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BARD

FINAL REPORT

PROJECT NO. IS-1926-90

Induced Plant Cell Wall Modifications: Use of Plant Cells with Altered Walls to Study Wall Structure, Growth and Potential for Genetic Modification

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1995

DATE: May 1, 1995BARD project No. IS-1926-90C

Title:

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genetic modification

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The Hebrew UniversityPurdue UniversityThe Institute for Cancer ResearchProject's starting date: October 1, 1991Type of Report: 1st Annual 2nd Annual Final X

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Abstract

Our previous work indicated that suspension-cultured plant cells show remarkable flexibility in altering cell wall structure in response either to growth on saline medium or in the presence of the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile (DCB). We have continued to analyze the structure of these modified cell walls to understand how the changes modify wall strength, porosity, and ability to expand. The major load-bearing network in the walls of DCB-adapted dicot cells that lack a substantial cellulose-xyloglucan network is comprised of Ca^{2+} -bridged pectates; these cells also have an unusual and abundant soluble pectic fraction. By contrast, DCB-adapted barley, a graminaceous monocot achieves extra wall strength by enhanced cross-linking of its non-cellulosic polysaccharide network via phenolic residues. Our results have also shed new light on normal wall structure: 1) the cellulose-xyloglucan network may be independent of other wall networks in dicot primary walls and accounts for about 70% of the total wall strength; 2) the pectic network in dicot walls is the primary determinant of wall porosity; 3) both wall strength and porosity in graminaceous monocot primary walls is greatly influenced by the degree of phenolic cross-linking between non-cellulosic polysaccharides; and 4) the fact that the monocot cells do not secrete excess glucuronoarabinoxylan and mixed-linked glucan in response to growth on DCB, suggests that these two non-cellulosic polymers do not normally interact with cellulose in a manner similar to xyloglucan. We also attempted to understand the factors which limit cell expansion during growth of cells in saline medium. Analyses of hydrolytic enzyme activities suggest that xyloglucan metabolism is not repressed during growth on NaCl. Unlike non-adapted cells, salt-adapted cells were found to lack pectin methyl esterase, but it is not clear how this difference could relate to alterations in wall expansibility. Salt-adapted cell walls contain reduced hup and secrete two unique PRPP-related proteins suggesting that high NaCl inhibits the cross-linking of these proteins into the walls, a finding that might relate to their altered expansibility.

I. Objectives of the Original Research Proposal

Prior to the initiation of this work, both the Israeli and the U.S. investigators on this project had initiated studies which indicated that plant cells respond to specific stresses by substantial alterations in the structure of their cell walls. The Israeli investigators had been working with suspension-cultured cells of tomato which were adapted to growth on an herbicide, 2,6-dichlorobenzonitrile (DCB), a specific inhibitor of cellulose synthesis in plants. In previous work supported by BARD, we showed that tomato cells adapted to growth on DCB were able to survive by virtue of remarkable changes in wall structure, including virtual loss of their cellulose-xyloglucan network (Shedletzky et al., 1990). At the same time, the US group at Purdue had shown that a variety of alterations occur in wall structure accompanied by loss of ability to expand normally in cells of tobacco adapted to growth on high concentrations of NaCl (Iraki et al., 1989a,b,c).

We were impressed by the surprising flexibility of plant cells to modify their wall structures in response to imposed stresses and proposed to continue studies on induced wall modifications in order to understand further the potential of plants for tolerating such changes, to understand the types of compensatory changes they make, and to determine to what extent such changes alter the strength of the wall and/or its ability to expand. We also predicted that such studies on the altered state might also lead to additional insights into normal wall structure and mechanisms of growth. [For a general review of cell wall structure as it relates to growth, the reader is referred to a recent review by Carpita and Gibeaut (1993).]

Our specific objectives were: 1) to attempt to determine what limits cell expansion during growth in saline medium with emphasis on studies of xyloglucan metabolism; 2) to understand how cells growing on the cellulose synthesis inhibitor DCB maintain wall integrity in the absence of a cellulose-xyloglucan network, with emphasis on the interconnections between the pectic components of the walls; 3) to understand how the protein networks are altered in salt- and DCB-adapted cells; 4) to assess the interconnections between the three networks in both the normal and adapted states and the relative roles of each network in determining wall tensile strength and porosity; and 5) to gain insights into wall structure in a graminaceous monocot (barley) adapted to growth on DCB.

