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Cell Walls of Tobacco Cells and Changes in Composition Associated with Reduced Growth upon Adaptation to Water and Saline Stress[1]

Naim M. Iraki[2], Narendra Singh[3], Ray A. Bressan, and Nicholas C. Carpita*

Department of Botany and Plant Pathology (N.M.I., N.C.C.) *and Department of Horticulture* (N.S., R.A.B.), *Purdue University, West Lafayette, Indiana 47907*

ABSTRACT

The relative mass of the cell walls of tobacco (*Nicotiana tabacum* L.) cells adapted to grow in medium containing 30% polyethylene glycol 8000 or 428 millimolar NaCl was reduced to about 50% of that of the walls of unadapted cells. Cellulose synthesis was inhibited substantially in adapted cells. The proportions of total pectin in walls of unadapted and adapted cells were about the same, but a substantial amount of uronic acid-rich material from walls of cells adapted to either NaCl or polyethylene glycol was more easily extracted with cold sodium ethylenediamine tetraacetic acid solutions (NM Iraki *et al.* [1989] Plant Physiol. 91: 39–47). We examined the linkage composition of the pectic and hemicellulosic polysaccharides to ascertain chemical factors that may explain this difference in physical behavior. Adaptation to stress resulted in the formation of a loosely bound shell of polygalacturonic acid and rhamnogalacturonan. Pectins extracted from walls of adapted cells by either cold sodium ethylenediamine tetraacetic acid or hot ammonium oxalate were particularly enriched in rhamnose. Compared to pectins of unadapted cells, rhamnosyl units of the rhamnogalacturonans of adapted cells were more highly substituted with polymers containing arabinose and galactose, but the side groups were of greatly reduced molecular size. Possible functional roles of these modifications in cell wall metabolism related to adaptation to osmotic stress are discussed.

Water stress slows the growth rate of glycophytes (22, 29). In 1982, we demonstrated (10) that cultured cells of tomato fail to enlarge after adaptation to water stress even though full turgor had been completely restored. Leaves or other organs fail to enlarge in several other species despite complete osmotic adjustment and reestablishment of adequate turgor (29, 36). The adaptive value of growth limitation under conditions of reduced water availability has been addressed (12, 33), but not known are the physiological or chemical cues by which the plant perceives stress or the mechanisms by which ultimate cell size is reduced. We suggested (4, 21) that altered cell wall properties or metabolism are directly involved in growth limitation in response to osmotic stress. Changes in the mechanical properties of cell walls of plants exposed to water stress have been documented (29), but only preliminary data have been reported on changes in the chemical composition of the walls of tobacco cells in liquid culture upon imposition of osmotic stress (8).

Cells in liquid culture offer excellent model systems to study the physiological and biochemical alterations induced by stress. Like whole plants, cells of tomato and tobacco adapted to grow in medium supplemented with either PEG 8000 or NaCl also exhibit a marked decrease in average cell volume despite an osmotic 'overcompensation' that results in turgor pressures severalfold higher than those of unadapted cells—a suggestion that the ability of the cell wall to extend is decreased (4, 5, 10, 11). Similarly, leaves of barley exhibit a reduced rate of expansion independent of turgor pressure after exposure to NaCl (36). Moreover, if the decrease in average cell size is a result of wall 'stiffening,' it occurs even though the synthesis of cell wall polymers is inhibited substantially at the expense of accumulation of osmotic solutes (4, 5, 10, 11, 21). In a companion study (25), we established that the absolute tensile strength of the cell wall is reduced substantially; this reduction is concomitant with a decrease in the mass of the cellulose-extensin network. The decrease in the ability of the cell wall to extend in adapted cells must be a result of different components than those of mechanical strength. The physical nature of wall expansion and control of growth rate through changes in cell turgor and wall extensibility are well studied (15, 34), but the biochemical alterations responsible for these changes are not known.

By fractionation of the major groups of polysaccharides and proteins based on their solubility in chemical solvents, separation of the individual polymers chromatographically, and linkage (methylation) analysis, a good catalog of the principal constituents of the primary walls of dicots has been established (2). Although information about the arrangement of the polymers and an accurate three-dimensional model of the wall is rudimentary, most models of the dicot wall envision a framework of cellulose microfibrils coated with hemicelluloses, mostly xyloglucans, which hydrogen bond tightly to cellulose and may span the distance between the microfibrils (2, 23). This semirigid framework is embedded in a gel matrix

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[2] Present address: Department of Biology, Bethlehem University, Bethlehem, Israel.

