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BARD

FINAL REPORT

PROJECT NO. IS-1386-87

**Induces Plant Cell Wall Modifications: Analysis
of Herbicide-Resistant Tomato Cells Possessing
Altered Cell Wall Composition**

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1992

Appendix VI

Standard BARD Cover Page for Scientific Reports

Date: September 17, 1990

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BARD project No. IS-1386-87

Title

Induced Plant Cell Modification+ Analysis of herbicide-resistant
tomato cells possessing altered cell wall composition

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Project's starting date: Oct. 1, 1988

Type of Report: 1st Annual 2nd Annual Final X

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ABSTRACT

In the first part of our work, we analyzed the nature of the adaptation of suspension-cultured tomato cells to grow on an herbicide which specifically inhibits cellulose synthesis (2,6-dichloro-benzonitrile, referred to hereafter as DCB). The mechanism of adaptation appears to rest largely on the ability of these cells to divide and expand in the virtual absence of a cellulose-xyloglucan network. Although cellulose synthesis is severely inhibited, the synthesis of xyloglucan is actually enhanced, but the majority of this polymer is secreted to the medium. These results support very nicely current wall models for the primary walls of dicots which propose that xyloglucan interacts via hydrogen bonding with cellulose to form a major load-bearing wall network. Walls of adapted tomato cells grown on DCB are very rich in uronic acids, but the nature of the remaining load-bearing networks was not determined.

In later work, we compared the adaptation mechanisms of two dicot cell lines (tomato and tobacco) with that of a Gramineous monocot (barley endosperm), since the latter type of cells are known to have very different wall structure. Indeed, we found that the compensatory changes in wall structure in response to growth on DCB are very different between the dicots and monocot. All types have reduced levels of cellulose in the walls. In the dicots, the major remaining load-bearing network is composed of Ca^{2+} -bridged pectates. Surprisingly, protoplasts can be prepared from these cells simply by treatment with the chelator CDTA and a purified endopolygalacturonase. These protoplasts can regenerate a new wall in the presence of DCB, a characteristic not observed previously with non-adapted cells. Tensile strength measurements indicate that these walls are much weaker than their non-adapted counterparts. In contrast, the monocot barley walls derived from cells growing on DCB are poor in pectic material, and are at least as strong as those from the non-adapted parent line in spite of the reduced levels of cellulose. This appears to be due to enhanced phenolic cross-linking of the hemicellulosic polymers of the walls.

In sum, these results point out the remarkable flexibility of plants to survive induced modifications in wall structure. The results also point out how studying such modifications can shed light on normal wall structure in plants, and also indicate that genetic engineering to alter wall structure may be feasible for agriculturally important plants.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

1. To determine the mechanism of adaptation of cells to growth on DCB (note, when proposal was written, we already had the tomato line adapted to growth on DCB and knew that the walls had reduced levels of cellulose).
2. To study how wall structure is modified in response to DCB; to determine what compensatory changes are made in wall structure to compensate for reduced levels of cellulose.
3. To ascertain what are the major load-bearing networks which exist in the walls of DCB-adapted cells. We predicted that there would be elevated levels of hydroxyproline-rich glycoproteins in the walls and proposed to study how these might contribute to wall strength.
4. To compare different cell types for ability to adapt to growth on DCB.
5. To assess the tensile strength and porosity of such walls.
6. To attempt to understand what, if any, are the stable genetic changes in such adapted cell lines.

WRITTEN REPORT

A comprehensive description of the results of our research can be found in the attached Appendices I and II. Appendix I is a paper already published in Plant Physiology. Appendix II is a draft of a paper which is to be submitted to Plant Physiology in about 2 months. We still have some analyses to complete before final submission; these are indicated in the current draft of the MS.

DESCRIPTION OF COOPERATION

All adaptation, growth, and preparation of cell walls was carried out by the Israeli group. This group also was responsible for all work on the carbohydrate and phenolic components of the walls. The US group was responsible for the work on the protein components of the walls. Prof. Delmer, the Israeli PI, spent every summer at the MSU-DOE Plant Research Laboratory at Michigan State University, where Prof. Lamport has his laboratory. During these periods, exchange of samples and data, discussion of results and plans for future work were made.