II. Relevant Data; Results and Discussion

A. Cell Wall Modifications Induced by Adaptation to Growth on DCB

i. General Findings; Comparison Between Adaptation in Dicots and a Graminaceous Monocot

These studies have been published in a paper which was featured on the cover of *Plant Physiology* (see V. Publications: Shedletsky et al., 1992), and the reader is referred to the attached reprint of this work for full details of the findings which are only summarized here briefly. [A portion of the work presented in this paper (adaptation of cell lines, development of some of the techniques, and some of the preliminary analyses) was conducted during the previous BARD grant period.] Our previous work (Shedletsky et al. 1990) had shown that tomato cells adapted to growth on DCB virtually lacked a cellulose-xyloglucan network. We extended these studies by showing that another dicot cell line tobacco, also has a greatly reduced cellulose-xyloglucan network, secretes its xyloglucan to the medium, and possesses elevated levels of pectins. In the dicots, the major polymers remaining in the wall were pectic in nature; cell wall integrity was found to be largely maintained via calcium-bridging between non-esterified pectic residues. (To our great surprise, protoplasts can easily be prepared from A12 tobacco cells just by addition of a calcium chelator and one purified polygalacturonase!). Since the graminaceous monocots are pectin-poor and also have very little xyloglucan, it was of interest to examine the nature of adaptation of these plants to growth on DCB. A cell line of barley was used for this comparison. Results indicated that the monocot had a very different mechanism of adaptation compared to the dicots tobacco and tomato. By contrast with the dicots, the barley cell line, while showing reduced levels of cellulose, did not compensate by increasing the level of pectic components. Instead, we found that the major compensatory change to provide strength in the absence of cellulose was to increase the level of phenolic cross-linking of the non-cellulosic polymers glucuronoarabinoxylan and/or mixed-linked glucan. We also found that all cells lines adapted to growth on DCB had very strong wall-membrane connections since they could not be plasmolyzed. We still do not understand the cause for this, but do know the connections can be severed by treatment with the chelator CDTA. Interestingly, cells adapted to growth on NaCl also show enhanced wall-membrane connections (Zhu et al., 1993).

Several other major conclusions could be drawn from these studies. Tensile strength studies with the dicot cells allowed us to conclude that the cellulose-xyloglucan network of normal dicot primary walls contributes about 70% of the total wall strength. Furthermore, studies on the porosity of the dicot walls strongly supported the suggestion of others that it is the pectin, and not the cellulose-xyloglucan, network which is largely responsible for determining wall porosity. By contrast, studies with the monocot cell line indicate that both wall strength and porosity in these cells is most probably determined by the degree of phenolic cross-linking of the non-cellulosic polymers. Thus, we made the surprising observation that the walls of the adapted cell line of barley that have less cellulose but are more highly cross-linked by phenolics, are less porous and actually stronger than the walls of non-adapted cells.

ii. Microscopic Studies on Structural Changes in Cell Walls

The results of these studies have also been published (see V. Publications: Wells et al., 1994) and therefore will only be summarized briefly here, and the reader is referred to this attached publication for a full report of these findings. This work was carried out in collaboration with scientists from The John Innes Institute, Norwich UK, who are very skilled in studies on the ultrastructure of plant cell walls (see McCann and Roberts, 1994). The major object of study involved a comparison in the ultrastructure of the walls of suspension-cultured cells of normal, non-adapted (NA cells) of tomato with those derived from cells adapted to growth on 12 μ M DCB (A12 cells). These results further confirmed and extended the biochemical studies described above and published by Shedletzky et al. (1990; 1992) as well as further chemical analyses performed to support the ultrastructural studies (Table 1; Wells et al., 1994). Studies using either Fourier Transform Infra-red microscopy or immunogold labeling with the JIM5 antibody which recognizes a non-esterified pectic epitope (Knox et al., 1990), showed that A12 walls contain much more non-esterified pectins than NA walls, and that most of this fraction can be extracted by a relatively mild DMSO treatment.

Perhaps the most interesting result concerned the positive staining of thin sections of cell walls with uranyl/lead. First, in spite of the very large differences in wall composition, the thickness of the walls was very similar. This led us to speculate that cells have some mechanism of regulating wall thickness which is independent of the cellulose-xyloglucan network. Furthermore, there was very little difference in the appearance of the two wall types at different stages of extraction, with both showing large quantities of fibrillar material even though the A12 walls virtually lacked the

cellulose-xyloglucan network. This led us to the conclusion that many people have in the past erroneously assumed that fibrillar material viewed after such staining is cellulose. Since the A12 cells contain very little cellulose, we have concluded that much of the fibrillar material is really pectic in nature. However, platinum shadowed replicas of the walls do reveal differences in that very long electron-opaque fibers (probably cellulose) are seen in NA walls but not in A12.