[3] Present address: Department of Biological Sciences, Virginia Common Wealth University, Richmond, VA 23284.

of pectic substances comprising polygalacturonic acid, rhamnogalacturonans, and several arabinans, galactans, and arabinogalactans (2, 26). During differentiation, the hydroxyproline-rich glycoprotein extensin accumulates in the primary wall and crosslinks the cellulosic framework to form a rigid, inextensible matrix (18, 28).

We found that cells adapted to media containing NaCl or PEG develop turgor pressures higher than normal cells yet diverted much more carbohydrate from the medium to osmotic solutes at the expense of synthesis of cell wall polysaccharides (25). In particular, the mass of the cellulose and hydroxyproline-rich glycoproteins, most likely extensin, was greatly lowered. Even though turgor pressures of adapted cells were higher than unadapted cells, the absolute tensile strengths of the cell walls of adapted cells were much lower. Consistent with loss in tensile strength was a reduction in the mass of the cellulose-extensin matrix (25). Hence, other determinants are likely to be responsible for the reduction in the ability of the cell walls of adapted cells to expand. We also had found large differences in the proportions of three fractions of pectins obtained by differential extraction with chelator solutions and weak alkali (25). In this report, we document specific alterations in the chemical composition and organization of the pectins that may be related to alteration in the ability of the cell walls of adapted cells to expand.

MATERIALS AND METHODS

Plant Material and Isolation of Wall Material

Cells of tobacco (*Nicotiana tabacum* L. cv W38) were maintained in liquid culture, and cell walls were purified and fractionated as described in a companion report (25).

Chemical Composition

Protein was assayed according to Bradford (9) (commercial reagents from Pierce) with BSA standards, total sugar by the phenol-sulfuric method (17) with glucose standards, and uronic acid by a carbazole method (16), as modified by addition of sulfamate (19), with galacturonic acid standards.

Determination of Sugar Composition and Linkage Analysis

Polymers (2–3 mg) were hydrolyzed in 1 mL of 2 M TFA[4] containing 1 μmol *myo*-inositol (internal standard) at 120°C for 90 min. The TFA was evaporated in a stream of N_2, and sugars were reduced and acetylated according to Blakeney et al. (6) as modified by Carpita and Whittern (14). Derivatives were separated by GLC in a 0.2-cm × 3-m column of 3% SP-2330 (Supelco) temperature programmed from 170°C to 240°C at 5°C/min. The injector and flame ionization detector temperatures were 260°C. One to three μL samples were injected, and the carrier N_2 flow was 30 mL/min.

For linkage analysis, the carboxyl groups of glycosyluronic acids were activated with carbodiimide and reduced by $NaBD_4$ according to Taylor and Conrad (35). Some samples of polysaccharides (1–3 mg) were per-*O*-methylated with the potassium methylsulfinylmethanide anion and CH_3I according to Hakomori (20) as modified by Carpita and Whittern (14). Other duplicate samples were permethylated with the Li^+ methanide anion (7) prepared by addition of *n*-butyllithium directly to the suspension of polysaccharide in DMSO (27). This latter method proved simpler and yielded consistently complete per-*O*-methylations. Per-*O*-methylated polymers were hydrolyzed with 2 M TFA, and partially methylated sugars were reduced and acetylated as described above. Partially methylated alditol acetates were separated in a 0.2-mm × 30-m vitreous silica wall-coated open tubular column of SP-2330 (Supelco) temperature-programmed from 160°C to 240°C at 2°C/min with a 10-min hold at the upper temperature. Samples (2–5 μL) were injected with a split ratio of about 50:1 and a H_2 carrier flow of 1.5 mL/min. Electron impact MS was performed with a Finnigan/MAT 4021 quadrupole MS interfaced to a Finnigan/MAT 2100 INCOS data system. Spectra were obtained at 70 eV and a source temperature of 160°C. General techniques for preparation, separation and identification of partially methylated alditol acetate derivatives were as described elsewhere (13). Samples were all run in triplicate, or sometimes duplicate, and variance was always less than ±5%.