EVALUATION OF THE RESEARCH ACHIEVEMENTS WITH RESPECT TO THE AIMS OF THE ORIGINAL RESEARCH PROPOSAL; BENEFIT TO AGRICULTURE

We consider that most of our original goals for this proposal have been met. We have confirmed that the major mechanism of adaptation rests largely on the ability of adapted cells to divide and expand under conditions where there is a substantial reduction in the level of cellulose in the wall. We have also shown that such cells maintain their cellulose-synthesizing machinery even in the presence of DCB; upon removal of DCB, these cells immediately start synthesizing cellulose; we have also confirmed that this pathway does not have an altered sensitivity to DCB. We have established to a great degree the nature of the remaining load-bearing networks in walls of DCB adapted cells. In the dicots, this is largely a Ca^{2+} -bridged pectin network, and the walls are much weaker than normal. These findings, as well as those concerning the secretion of xyloglucan, have generated much interest in the scientific community, as they are quite unique and, at the same time, confirm many aspects of current wall models. (See, for example, the enclosed quotation about our work taken from a recent review by Keith Roberts; Appendix III). The newer findings on barley are also of interest in that they show a very different strategy for wall modification which is so successful that the walls of adapted cells with reduced cellulose are at least as strong, or even stronger, than normal walls!

We have not come to any final conclusions concerning the status of the protein network in these cells. In contrast to our original expectations, we actually found reduced levels of hydroxyproline-rich glycoproteins in the adapted dicot walls, and no significant increase in total protein. In more recent work with the dicots, we have found that as they are grown for very long times on DCB, the total protein does rise. However, our finding that simple cleavage of the pectic network with chelators and endopolygalacturonase can lyse cells indicates to us that the protein network does not play a major role in providing extra strength in the absence of cellulose and xyloglucan. Similarly, in the monocot, the levels of hyp-rich proteins is quite low, and does not seem to be of great importance as a contributor to overall wall strength.

We had hoped to be able to regenerate plants from one or more of our adapted lines to attempt to assess the heritability of DCB-resistance. However, we have not been able to do so with any of the lines, including tobacco which is usually rather easy to regenerate. However, we have ascertained that resistance is very stable in culture. Thus, adapted cells can be grown for many generations in the absence of DCB, and, when returned to medium containing DCB, can grow at concentrations which would kill non-adapted cells. Similarly, protoplasts derived from adapted cells can regenerate a wall in the presence of DCB, a trait non-adapted cells do not possess. Thus, some memory mechanism does exist which does not depend upon the presence of the cell wall which is involved in the resistance; however, we have not been able to assess whether this is genetic or epigenetic in nature.

possess extremely strong connections between the wall and plasma membrane and cannot be plasmolyzed. This is reminiscent of wall-extracellular matrix connections in animal cells mediated by transmembrane proteins called integrins. There is now evidence that similar proteins exist in plants and could be involved in signal transduction to the cytoskeleton. Therefore, this is one area we wish to continue to investigate with these cells.

Our current results have no immediate benefits to agriculture. However, a number of long-term benefits might be envisioned. Since the results indicate that plant cells, and particularly the Gramineous monocots, can adapt to growth with much reduced levels of cellulose and still maintain walls with great integrity and strength. This suggests to us that it is indeed feasible to think about the use of genetic engineering to modify wall structure in useful ways. For example, reduced levels of cellulose may be beneficial to the tenderness, digestibility, and nutritional quality of a variety of plant parts (e.g. fruits, vegetables, forage crops). Coupled with recent advances in cloning genes involved in cellulose synthesis, the time is approaching when it should indeed be feasible to attempt alterations in the timing and/or extent of cellulose deposition in specific plant tissues. Our work with the DCB-adapted cells provides incentive that such attempts may succeed in ways not believed to be possible prior to this work.

