 ± 2	53 ± 6	25 ± 1	28
	9	19 ± 2	78 ± 1	51 ± 4	27
	16	21 ± 1	68 ± 4	48 ± 2	20
NaCl-adapted cells	10	37 ± 1	54 ± 2	20 ± 3	34
	19	34 ± 2	53 ± 15	29 ± 1	25
	26	35 ± 2	55 ± 1	43 ± 7	12

[a] Uronic acid content as mol% of total cell wall non-cellulosic sugar. Values are the mean ± SD of four samples.

[b] Total esterified GalA was determined after selective reduction of GalA units to 6,6-dideuteriogalactosyl residues and subsequent GLC-MS. Values are the mean ± variance of two samples each from two experiments, with each MS determination the mean of four diagnostic mass ratios (Kim and Carpita, 1992).

[c] Methyl-esterified GalA was determined by measurement colorimetrically of total uronic acid (Filisetti-Cozzi and Carpita, 1991) and MeOH released by saponification (Wood and Siddiqui, 1971). Values are the mean ± variance of two samples. The proportion of GalA in total uronic acid was calculated after reduction of both GalA and GlcA with $NaBD_4$ and estimation of uronic acid by GLC-MS. The percentage of non-methyl esters of GalA is the difference of the means of total ester and methyl ester.

FTIR difference spectra

FTIR difference spectra generated by digital subtraction of the spectra of unadapted tobacco cell walls from the spectra of NaCl-adapted tobacco cell walls at equivalent growth stages show that walls of adapted cells have more protein, saturated and phenolic esters (absorbances at 1740 and 1720 cm^{-1}) and unesterified pectin in a given area of cell wall (Figure 7). From estimates of total non-cellulosic sugar and GalA by gas chromatography-electron impact mass spectroscopy and colorimetric assays, walls from adapted cells have much higher proportions of GalA than those from unadapted cells, but this varies little during the culture period, from 37 to 34%.