iii. An Unusual Pectic Fraction in DCB-Adapted Cells of Tobacco

The discovery of this fraction occurred by accident while we were investigating a different phenomenon--the inability to plasmolyze any of our cell lines adapted to growth on DCB (except in the presence of high concentrations of CDTA which chelates Ca^{2+} and solubilizes pectins; see Shedletzky et al., 1992). We were interested to investigate the nature of the very strong connections between wall and membrane in these lines. We began with the hypothesis that the connection might be mediated by some unique form of arabinogalactan protein (AGP) since AGP's have been found to be associated with the plasma membrane and have lectin-like properties (Pennell et al., 1989). We therefore began by assaying for the presence of AGP's as summarized below:

a) AGP's in Tobacco Cell Cultures

Following standard protocols for AGP isolation, non-adapted and DCB-adapted tobacco cells were harvested, washed, and disrupted in an extraction buffer containing 50 mM Tris/HCl buffer, pH 8.0 containing 1% Triton X-100 and 10 mM EDTA. Cell walls and membranes were removed by centrifugation, and the AGPs were precipitated from the supernatant by addition of Yariv antigen (a red compound which contains β -glucosyl residues which interact with AGPs and cause precipitation; see Jermyn and Yeow, 1975) in the presence of 1% NaCl. We repeatedly obtained a very large precipitate from the T-12 cell extract and only a very small one from the T-NA extract. However, when these fractions were run on SDS-PAGE gels and stained with Yariv antigen to detect AGPs, we observed only similar low levels of AGPs from the fractions of either T-NA or T-12 (not shown). We then repeated the precipitation step but this time omitted the Yariv antigen and added only NaCl to 1%; to our surprise, we obtained the same large precipitates. This indicated that the fraction contained something other than AGPs that was precipitated from solution only by NaCl. This fraction has been designated SIF (salt-insoluble fraction), and contains the unusual pectic components described in the next section iii (b).

We further studied the true AGP fraction by the use of monoclonal antibodies directed against

AGPs and donated to us by Prof. Keith Roberts of the John Innes Institute. Three different monoclonals which recognize different AGP epitopes were tested: MAC, JIM 4, and JIM 13 (Knox et al., 1991). We found that only JIM 13 gave a positive response when Triton-soluble proteins of the two cell lines were analyzed by Western blotting. The reaction was similar for both T-NA and T-12 cells (Fig. 1, below), and the AGP level did not vary with culture age (not shown). We also did *in situ* immunolocalization with the two cell lines using the JIM13 antibody and a second antibody labeled with a fluorescent probe (FITC). Once again, we saw a similar reaction with both cell lines (not shown). From all these results, we conclude that there are no major differences in AGP content between the T-NA and T-12 cell lines.

Figure 1. AGPs in Suspension-Cultured Tobacco Cells as Detected by the JIM 13 Antibody. Western blot showing reaction of JIM 13 antibody from Triton-X100 soluble proteins extracted from non-adapted T-NA and T-12 cells. Equal amounts of protein were loaded per lane. Second antibody was anti-mouse alkaline phosphatase.

b) Analysis of SIF--The Unusual Pectic Fraction

To reiterate, this fraction, defined as SIF ("salt-insoluble fraction"), is obtained from T-NA or T-12 cells by breaking the cells in buffer containing 1% Triton X-100 followed by centrifugation to remove cell walls and membranes. (More recent work has used a different extraction buffer of 50 mM BTP-HEPES, pH 7.5, 5 mM EDTA, 5 mM DTT, 330 mM sucrose, and the protease inhibitors leupeptin (0.5 mM) and Pefabloc 20 uM). NaCl is added to the supernatant to a final concentration of 1%, followed by incubation for 1 hr at 4C, and centrifugation at 10,000 rpm. The resulting pellet, SIF, is gelatinous in nature and much more abundant in extracts of T-12. It

can be further purified either by re-dissolving in buffer and re-precipitation in 1% NaCl or by prolonged dialysis against water followed by lyophilization. Dried SIF can only be solubilized in buffers which contain chelating agents, and the ideal buffer for solubility and column chromatography has been found to be 50 mM imidazole, pH 7.0, containing 10 mM EDTA. Table 1 below summarizes some of the general properties of SIF. The analyses described below have been done in collaboration with Prof. Alan Darvill and his colleagues at the Complex Carbohydrate Research Center (CCRC) at the University of Georgia whose collaboration we sought since they are experts on determination of pectin structures in dicot primary walls (O'Neill et al., 1990).

Table 1. General Properties of SIF

Source	mg SIF/g dry wt cells	Composition (uronic acid: neutral sugar: protein)	% Total Cell Uronic Acids Found in SIF
T-NA	11.9	1.0: 0.59: 0.07 ^a	29
T-12	74.2	1.0: 0.19: 0.05 ^b	28

^aAverage of two experiments

^bAverage of 5 experiments

The quantity of SIF obtained from T-12 cells is usually at least 6 times greater than that obtained from T-NA cells. Compositional analyses indicate that SIF is very rich in uronic acids, but also contains neutral sugars and some protein. The composition of SIF from T-12 cells appears somewhat different from that of T-NA cells in that it contains relatively more uronic acids (perhaps indicative of more homogacturonan), although the sugar composition is fairly similar (see Table 2 below). Note that this fraction, both in T-NA and T-12 cells contains almost 30% of the total uronic acid of the cell (including walls); it seems to be a soluble pectic fraction which has been ignored in the past. The T-12 cells are presumably a rich source of this fraction since they synthesize in general elevated levels of pectins.