RESULTS AND DISCUSSION

Growth

Unadapted cells and cells adapted to desiccation or salt stress used in this study have been maintained in liquid culture for several years and accumulated dry weight at about the same rate (25), indicating that the cells had adapted to osmotic stress. The fresh weight gain, however, was much greater in unadapted than in either PEG- or NaCl-adapted cells. This difference was also apparent in the fresh weight/dry weight ratio (25). These results and measurements of rates of cell enlargement indicated that, upon adaptation to water stress, cell expansion was markedly reduced (25).

Decreased cell expansion might be a mechanism for water conservation by allowing the plant to survive when amounts of available water are limited (12, 33). On the other hand, the observed inhibition of growth may simply result from metabolic alterations necessary for the plant to cope with stress and may be unrelated to adaptation (22). Cells adapted to water stress accumulate large amounts of solutes either by active uptake of ions or by synthesis of organic solutes (22, 29). These processes demand input of energy and intermediates that could otherwise be used for growth.

Chemical Composition of the Pectic Polysaccharides

The total amount of cell wall was greatly reduced upon adaptation to saline or water stress, and there were differences between adapted and unadapted cells in the distribution of the various wall fractions (25). Cell walls of adapted cells had much lower proportions of cellulose and, consequently, higher proportions of hemicellulose than those of unadapted cells (25). Examination of the chemical composition of the various pectic fractions also revealed substantial differences among the cell lines. The large amounts of material extracted from

[4] Abbreviation: TFA, trifluoroacetic acid; PEG, PEG 8000.

the adapted cells by cold EDTA were particularly enriched in uronic acids compared to the unadapted cells, whereas the small amounts of material extracted from unadapted cells mostly comprised polymers containing neutral sugar (Table I). The proportions of uronic acids in the material extracted with hot ammonium oxalate from all cell lines were substantially higher than those in the EDTA fraction (Table I). Linkage analyses described later indicated that the increased proportion of uronic acid is largely due to galacturonan.

The proportions of several neutral sugars in these polymers differed as well. Arabinose and galactose were the predominant sugars in all fractions, perhaps from arabinans, galactans, and arabinogalactans that are ubiquitous constituents of the side chains of rhamnogalacturonans in primary walls of dicotyledonous species (1, 2, 26). The ratios of arabinose and galactose were quite variable in each fashion, and the fractions extracted with dilute alkali were particularly enriched in arabinose (Table I). Most notably, however, amounts of rhamnose in material extracted by hot ammonium oxalate from walls of PEG-adapted cells were substantially higher than in any corresponding fractions from unadapted cells or even cells adapted to NaCl (Table I).

Linkage Analysis

Linkage analysis of the pectic fractions showed several differences in composition among the fractions as well as among the cell lines. First, the same amount of material extracted by cold EDTA from unadapted cells contained higher proportions of total galactosyl and arabinosyl units, particularly 3,6-galactosyl and terminal-arabinofuranosyl units, (Table II), the expected diagnostic linkages of type II arabinogalactan (1, 2). In contrast, the cold EDTA fractions from the PEG-adapted cells contained, in addition to arabinogalactan, polymers with more 2- and 2,4-linked rhamnosyl and 4-linked galactosyluronic acid units; these linkages are expected from rhamnogalacturonan, a substituted polymer in pectins of many dicots (1, 2, 26). The material extracted by hot ammonium oxalate from walls of cells adapted to 428 mM NaCl was enriched in 4-galactosyluronic acid but not in rhamnosyl units (Table II). Pectins extracted by hot ammonium oxalate from walls of all cell lines were enriched with the 2- and 2,4-linked rhamnosyl units expected from rhamnogalacturonan compared to materials from EDTA extracts. The fraction from PEG-adapted cells was particularly enriched in these rhamnosyl units (Table II). The material from walls of NaCl- and PEG-adapted cells extracted by dilute KOH was similar to that extracted by hot ammonium oxalate except that arabinans containing 5-linked arabinosyl units were more abundant (Table II).