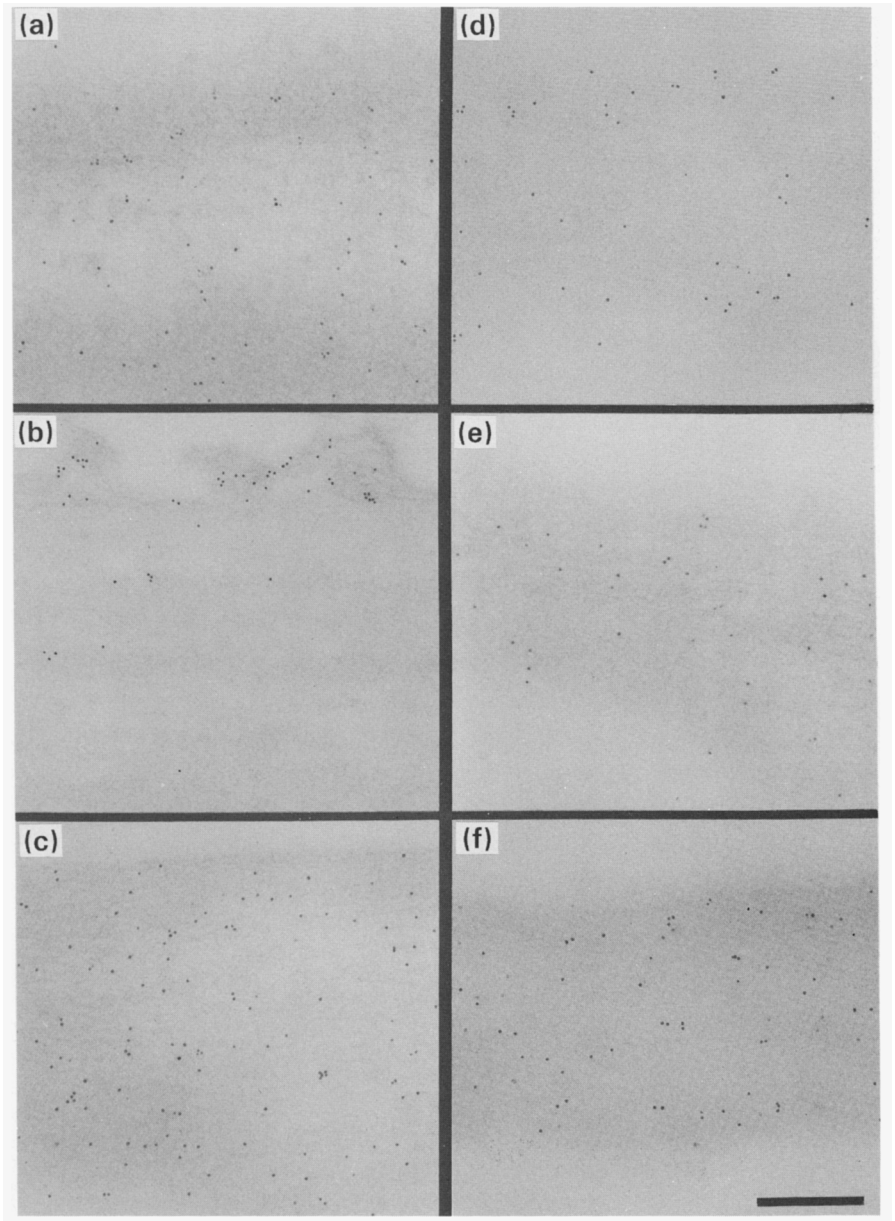

Figure 5. Immunogold labelling on thin sections of low-temperature embedded cell walls from unadapted and NaCl-adapted tobacco cells with JIM 5, a monoclonal antibody that recognizes a relatively unesterified pectic epitope.
(a) Cell walls of 5-day-old unadapted cells label strongly.
(b) Cell walls of 9-day-old unadapted cells label very weakly, but material that is being secreted into the culture medium labels strongly. The old part of the wall is labelled but new wall material is not.
(c) Cell walls of 16-day-old unadapted cells label strongly.
Cell walls of NaCl-adapted cells (d) 10 days old, (e) 19 days old and (f) 26 days old all label strongly.
Scale bar represents 200 nm.

The proportion of GalA in walls of unadapted cells is highest during cell division (26%) and decreases to about 20% of the total sugar during the remainder of the culture period (Table 1). A strong absorbance at 1620 cm^{-1} is also present in spectra of adapted cell walls which may be assigned as a phenyl-conjugated C=C, such as occurs in all hydroxycinnamic acids and their esters. The FTIR difference spectra also revealed the differences in pectin ester dynamics between unadapted and adapted cells (Figure 7b) with increased absorbances at 1600 and 1414 cm^{-1} showing the relatively greater proportion of unesterified pectin in the walls of adapted cells because of the decrease in the proportion of unesterified pectin in walls of unadapted cells at maximal cell elongation.

Discussion

In previous studies, we documented that, paradoxically, NaCl-adapted cells fail to expand fully despite more than adequate turgor and a structurally weaker cell wall (Iraki *et al.*, 1989a). However, turgor pressures in growing cells are well below the tensile strength of the wall. Cells expand because of a change in 'extensibility', discrete biochemical loosening of the matrix to permit microfibril separation together with carefully coordinated cell wall synthesis and deposition; extensibility is independent of tensile strength. In this study we focused on differences in pectin structure and its localization between unadapted and NaCl-adapted cells, and how these may relate to differences in cell expansion.