Table 2. Neutral Sugar Composition of SIF

	mol% ^a		mol% ^b	
	T-NA	T-12	T-NA	T-12
RHA	10	9	25	9
FUC	<1	<1	4	3
ARA	26	20	27	18
XYL	<1	2	<1	<1
MAN	<1	<1	<1	7
GAL	21	20	15	22
GLC	15	14	10	31
meFUC	8	6	11	4
meXYL	<1	<1	3	1
APIOSE	ND ^c	ND	5	5
ACERIC ACID	ND	ND	0	0
KDO	ND	ND	+ ^d	+ ^d
Unidentified	18	30		

^aDetermined at Hebrew University^bDetermined at CCRC. T-12 was not dialyzed and probably contained excess free sucrose which could account for the higher level of glc and man observed.^cND: not determined^d+ : detected by color test but not quantified as mol %

The high uronic acid content, as well as the preliminary neutral sugar composition shown above in Table 2, indicate that SIF is largely composed of pectic polymers such as homogalacturonan and Rhamnogalacturonan I (RG I). The presence of the more rare sugars apiose, meFUC, meXYL, and KDO suggest that the fraction may also contain Rhamnogalacturonan II (RG II) (O'Neill et al., 1990).

Our collaborators at the CCRC have chromatographed SIF on a P-30 gel filtration column; under these conditions SIF can be resolved into a peak A which voids the column and a second peak B which elutes in a position consistent with the smaller RG II polymer. Sugar analyses of this latter fraction indicated large amounts of galacturonic acid with smaller amounts of GLC, ARA, RHA, and XYL. However, the amount analyzed was insufficient to allow detection of other sugars characteristic of RG II. Larger amounts are now being further analyzed to verify our

suspicion that this fraction probably contains at least some RGII. Further analysis of Peak A on a Bio-Gel Agarose 5 M column shows it can be resolved into two further peaks with mol wts of about 5,000,000 and 500,000. No KDO was detected in either peak, and glycosyl residue composition analyses gave similar results for both peaks. Both contained about 75% galacturonic acid with lesser amounts of GAL, ARA, and RHA. This is consistent with both fractions containing homogalacturonan and RG I.

Amino acid analysis of SIF from T-12 cells shown in Table 3 below. There is no indication of high contents of hyp or pro, once again indicating the fraction is not rich in AGPs, extensins or proline-rich proteins.

Table 3. Amino acid composition of SIF from T-12 Cells

	mol %
ASP	10.5
HYP	0.9
SER	7.8
GLU	12.0
THR	5.5
GLY	15.5
ARG	3.1
ALA	10.0
TYR	0.5
PRO	3.8
VAL	7.5
PHE	2.1
ILEU	4.5
LEU	6.5
HIS	2.1
LYS	7.9

In sum, we have discovered a novel fraction which is not bound to cell walls but nevertheless contains pectic polymers such as RG I, RGII, and homogalacturonan. SIF can only be detected in the "soluble" fraction of cells and is not detectable in the growth medium as a secreted polymer. We suspect that it represents either: a) cytoplasmic polymers en route to secretion and integration into the cell wall; or b) polymers which are already secreted to the periplasmic space but not further covalently linked to the wall. Since T-12 cell walls are highly enriched for pectins, it is perhaps not surprising that there is a large excess of these polymers as well in these cells. It is tempting to speculate that these polymers might be involved in the very tight wall-membrane connections in A-12 cells which can only be severed by treatment of whole cells with chelators

such as CDTA. However, we have not found a way yet to test this hypothesis in any firm way. Finally, SIF may have value for analyses of general pectin structure in plants. To date, RG I and II have only been solubilized and analyzed for structure after release from walls by hydrolase digestions. SIF contains these same polymers yet can be solubilized and analyzed without enzymatic treatments which certainly alter polymer compositions. Thus it may provide a clue as to how pectins are synthesized--first as individual polymers of homogalacturan and RG I and II as represented in SIF and only later fully integrated into the insoluble matrix. Pulse-chase experiments might be possible in the future to test the hypothesis that polymers in SIF are truly precursors to the insoluble pectic components of the wall or whether it is a distinct fraction which is never highly integrated.