To gain a better idea of the composition of the total pectin and the changes induced upon adaptation to water or saline stress, we calculated the contribution of the rhamnosyl units (both branched and unbranched), the combined arabinosyl and galactosyl (from polymers which comprise the principal side group substitutions of the rhamnogalacturonan), and galactosyluronic acid to the total pectin. The total proportion of rhamnosyl units in the wall increased over unadapted cells by about 3-fold in PEG-adapted cells, but only by about 30% in the NaCl-adapted cells (Table III). In both of the adapted lines, however, the proportion of the 2,4-linked rhamnosyl branch residue increased about 2- to 3-fold over that in walls of unadapted cells (Table III). These data indicated that walls of adapted cells were preferentially enriched in rhamnogalacturonan with increased side group substitution, and those of unadapted cells contained more homogalacturonan. The relative increase in proportion of rhamnose was due largely to a marked reduction in the average size of the side groups. From comparison of the total arabinosyl and galactosyl units to available 2,4-linked rhamnosyl units, the average size of the substitutions was about 26 sugar residues for unadapted cells compared to only 10 to 12 residues for the adapted cells (Table III). In the hot ammonium oxalate fraction specifically, the average size was about 15 residues for unadapted cells but only about 6 residues for the adapted cells (Table III). Of course, these simple calculations cannot provide specific information on the actual composition and diversity of these substitutions, but they do reflect subtle but consistent altera-

Table I. *Sugar Composition of the Cell Wall Pectic Fractions Extracted from Unadapted Tobacco Cells (S0) and Cells Adapted to Medium Containing 428 mM NaCl (S25) or 30% PEG (P30)*

Pectins were extracted sequentially with ice-cold 5 mM EDTA, hot 0.5% ammonium oxalate, and 0.1 M KOH as described in the "Materials and Methods"; values in brackets are weight percent of the total cell wall. Values are mol % of the sugar recovered; for comparison, those in parentheses denote mol % of neutral sugars calculated with omission of the uronic acids.

Cell Line	Extractant	Rhm	Fuc	Ara	Xyl	Man	Gal	Glc	Uronic Acid
					mol %				
S0	EDTA [4]	5 (6)	tr (1)	32 (36)	8 (9)	4 (5)	28 (31)	11 (12)	11
	Ammonium oxalate [34]	5 (18)	1 (3)	12 (44)	1 (4)	1 (3)	5 (20)	2 (8)	73
	KOH [12]	3 (4)	1 (1)	54 (73)	2 (3)	2 (3)	9 (12)	3 (4)	26
S25	EDTA [26]	5 (10)	1 (2)	20 (41)	3 (7)	2 (4)	11 (23)	6 (13)	52
	Ammonium oxalate [12]	5 (18)	tr (tr)[a]	10 (36)	2 (6)	1 (3)	9 (32)	1 (5)	72
	KOH [10]	10 (13)	tr (tr)	38 (49)	5 (6)	2 (2)	17 (22)	6 (8)	22
P30	EDTA [12]	9 (15)	1 (2)	20 (32)	6 (9)	3 (5)	16 (26)	7 (11)	37
	Ammonium oxalate [29]	17 (34)	1 (1)	12 (24)	3 (5)	2 (5)	12 (24)	3 (7)	51
	KOH [8]	8 (11)	2 (2)	38 (51)	5 (7)	3 (4)	14 (19)	4 (6)	28

[a] tr = trace.

Table II. *Distribution of Sugar Linkages in the Chemically Reduced Pectic Fractions from Walls of Unadapted Cells (S0) and Cells Adapted to 428 mM NaCl (S25) and 30% PEG (P30)*

Values in brackets are the weight percent of the total cell wall material (25).