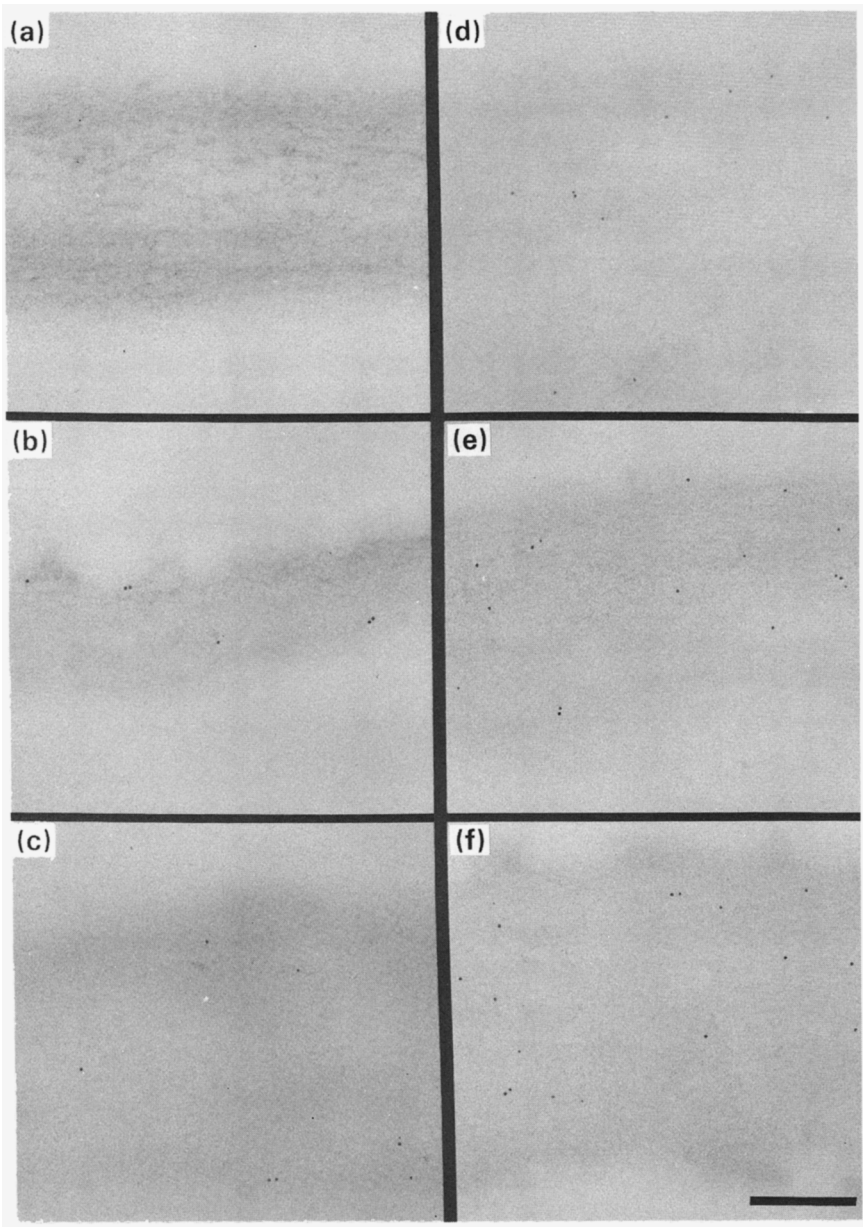

Figure 6. Immunogold labelling on thin sections of low-temperature embedded cell walls from unadapted and NaCl-adapted tobacco cells with JIM 7, a monoclonal antibody that recognizes a relatively methyl-esterified pectic epitope.
(a) Cell walls of 5-day-old unadapted cells do not label.
(b) Cell walls of 9-day-old unadapted cells label weakly.
(c) Cell walls of 16-day-old unadapted cells label weakly.
Cell walls of NaCl-adapted cells (d) 10 days old label weakly whilst labelling is slightly increased at (e) 19 days old and (f) 26 days old.
Scale bar represents 200 nm.

Cell shape is essentially predetermined by the net orientation of cellulose microfibrils within the cell wall and this in turn appears to depend on the net orientation of cortical microtubules within the cell. In the switch from isodiametric to elongation growth, transversely oriented cortical microtubule arrays may determine the orientation of newly deposited cellulose microfibrils. Correlated with the cell's ability to elongate, polarized FTIR micro-spectroscopy shows that both cellulose and free acid stretches attributable to pectin are oriented transversely to the direction of cell elongation during growth of unadapted cells. Neither isodiametrically expanding tobacco cells nor NaCl-adapted cells exhibit polarization.

Recent work has suggested that polymers not previously thought to be ordered such as pectin and protein are strictly oriented in some elongating cell types (McCann *et al.*, 1993), and it is changes in pectin structure and orientation as well as cellulose that correlate with the switch to elongation in the tobacco cells. However, the reduced expansion of the adapted cells is not simply a failure to elongate. Isodiametric expansion after cell division is slowed, and at stationary phase, the adapted cells become elliptical but with diameters less than those of unadapted cells (Iraki *et al.*, 1989a). Unadapted cells both expand and elongate at much higher rates. Direct visualization of wall architecture by the FDR replica