B. Cell Wall Modifications in Tobacco Cells Adapted to Growth on NaCl

i. Alterations in Levels of Cell Wall Hydrolytic Enzymes

One of the major questions we proposed to address concerned the curious inability of cells adapted to growth in NaCl to expand normally. We initially proposed that alterations in xyloglucan metabolism might be responsible for this phenomenon. Therefore, Dr. Shedletzky from the Israeli group spent the second year of this project in Prof. Carpita's lab at Purdue with the goal of investigating this possibility. She began by studies designed to measure possible differences in levels of xyloglucan endo-transglycolyase (XET) between the non-adapted (S0) and salt-adapted (S25) cells. Recent studies with XET (de Silva et al., 1994) indicate it may play an important role in cleavage and re-formation of xyloglucan chains which may play a role in cell expansion. Dr. Shedletzky prepared, purified, and labeled xyloglucan oligosaccharides for use as XET substrates and searched for XET activity in the two cell lines. Unfortunately, no reproducible activity was detected in either cell line and this approach was not pursued further.

Other proteins which have received much attention lately are the expansins--polypeptides of about 30 kD found in the cell walls of plants which have been shown to promote cell expansion without accompanying hydrolysis of polymers (McQueen-Mason and Cosgrove, 1994; Taiz, 1994). We obtained from Prof. Dan Cosgrove a polyclonal antibody prepared against purified expansins and used this to test for the presence of this protein in walls or the growth medium derived from the two cell lines. Initial results, involving Western blotting of salt-extractable walls proteins, were very promising. Dr. Shedletzky detected a very strong reaction with a protein of

about 32 kD which was extractable from walls of S0 but not S25 walls. However, after many further studies, we have concluded that this reaction is not specific for expansins. There is indeed a difference in the level of this protein between the two cell lines, but this protein has the peculiar property of interacting with almost any rabbit polyclonal antibody, and the reaction is amplified after reaction with anti-rabbit second antibody. We do not know the nature of this protein and have not pursued this further.

Dr. Shedletzky then examined the levels of a variety of other hydrolytic enzymes which could be extracted from cell walls and might play a role in regulation of cell wall turnover and/or expansion. These results are summarized in Table 4 below

Table 4. Cell Wall Hydrolytic Enzyme Activities in Tobacco Cells

Units for enzyme activities are: cellulase: change in viscosity of CMC/min/mg protein; PME: nmol methanol released/min/mg protein; for all other enzymes: nmol p-nitrophenol released/min/mg protein. CW: proteins released from intact cells (cell walls) by 200 mM CaCl₂; Med: proteins secreted to the medium. Assays were performed with proteins harvested from cells at early stationary phase (14 days for S0 cells and 21 days for S25 cells).

Source	Cellulase	β -Xyl-ase (pyr)	β -Glc-ase (pyr)	β -Gal-ase (pyr)	α -Ara-ase (pyr)	α -Ara-ase (fur)	α - or β -fuc-ase	PME
S0-CW	1	22	25	5	1	53	<1	2,470
S25-CW	25	306	1	10	17	545	<1	200
S0-Med	none	3	2	<1	1	9	<1	none
S25-Med	none	417	1	13	22	725	<1	none

Taken together the data show that there are surprisingly large differences in the levels of a variety of cell wall hydrolytic enzymes found in walls or secreted by S0 cells compared to S25 cells. The cellulase activity, measured as ability to alter the viscosity of carboxymethylcellulase (CMC), was found to be higher in the walls of S25 cells. This result does not seem consistent with a model in which these cells expand poorly due to inability to degrade xyloglucan. Furthermore, a tobacco cell line adapted to growth on high NaCl in Israel and which also did not show good cell expansion, did not show such a high elevation of cellulase levels (not shown). Also the exo-hydrolase activities which could degrade the terminal xylose, galactose, or arabinose residues of xyloglucan were also higher, not lower, in S25 compared to S0 walls or medium. The only exo-hydrolase activity which we found not be elevated, but reduced, upon adaptation to growth on NaCl was that of β -glucosidase. Tobacco xyloglucan is unusual and does not have t-fuc residues (see Hayashi, 1989), but rather has t-ara residues substituting the gal residues, and

no fucosidase activities were detected either in the salt-extractable wall protein fraction nor among the proteins secreted by either cell line. In sum, the S25 cell line seems to have, on the whole, a relatively greater potential for xyloglucan degradation than that of S0 cells, and the data do not support a model in which reduced xyloglucan metabolism is a cause for reduced cell expansion. The higher level of the arabinosidase and galactosidase activities might, however, be related to the previous observation of Iraki et al. (1989b) that the pectins of S25 cells have a larger number, but shorter, branches than those found on pectins of S0 cells.