Deduced Linkage[a]	5 mM EDTA			0.5% Ammonium Oxalate			0.1 M KOH		
	S0 [4]	S25 [26]	P30 [12]	S0 [34]	S25 [12]	P30 [29]	S0 [12]	S25 [10]	P30 [8]
					mol %				
Rhamnosyl									
t-	2	3	3	2	1	4	2	1	1
2-	2	1	5	8	1	17	6	2	4
2,4-	1	1	1	3	4	6	3	4	4
	5	5	9	13	6	27	11	7	9
t-Fucosyl		1		1	1	1		tr	
Arabinosyl									
t*f*-	29	11	18	16	8	9	30	20	15
t*p*-	1	tr[b]	1	1	1	1		1	
2*f*-	1	1	6	1	2	tr	5	1	5
3*f*-	2	1	4	1		1	7	1	3
5*f*-	7	9	5	19	17	11	15	23	14
2*p*-	tr			tr	2	tr		1	
2,5*f*-							1	tr	1
3,5*f*-	1	tr		5	2	1	5	2	1
	41	22	34	43	32	23	63	49	39
Xylosyl									
t*p*-	3	4	2	2	3	4	1	1	2
2*p*-	2	1	1	1	1	tr	1	tr	2
4*p*-	2	tr	3	tr	1	1	1	3	8
2,3+3,4*p*-	2	tr	5	1	1	tr	1	tr	
2,4*p*-	tr	1	1	tr	tr	tr		tr	
	9	6	12	4	6	5	4	4	12
t-Mannosyl	tr			3	1	1		tr	
Galactosyl									
t-	2	2	2	3	4	7	2	3	3
3-	1	2		1	1	1	1	1	2
2-				2					
4-	1	4	1	4	3	4	tr	2	3
6-	1	2		2	3	1	1	1	2
3,6-	10	2		5	2	1	4	9	3
	15	12	3	17	13	14	8	16	13
Galactosyluronic									
t-	3	1	7	3	2	5	3	2	1
4-	14	49	17	12	33	17	4	1	9
	17	50	24	15	35	22	7	3	10
Glucosyl									
t-	1	1	12	1	1	2	3	11	1
2-	tr	1			tr	1			tr
4-	4	2	6	2	4	1	1	8	10
4,6-	5	tr		tr	1	1	2	tr	1
	10	4	18	3	6	5	6	19	12
Glucosyluronic									
t-	1			1	tr	1	1	1	2
4-	2	tr			tr	1		1	3
	3	tr	—	1	tr	2	1	2	5

[a] Linkages were deduced from electron-impact MS of partially methylated alditol acetate derivatives. t-=nonreducing terminal sugar; 2*p*-=2-linked rhamnopyranosyl unit deduced from 1,2,5-tri-*O*-acetyl-(1-deuterio)-3,4-di-*O*-methyl-6-deoxyhexitol identified by GLC-MS (13), and so forth. [b] tr=trace amounts less than 0.5%.

Table III. *Proportions of Neutral Sugars and Uronic Acids in Pectin Fractions from Unadapted Cells (S0) and Cells Adapted to 428 mM NaCl (S25) and 30% PEG (P30) Calculated from the Contribution of Each Sugar to the Total Sugar Content of Each Fraction and from the Contribution of Uronic Acids to the Total Polysaccharide Content of the Fraction (Table I)*

Values are mol % of the sugars based on the weight percent contribution of each pectin fraction to the total cell wall. The proportions of the 2,4-linked rhamnosyl residues in each pectic fraction were calculated from the proportions of these residues obtained from the methylation analysis (Table II).

Sugar	Cell Line and Fraction											
	S0				S25				P30			
	EDTA	Ammonium oxalate	KOH	Total	EDTA	Ammonium oxalate	KOH	Total	EDTA	Ammonium oxalate	KOH	Total
						mol %						
Rhamnosyl	0.2	1.7	0.4	2.3	1.3	0.6	1.0	2.9	1.1	4.8	0.6	6.5
2,4-Rhamnosyl	0.1	0.4	0.1	0.6	0.3	0.4	0.6	1.3	0.1	1.1	0.3	1.5
Ara+Gal	2.4	5.9	7.6	15.9	8.0	2.3	5.5	15.8	4.4	6.8	4.0	15.2
Other sugars	1.0	1.7	1.0	3.7	3.2	0.5	1.3	5.0	2.0	2.6	1.1	5.7
Uronic acids	0.4	24.8	3.1	28.3	13.5	8.6	2.2	24.3	4.4	14.8	2.2	21.4
Ara+Gal/2,4-rhamnosyl	24.0	14.8	76.0	26.3	26.7	5.8	9.2	12.2	44.0	6.2	13.3	10.1

Table IV. *Distribution of Sugar Linkages in Hemicellulosic Material Extracted with 4 M KOH from Cell Walls of Unadapted Cells (SO) and Cells Adapted to 428 mM NaCl (S25) and 30% PEG (P30)*