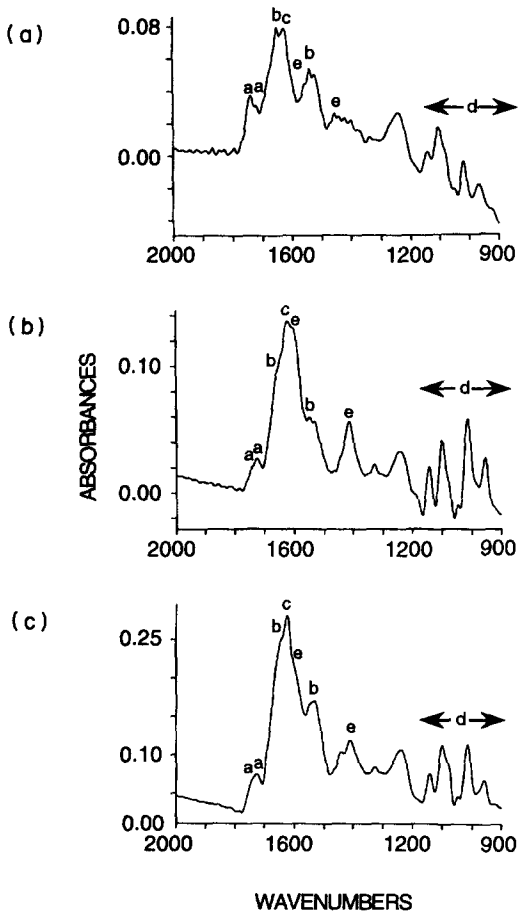

Figure 7. Difference spectra generated by digital subtraction of the spectra of unadapted tobacco cell walls from the spectra of NaCl-adapted tobacco cell walls at equivalent growth stages.
(a) Difference spectrum from unadapted walls (5 days old) and NaCl-adapted walls (10 days old) at maximal cell division.
(b) Difference spectrum from unadapted walls (9 days old) and NaCl-adapted walls (19 days old) at maximal cell expansion.
(c) Difference spectrum from unadapted walls (16 days old) and NaCl-adapted walls (26 days old) at stationary phase.
All three difference spectra show that NaCl-adapted walls contain more protein (amide stretches at 1550 and 1650 cm^{-1}) (labelled b), esters (a saturated ester stretch at 1740 cm^{-1} and a phenolic ester at 1720 cm^{-1}) (labelled a), pectin (carboxylic acid stretches at 1600 and 1414 cm^{-1} (labelled e) and a 'fingerprint' in the 1200–900 cm^{-1} region of the spectrum (labelled d) characteristic of polygalacturonic acid (McCann *et al.*, 1992b), and a band at 1620 cm^{-1} (assignable to a phenyl-conjugated C=C in, for example, ferulic esters) (labelled c), in a given area of cell wall than in the same area of wall from unadapted cells.

technique shows very different architectures of walls of NaCl-adapted and unadapted cells with much bundling of fibres in the adapted walls.

The mole fraction of uronic acid in polymers in the walls of unadapted cells decreases slightly during the culture period. However, there is a large increase in the mass of all polymers during cell expansion and elongation, and the total uronic acid ester increases markedly during this time from about 50 to nearly 80%. The proportion of total GalA esters also increases transiently from 65 to 80% during maximal elongation of maize coleoptiles (Kim and Carpita, 1992). This transient increase is a result of formation of an unidentified ester, whereas methyl esters decreased slightly in proportion throughout elongation. FTIR spectra indicate that there are phenolic and saturated esters in the walls of tobacco cells: phenolic esters giving rise to a peak at 1720 cm^{-1} and saturated esters absorbing at 1740 cm^{-1}, and a strong absorbance at 1620 cm^{-1} is also present in spectra of adapted cell walls which may be assigned as a phenyl-conjugated C=C, such as occurs in all hydroxycinnamic acids and their esters. GLC-MS analysis shows that only a proportion of the ester can be accounted for by methyl esters in both cell types. In contrast with the decrease observed in elongating maize coleoptiles, the proportion of methyl ester increases markedly during cell expansion, from 25 to about 50%, and accounts for all of the increase in total ester (Table 1). In tobacco, the proportion of the unidentified esters remains unchanged as the cells transit from division to expansion, but, as in maize coleoptiles, decreases in these esters accompany culmination of growth events. Carrot suspension cells also show a decrease in esterification after elongation (McCann *et al.*, 1993). In the walls of NaCl-adapted cells, uronic acid-containing polymers make up about one-third of the total non-cellulosic sugar throughout the culture period. Slightly more than one-half of the GalA is esterified and the proportion of methyl esters increases steadily throughout the culture period whilst the unidentified non-methyl esters decrease in the same proportion, such that total ester remains constant. In unadapted cells, a substantial proportion of the pectin is secreted into the medium (Iraki *et al.*, 1989c), so the changes in proportion of esterified GalA in the wall may reflect loss of specific polymers. In summary, marked increases in methyl esters accompany cell expansion and elongation of unadapted cells but loss of non-methyl esters is associated with cessation of expansion in both unadapted and adapted cells.