One of the most dramatic changes we observed between the cell lines was in the level of a salt-extractable, wall-localized pectin methyl esterase (PME) which was consistently much higher in the S0 line than that of S25 cells. This activity was examined further. The activity was measured by 3 different techniques: a) release of methanol from a commercial citrus pectin substrate; b) titration of the pH change induced by cleavage of the uronic methyl ester from citrus pectin; 3) an in situ assay using native PAGE. All 3 assays gave similar results--i.e., that the S25 line was almost devoid of PME activity while the S0 line contained high activity, and the differences are observed when the results are expressed either on the basis of total protein, fresh, or dry weight of cells (see Fig. 2A below). This activity was studied as a function of culture age in the S0 cell line; the results (Fig. 2B below) show that the cells contain very low activity in the early stages of subculture and the level rises to very high values as the cells enter stationary phase.

Figure 2. PME Activity in the Salt-extractable Protein Fraction from Suspension-cultured Tobacco Cells. A. A. Comparison of PME activity between S0 and S25 cell lines. Activities were measured on the protein fraction extractable from intact cells by 200 mM CaCl_2 . Activity is expressed in three different ways: a) as a function of protein content; b) per gr fresh wt (fw); or, c) per gr dry weight (dw). B. PME activity in CaCl_2 extractable proteins of S0 cells as a function of culture age.

These results can be interpreted in light of other studies by Carpita's group (McCann et al., 1994) on pectin structure in salt-adapted tobacco cells. The most relevant finding in that study was that, as measured either by FTIR microscopy or immunogold labeling with the JIM 5 antibody which recognizes unesterified pectic epitopes, there was virtually no change in the level of esterified uronic acids in the pectin fraction of S25 walls, whereas there were age-dependent changes in uronic acid esters in S0 cells. In S0 the level rose during elongation then fell again at stationary phase. These results would thus correlate well with the finding of very little PME in S25 cells and a large increase in PME in S0 cells as they enter stationary phase. However, at present, it is still unclear how these differences in level of PME during growth of S0 or between S0 and S25 cells relate to differences in ability of the walls to expand. A higher level of PME would presumably lead to more rigid walls as a result of increased Ca-bridged pectates. Therefore, the rise in PME at the end of growth of S0 cells may well be correlated with cessation of growth. However, it is difficult to make a case that lack of substantial PME activity in the S25 cells would be directly related to the inability of these walls to expand normally. Thus, having found no evidence that would support a role for alterations in metabolism of either pectins or xyloglucan in altering wall expansibility during growth on high salt, we turned our attention to the protein components of these walls as described below.

ii. PRPP-Related Proteins in Salt-adapted Tobacco Cells

In initial studies on tobacco cells adapted to growth on 428 mM NaCl, Iraki et al. (1989a) reported that the cell walls of adapted cells contained a lower content of hydroxyproline (6.9% mol% hyp in non-adapted vs. 1.8% hyp in adapted cells; proline content was not changed). Furthermore, they noted that adapted cells secrete an abundant 29 kD polypeptide to the medium (Iraki et al., 1989c), although there was no indication that this polypeptide was rich in hyp. In the past two years, we have further characterized this 29 kD secreted polypeptide (referred to hereafter as 29 MS) and found that it strongly cross-reacts with a polyclonal antibody prepared by one of us (A.M.) against proline-rich repetitive proteins (PRPPs) from soybean (Marcus et al., 1991). This abundant polypeptide was blotted to Immobilon membranes, hydrolyzed, and examined for amino acid composition. Results (see Table 5 below) show that the protein does indeed contain reasonably high levels of hyp, although the composition does not in other respects closely resemble other PRPPs (e.g., not especially rich in pro, val, tyr, or lys), even though it is similar in mol wt. Because it does contain hyp and cross-reacts with the anti-PRPP antibody,

we refer to it as a PRPP-related protein, although we recognize that it requires further characterization and may differ in significant ways from other PRPPs.

Table 5. Amino acid composition of 29 kD polypeptide secreted by salt-adapted tobacco cells

29 MS: 29 kD polypeptide secreted to medium of salt-adapted, but not non-adapted cells.

	mol%
	29 MS
ASP	13.7
HYP	8.6
SER	4.7
GLU	11.5
CYS	0.0
THR	5.9
GLY	15.8
ARG	4.3
ALA	9.6
TYR	3.3
PRO	5.2
MET	0.9
VAL	4.0
PHE	4.1
HIS	5.3
LYS	3.9

A second hyp-rich protein which also cross-reacts with this same soybean anti-PRPP antibody was discovered as a 24 kD polypeptide which can be eluted from cell walls of salt-adapted cells; it can also be eluted, but at much lower levels, from the walls of non-adapted cells. The amino acid composition of this polypeptide is currently under investigation.