Cell Line	Rhm			Fuc	Ara								Xyl					Man			Glc				Gal				
	t-[a]	2-	2,4-	t-	tf-	tp-	2f-	3f-	5f-	2p-	3,5f-	2,5f-	t-	2-	4-	2,3-3,4-	2,4-	t-	4-	4,6-	t-	2-	4-	4,6-	t-	3-	4-	6-	3,6-
													mol %																
S0	1	1	2	1	11	2	1	1	11	tr[b]	4		7	2	12	tr	3	tr	1	6	1	tr	16	7	5	1	1	2	1
S25	1	1	2	1	11	1	tr	1	36	tr	8	1	3	1	6	tr	1	2	1	2	tr	1	9	2	4	2	1	1	1
P30	1	2	1	1	12	2	2	3	7	tr	1		6	4	8	tr	3	1	1	7	1	tr	19	8	6	1	1	1	1

[a] Sugar designations as in Table II. [b] tr = trace amounts less than 0.05%.

tions in the organization and composition of the wall upon adaptation to saline and water stress.

The organization of the various pectic components depends on many factors not directly apparent from chemical analysis. Current models of the pectin gel matrix suggest that homogalacturonans are immobilized and cross-linked by Ca^{2+} into rigid "egg-box" structures (32). The size and distribution of the egg-box junction zones are possibly controlled by incorporation of the contorted rhamnogalacturonan polymers (26). Pectins constitute an important determinant of wall porosity (3), and fine tuning of this porosity of the matrix might be controlled by size and extent of the side group substitution upon the rhamnogalacturonan. Further, galacturonans are possibly secreted as more neutral polymers with carboxyl groups of the glycosyluronic acids blocked as methyl esters (30). The size and extent of the Ca^{2+}-stabilized junction zones could also depend on enzymic deesterification of the homogalacturonan (30, 31). An additional group of pectins may be organized through ester linkages between chains, perhaps through ether linkage of aromatic cross-links provided by ferulic, *p*-coumaric, and other hydroxycinnamic acids (18). The relevance of the changes in the organization of the pectins to the mechanical properties of the walls of adapted cells is not readily apparent, but others have observed changes in pectin structure upon changes in growth status. Treatment of epicotyl sections with auxin caused a marked increase in the solubility of arabinogalactans and galacturonans in addition to the more widely acknowledged release of xyloglucan fragments (37).

Linkage analysis of the material extracted by 4 M KOH showed that arabinogalactans still contributed a substantial part of the walls of both unadapted and adapted cells (Table IV). The proportions of terminal- and 2-linked xylosyl and 4,6-linked glucosyl residues in the fraction from NaCl-adapted cells were much lower than those in the corresponding fraction from unadapted cells (Table IV). These xylosyl and glucosyl linkages are typical of hemicellulosic xyloglucan in primary walls of dicots (2), and the reduced proportion of xyloglucan in the walls of the NaCl-adapted cells may be a consequence of a selective inhibition of its synthesis. This hemicellulosic fraction of the saline-adapted cells was remarkably enriched in polymers containing 5-linked arabinosyl units (Table IV). The 2-xylosyl and 4,6-glucosyl units indicative of xyloglucans were particularly enriched in fractions extracted by cold EDTA as well (Table II). In the companion paper, we report that substantial amounts of xyloglucan were actually released into the incubation medium from unadapted cells, but not adapted cells (24). We suggest that inhibition of the hydrolysis of the cell wall xyloglucan contributed to the decrease in the ability of walls of adapted cells to extend. We do not know whether or not this results from inhibition of secretion of hydrolases responsible for the cleavage of xyloglucans, but changes in pectin structure certainly could contribute to control of the activity of these enzymes *in vivo*. Ratios of deester-

ified homogalacturonan to rhamnogalacturonan and the extent and size of the side group substitutions upon these polyuronans each could control porosity of the wall and even the movement of protein and hydrolytic enzymes within the wall matrix. Observations of the kinds of material secreted or released into the incubation medium will give further insight to changes that have occurred in the cell wall as a result of adaptation to saline stress, and this is the subject of the companion paper.

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