The alterations in methyl and total esterified uronic acids measured by GLC-MS are consistent with results from immunolabelling of thin sections with monclonal antibodies that recognize specific pectic epitopes. Immunolabelling density with JIM 5, a monoclonal antibody that recognizes a relatively unesterified pectic epitope, is almost abolished in all walls during elongation of unadapted cells whilst labelling with JIM 7, a monoclonal antibody that recognizes a relatively methyl-esterified pectic epitope, is increased. Although JIM 7 labels walls of cells at stationary phase reflecting the lack of change in methyl ester content, JIM 5 labelling density is substantially increased by only a 10% decrease in total ester. Hence the pectin in the wall is close to the limit of

pectin esterification at which the antibody will still bind and de-esterification is due to loss of other esters implying the activity of a novel esterase. Methyl esterification during elongation must occur uniformly along newly synthesized pectin molecules as no JIM 5-reactive epitopes are available for antibody binding. It seems likely that fine control of gel matrix properties can only be achieved if positions of esterification on long pectin molecules (some range up to 700 nm in length) (McCann *et al.*, 1992a) are very precisely defined. We also showed previously that both xyloglucans and pectins were released from the walls of growing tobacco cells, and this release was markedly attenuated in the very slowly expanding NaCl-adapted cells (Iraki *et al.*, 1989c). At maximum elongation, it appears that the unesterified pectin is secreted into the medium and that therefore an entire pectic network is being replaced. This raises some interesting questions. How does the new network of more methyl-esterified pectin become inserted into the wall? Is the whole network replaced before elongation can occur or are there domains that are capable of wall loosening?

It has been suggested that pectin methylesterase (PME) may have a regulatory role in cell expansion: this enzyme is maximally active at pH 8 and may de-esterify pectins in the wall thus generating fixed negative charges (Ricard and Noat, 1986). During acid growth, PME activity is inhibited, and wall glucosyl transferases may be active, resulting in changes in pectin gel rheological properties by increasing the proportion of methyl-esterified pectin, and permitting movement of cellulose microfibrils by cleavage and religation of non-cellulosic polymers that span between microfibrils. However, gels of isolated methyl-esterified pectins are mechanically stronger than gels of isolated unesterified pectins (Morris *et al.*, 1980). Furthermore, the proportion of methyl-esterified galacturonic acid increases from 40 to finally 80% of total ester in the adapted cell walls, but there is no change in the proportion of total ester. The enormous number of cell wall enzymes may include as yet uncharacterized transesterases and pectin trans-glycosylases.

Measurements of yield stress on gels of isolated pectins have shown that the optimum degree of esterification (d.e.) for maximum gel strength is around 70% (Morris *et al.*, 1980). The gel strength is not dependent on divalent cation concentration, and may be a result of non-covalent interactions with ester groups helping to stabilize interchain junctions. Morris *et al.* (1980) note a sharp reduction in gel strength at around 80% d.e. which they speculate is due to disruption of these non-covalent interactions. Maximal elongation of both unadapted tobacco cells and maize coleoptiles occurs at 80% d.e. of pectin. Whilst isolated pectin gels are clearly abiotic systems, gelation characteristics of pectins in the wall may well be greatly modified by the presence of other components, and the isolated pectin gels were made under conditions of low water activity, nevertheless, aggregation of pectins by non-ionic interchain associations has also been documented by gel permeation chromatography (Davis *et al.*, 1980) in aqueous pectin solutions, and as a contributing mechanism to network formation in calcium pectate gels (Gidley *et al.*, 1980). High-performance size exclusion chromatography measurements (Fishman *et al.*, 1989) and direct visualization in the electron microscope (McCann *et al.*, 1992a) have also demonstrated aggregation of isolated pectins. It has also been shown that the pectin matrix determines wall porosity (Baron-Epel *et al.*, 1988). It is possible that disruption of the non-covalent interactions, which may be analogous to tertiary interactions in protein folding, allows increased accessibility of expansins (McQueen-Mason *et al.*, 1992) or transglycosylases (Fry *et al.*, 1991) or hydrolases (Taiz, 1984) to the cellulose-xyloglucan network in the cell wall as well as providing a suitable ionic environment for relevant enzymes. A 10% reduction in esterification results in a gel of optimum strength (Morris *et al.*, 1980). By stationary phase, unadapted tobacco cells have 70% d.e. Artificial gels show reversible volume changes induced by small changes in temperature, type and concentration of salts, pH and electrical fields (Tanaka *et al.*, 1980, 1982). Further, the complexity and diversity in pectin structure and localization in cell walls of different species, tissues, and in different wall domains around a single cell (McCann *et al.*, 1992a) may reflect a functional diversity in the fine control of gel rheology .