Figure 3 below shows Coomassie Blue stained profiles of SDS-PAGE gels as well as results of Western blotting of proteins from various fractions of non-adapted and salt-adapted cells. Coomassie Blue profiles of proteins secreted to the medium (lanes 1 and 2) show the abundant 29 MS polypeptide secreted to the medium by S25, but not S0, cells. Lanes 3 and 4 show the corresponding Western blots of these fractions probed with the soybean anti-PRPP antibody and show the very strong reaction of 29 MS. Lanes 5 and 6 compare the Coomassie Blue Profile of proteins extracted from the walls of S0 and S25 by LiCl. The polypeptide at 24 kD (24 CWS)

is more abundant in the S25 extract, and this protein also cross-reacts with the anti-PRPP antibody (lane 7 for S0 and lane 8 for S25).

Figure 3. SDS-PAGE and Western Blotting of Proteins from Cultured Tobacco Cells. Lanes 1-2 and 5-6 show Coomassie Blue staining. Lanes 3-4 and 7-8 are results of Western blotting of proteins probed with the soybean anti-PRPP antibody; reaction was detected by the ECL method. Lanes 1-4 contain proteins secreted by S0 (lanes 1,3) and S25 (lanes 2,4) cells, respectively. Lanes 5-8 contain proteins extracted by LiCl from walls of S0 (lanes 5,7) and S25 (lanes 6,8) cells.

Further studies (not shown) indicate that the level of 29 MS secreted by S25 cells does not vary significantly as a function of culture age. We know less in general about 24CWS as its level in both S0 and S25 cells is more variable. However, we do know that these species do not appear after just short-term incubation of S0 cells in salt; it appears that their appearance is a function of long-term adaptation of the cells to salt. These results suggest interesting alterations in the levels and/or localization of several hyp-and/or pro-rich cell wall proteins which should definitely be pursued in future studies. These results could be correlated well with the previous results of Iraki et al. (1989a,c) which show reduced levels of bound hyp in the cell walls of salt-adapted cells. Since we find both 24 CWS and 29 MS appearing in abundance as non-cross-linked species (either salt-extractable from walls or secreted), this might suggest that high salt inhibits the cross-linking of these species into the walls. If so, one would predict that these proteins should be

present as cross-linked species in non-adapted cells. Alternatively, high salt might induce expression of these genes which are not expressed under normal growth conditions. We are in the process of trying to distinguish between these alternatives. We have prepared RNA from both cell types and hope soon to perform Northern blots using soybean PRPP cDNA clones as probes. The genes for 24 CWS and 29 MS may or may not be similar enough to allow detection of the correct mRNA's, but we believe this is worth trying. Second, we are now trying immunolocalization studies with the anti-PRPP antibody and isolated tobacco cell walls. One might predict a higher reaction of the Ab with non-adapted walls if high salt is really inhibiting the cross-linking of these proteins and, in fact, this is what we have recently observed.

A longer-term question is whether the difference in levels of these proteins can be related to the inability of salt-adapted cells to expand normally during growth. Eventually this might be answered by: 1. Use of other means to inhibit protein cross-linking in non-adapted cells and observe effects on cell expansion; 2. Isolation of genes which code for these proteins and use of anti-sense technology to see if inhibition of gene expression has effects on cell expansion. We do note that one of the soybean PRPPs is expressed at high levels in expanding tissues (Kleis-San Francisco and Tierney, 1990; Marcus et al., 1991); thus, there is some reason to speculate that these proteins may play a role in the process.

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III. Description of Cooperation

We have tried to indicate above for each part of the overall project how the collaboration was carried out. In general, the work on cell wall structure in the DCB-adapted cells was carried out in Delmer's lab in Israel. However, they drew on the techniques and advice from Carpita's laboratory for the studies on tensile strength and plasmolysis. In addition, once the unusual pectic fraction (SIF) was identified in these cells, we sought the collaboration of Prof. Alan Darvill (CCRC) for more detailed analyses, as his group is an expert in analyses of RGI and II-type of polymers. The collaboration on the ultrastructural studies of these walls between Delmer's lab and the group at The John Innes Institute was carried out in parallel with another collaboration between this group and Carpita's on similar ultrastructural studies with the salt-adapted tobacco cells (see McCann et al., 1994). In general, all groups were involved in discussions and interpretations of these data.

Dr. Shedletsky from the Israeli group initiated the studies on cell wall lytic enzymes while a visiting scientist at Purdue in the second year of this project, and continued these studies when she returned to The Hebrew University with the aid of the skilled technician Miri Shmuel. Dr. Marcus and Prof. Delmer have directed the studies on cell wall proteins, and work was carried out both at The Institute for Cancer Research and while Prof. Marcus was Visiting Professor with The Israeli group with the help of a student Nimrod Blau. The Israeli group has kept in constant

communication with the US via e-mail, and Delmer, Carpita, and Marcus have also had the opportunity for further communication during their participation in several international meetings.

IV. Research Achievements Relative to Original Goals

We have achieved many, but certainly not all, of our original goals. Of the original 5 specific objectives, we have met most of our goals for objectives 2-5 (see page 3). Thus, for the DCB-adapted cells, we have provided strong evidence that the major load-bearing network in the walls of adapted dicot cells is comprised of Ca^{2+} -bridged pectates (objective 2). As we suspected, a graminaceous monocot has a very different strategy for compensatory changes upon reduction in cellulose level in the wall. Unlike the dicot cells, these cells do not elevate their pectic network, but rather achieve extra strength by enhanced cross-linking of their non-cellulosic polysaccharide network via phenolic residues (objective 5). As predicted, our results have also shed new light on normal wall structure including the function of, and relationship among, the different wall networks (objective 4). The major findings in this regard were: 1) the cellulose-xyloglucan network in normal dicot primary walls accounts for about 70% of the total wall strength; 2) the pectic network in dicot primary walls is the primary determinant of wall porosity; 3) both wall strength and porosity in graminaceous monocot primary walls is greatly influenced by the degree of phenolic cross-linking between non-cellulosic polysaccharides; 4) the finding that the bulk of the xyloglucan is secreted in dicot cells which synthesize very little cellulose, strongly supports the notion that cellulose and xyloglucan are an interacting network; furthermore, it suggests that xyloglucan may not be further linked to other components of the wall; and 5) the fact that the monocot cells do not secrete excess hemicellulose (glucuronoarabinoxylan and mixed-linked glucan) in response to growth on DCB, suggests that these two non-cellulosic polymers do not normally interact with cellulose in a manner similar to xyloglucan.

The most difficult, and perhaps least successful, of our studies concerned objective 1: to attempt to understand the factors which limit cell expansion during growth of cells in saline medium. None of our results concerning hydrolytic enzymes supports the hypothesis that xyloglucan metabolism is repressed during growth on NaCl; levels of most lytic enzymes examined were, if anything, higher in salt-adapted cells. (However, since we were not successful in detecting either expansins or XET activity, we still cannot rule out alterations in level and/or activity of these proteins as playing a role.) We observed a striking difference in level of pectin

methyl esterase (PME) which was much higher in S0 than S25 cells, but, at this point, it is difficult to envision how this difference could relate to alterations in wall expansibility. Perhaps the most important clue could come from our studies on the protein network of these cells (objective 3). We have clearly seen differences in the level of two PRPP-related proteins which occur in response to growth on saline medium. Thus, we have observed appearance of an abundant 29 kD PRPP-like protein which is secreted to the medium and another of 24 kD which is extractable from walls by high salt. Our best interpretation at present is that high NaCl inhibits the cross-linking of these proteins into the walls. Since it has also been observed that the total hyp level in the S25 cells is greatly reduced, it would appear that S25 walls contain reduced levels of cross-linked proteins such as PRPPs (and perhaps also extensins). On the surface, one would expect this to favor cell expansion as cross-linking of extensins, at least, is believed to decrease wall expansibility. However, the expression of the gene for at least one member of the PRPP family is associated with elongating cells (Kleis-San Francisco and Tierney, 1990; Marcus et al., 1991). Thus, this project has opened up a new area for future investigation: the effect of NaCl on cross-linking and or expression of genes for wall proteins and the potential relationship to inhibition of cell expansion.

V. Publications Resulting from the Project

Shedletzky, E., Shmuel, M., Trainin, T., Kalman, S., and Delmer, D.P. (1992) Cell wall structure in cells adapted to growth on the cellulose-synthesis inhibitor 2,6-dichlorobenzonitrile. A comparison between two dicotyledonous plants and a graminaceous monocot. *Plant Physiol.* 100: 120-130. (Featured on cover of journal)

Wells, B., McCann, M.C., Shedletzky, E., Delmer, D., and Roberts, K. (1994) Structural features of cell walls from tomato cells adapted to grow on the herbicide 2,6-dichlorobenzonitrile. *J. Microscopy* 173: 155-164.

Pending the completion of a few more experiments, the following will be prepared and submitted for publication:

Darvill, A., Shmuel, M., Shedletzky, E., and Delmer, D.P. Characterization of a novel soluble pectic fraction from suspension-cultured tobacco cells adapted to growth on the cellulose-synthesis inhibitor 2,6-dichlorobenzonitrile.

Shedletzky, E., Shmuel, M., Carpita, N.C., and Delmer, D.P. Alterations in level of cell wall lytic enzymes in response to adaptation of cultured tobacco cells to growth on NaCl.

Marcus, A., Shmuel, M., Blau, N., Shedletzky, E., Carpita, N.C., and Delmer, D.P. Alterations in level and/or localization of cell wall structural proteins in response to adaptation of cultured tobacco cells to growth on NaCl.