At present, we can only speculate on why the NaCl-adapted cells fail to expand fully. These cells do not have oriented cellulose, and it is possible that the presence of half molar NaCl affects microtubule rearrangements. The wall does not change in thickness during the growth period; with continued biosynthesis of new wall polymers but reduced extensibility of the matrix, the wall would increase in thickness, whereas with normal extensibility but reduced polymer synthesis, the wall would thin during growth. The rate of wall loosening and the rate of deposition of new polymers must be tightly coupled with feedback mechanisms from wall to cell and it seems likely that both of these rates are reduced in the NaCl-adapted cells. One contributing factor to the lowered extensibility may be the effect of half molar salt on pectin gel rheology, which may then affect loosening of the cellulose-xyloglucan network.

Given the variety in cell wall composition between tissues, and even between walls bordering a single cell (McCann and Roberts, 1991), it is important to demonstrate that data obtained from bulk chemical analysis are applicable to the walls of all cells in the

population sampled. Using FTIR microspectroscopy and electron microscopy, two methodologies which permit analysis at the level of a single cell wall, we have shown that the major variation in the cell populations occurs in the amount of protein in adapted cell walls at very late stages of growth, and in the presence of a subpopulation of cells expressing the JIM 13-reactive epitope at all stages in both adapted and unadapted cells. The observed changes in esterification at different growth stages occur, however, in the entire cell population, thus validating the conclusions from the bulk analysis.

Experimental procedures

Biological material

Tobacco (*Nicotiana tabacum* L. cv. W38) suspension cells were maintained in a liquid medium (pH 5) containing Murashige and Skoog (1962) salts (prepared commercially by GibCo) and (per litre): 30 g sucrose, 1 g casein enzymic hydrolysate (Sigma), 100 mg *myo*-inositol, 3 mg 2,4-D, 0.1 mg kinetin, and 0.4 mg thiamine-HCl. Cells were adapted to grow in 428 mM NaCl (25 g l^{-1}) as described (Binzel *et al.*, 1985), and have been maintained in liquid culture for several years prior to use in experiments described here (Iraki *et al.*, 1989a). Unadapted cells were subcultured fortnightly, and NaCl-adapted cells monthly, at 0.8–2 g fresh weight per 100 ml of fresh medium in 500 ml Erlenmeyer flasks. Cultures were incubated at 26°C on a gyratory shaker at 110 r.p.m. with a 2.5 cm displacement.

Preparation of cell walls

Unadapted and NaCl-adapted cells were harvested at days representing maximal cell division (peak mitotic index or minimum cell volume), cell expansion or elongation, and stationary phase; unadapted cells were harvested 5, 9 and 16 days after subculture, and adapted cells were harvested at the corresponding 'physiological states' at 10, 19 and 26 days after subculture. Cells were collected by filtration on coarse sintered-glass funnels and frozen in liquid nitrogen. Frozen cells were suspended in 80% aqueous ethanol at 70°C for 30 min to inactivate pectin methyl esterase (Koch and Nevins, 1989). The cells were homogenized in a glass–glass Duall grinder in ice-cold 50 mM Tes (NaOH) supplemented with 30 mM ascorbate (pH 7.2). The cell wall material was pelleted at 1200 ***g*** for 5 min, resuspended and washed sequentially twice with Tes-ascorbate buffer, once with water, twice with ethanol, and four times with water. The walls were suspended in 90% aqueous DMSO and stirred vigorously overnight to extract starch (Carpita and Kanabus, 1987). After DMSO extractions, the walls were washed thoroughly with water and lyophilized.

Fast-freeze, deep-etch, rotary-shadowed (FDR) replica technique

Replicas of cell wall preparations from populations of NaCl-adapted and unadapted tobacco suspension cells were prepared, examined and photographed as described previously (McCann *et al.*, 1990).

Fourier transform infrared microspectroscopy

Spectra were obtained on a Bio-Rad FTS40 FTIR spectrometer equipped with a Spectra-Tech IR-Plan microscope accessory. Spectra were obtained at a resolution of 8 cm^{-1}, with 256 co-added interferograms. Ten spectra of each sample were collected; all spectra were highly reproducible. Cell walls were dried in a layer on the barium fluoride window (13 mm diameter × 2 mm) (McCann *et al.*, 1992b). A single beam of each sample was collected in transmission mode and ratioed to a single beam of the background collected from a clean region of the barium fluoride window. Insertion of a single polarizer (KRS5, Specac Ltd, UK) into the path of the IR beam before it passes through the cell wall sample mounted on the stage of the microscope accessory permitted polarization of the IR beam during micro-spectroscopy (McCann *et al.*, 1993). Difference spectra were generated by digital subtraction of a spectrum from a chosen reference spectrum using a subtraction factor to compensate for differing absorbances as discussed previously (McCann *et al.*, 1992b).

Immunogold labelling

Purified cell walls and suspension culture cells were fixed in 2% glutaraldehyde, pelletted, and re-suspended in a drop of low-gelling-point agarose before re-fixing for low-temperature embedding. Immunogold labelling was carried out as described previously (McCann *et al.*, 1992a).

Determination of ester content

Total uronosyl ester was determined by a modification of a selective reduction procedure (Maness *et al.*, 1990) where esterified uronosyl units are converted to their 6,6-dideuteriosugar derivatives (Kim and Carpita, 1992). Samples of the material from the primary reduction were saved for analysis of uronic acids. The remaining duplicate samples were activated with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate powder as described by Taylor and Conrad (1972) except that when the pH stabilized after about 2 h, the solutions were chilled to ice temperature and 2.5 ml of 4 M imidazole(HCl), pH 7.0, were added. Two batches of 200 mg each of $NaBH_4$ or $NaBD_4$ were added to the paired samples, and the suspensions were stirred in an ice bath for at least 1 h. The excess borohydride(borodeuteride) was destroyed by drop-wise addition of glacial acetic acid, and the mixtures were dialyzed against running deionized water for at least 40 h. Samples were frozen and lyophilized.

Chemical analyses

Uronic acid in 0.5 mg ml^{-1} aqueous suspensions was determined by a method where sulfamate and *m*-hydroxy-diphenyl were used to eliminate interference by neutral sugars completely (Filisetti-Cozzi and Carpita, 1991). Methanol from 5–10 mg samples of cell wall (or 1–2 mg of material from ammonium oxalate extracts) was released by saponification essentially as described by Wood and Siddiqui (1971) but with some slight modifications that increased sensitivity and reproducibility (Kim and Carpita, 1992). The assay was carried out as described by Wood and Siddiqui (1971) except that dilution of the arsenite with water was omitted and fresh pentane-2,4-dione reagent was always used.

Alditol acetate derivatives of the uronosyl-reduced wall material were prepared as described previously (Kim and Carpita, 1992). Derivatives were separated in a 0.25 mm × 30 m column of SP-2330 (Supelco) temperature programmed from 160°C to 240°C at 5°C min^{-1} with a 5–10 min hold at the upper temperature. Helium flow was 1 ml min^{-1} with a splitless injection. Electron-impact mass spectrometry was with a Hewlett-Packard MSD at 70 eV and a source temperature of 250°C. The system was autotune-programmed on the day of the sampling, and all samples were run in duplicate under the same tune. The proportion of 6,6-dideuterioalditol acetate was calculated as the mean of ratios of *m/z* 187/189, 217/219, 259/261 and 289/291. The proportions of uronic acid and neutral sugars were determined as previously described (Kim and Carpita, 1992).

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