LIST OF PUBLICATIONS

Shedletzky E, Shmuel M, Delmer DP, Lamport DTA (1990) Adaptation and growth of tomato cells on the herbicide 2,6-dichlorobenzonitrile leads to production of unique cell walls virtually lacking a cellulose-xyloglucan network. Plant Physiol 94: 980-987

Shedletzky E, Shmuel M, Trainin T, Delmer DP . Cell wall structure in cells adapted to growth on the cellulose-synthesis inhibitor 2,6-dichlorobenzonitrile (DCB): A comparison between two dicots and a monocot grass. Draft of manuscript is enclosed as part of description of results. We are completing a few details for this MS, and expect to submit it to Plant Physiology in February, 1992

Posters and Lectures Presented at International Meetings

Poster:

Esther Gedalovich-Shedletzky, Miri Shmuel, Deborah P. Delmer, and Derek T.A. Lamport. Properties of DCB-adapted cell suspension cultures of tomato. Presented at the Fifth Cell Wall Meeting, Edinburgh, UK, September 1989

Poster:

E. Shedletzky, M. Shmuel, D. Delmer, and D. Lamport. DCB-adapted plant cells possess unique wall structure. Presented at the Annual Meeting of the American Society of Plant Physiologists, Indianapolis, Indiana, July, 1990

Invited lectures on this work were given by Prof. Delmer at the following international meetings:

25th Anniversary Symposium, MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing MI USA, May, 1990

American Society of Cell Biology Symposium on the Biology of the Extracellular Matrix of Plants and Animals, Airlie, VA, USA, June 1990

UCLA Keystone Symposium on The Genetic Dissection of Plant Cell Processes, Keystone, CO, USA, Jan. 1991

Annual Meeting of the German Society of Biochemistry, Bayreuth, Germany, September, 1991

Cell Wall Structure in Cells Adapted to Growth on the Cellulose-Synthesis Inhibitor 2,6-Dichlorobenzonitrile (DCB): A Comparison Between Two Dicotyledonous Plants and a Graminaceous Monocot¹

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Footnotes:

¹This work was supported by Grant No. IS-1386-87 from BARD, The United States-Israel Binational Agricultural Research & Development Fund, and by Contract No. DE-AC02-76ERO-1338 from the U.S. Department of Energy.

²Abbreviations: DCB: 2,6-dichlorobenzonitrile; GAX: glucuronoarabinoxylan; HRGP: hydroxyproline-rich glycoprotein; CDTA: trans-1, 2-diamino-cyclo-hexane N',N',N',N'-tetaacetic acid.

ABSTRACT

Our previous work (E. Shedletzky, M. Shmuel, D.P. Delmer, D.T.A. Lamport [1990] *Plant Physiol* 94:980-987) showed that suspension-cultured tomato cells adapted to growth on the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile (DCB) have a markedly altered cell wall composition, most notably having a markedly reduced level of the cellulose-xyloglucan network. This study compares the mechanism of adaptation to DCB of two cell lines from dicots (tomato and tobacco) and a Gramineaceous monocot (barley endosperm), the last having a markedly different wall composition from that of the dicots. This difference is also reflected in the very different types of wall modifications induced by growth on DCB. The dicots, having reduced levels of cellulose and xyloglucan, possess walls, the major integrity of which is provided by Ca^{2+} -bridged pectates. The tensile strength of these walls is considerably less than walls from non-adapted cells. Protoplasts can be prepared from these walls simply by treatment with divalent cation chelator and a purified endopolygalacturonase; these protoplasts can then generate a new wall in the presence of DCB. In contrast, the barley walls contain very little pectic material, normal levels of glucuroarabinolyan, and elevated levels of mixed-linked glucan. Surprisingly, they have tensile strengths as high or higher than their non-adapted counterpart, even when cellulose levels are reduced by 70%. Evidence is presented that these walls obtain their additional strength by an altered pattern of cross-linking of polymers involving phenolic components. Cells of all adapted lines are resistant to plasmolysis suggesting that they possess very strong connections between the wall and the plasma membrane.

INTRODUCTION

Our previous work (28) has shown that suspension-cultured tomato cells can be adapted to growth on the cellulose synthesis inhibitor DCB². The mechanism of adaptation is somewhat surprising, in that the ability to survive on DCB can be explained by the fact that adapted cells develop a capacity to divide and expand under conditions where cellulose synthesis is inhibited. As a result, the cell walls of tomato cells adapted to growth on 12-25 μ M DCB contain markedly reduced levels of cellulose. In addition, they show other unique characteristics including a reduced level of xyloglucan and a significant enrichment in pectic polymers. The finding of a reduced level of xyloglucan in the walls, coupled with excess secretion of this polymer to the medium, lends further support to current wall models for primary walls of dicots, which propose that one major load-bearing network is comprised of a cellulose-xyloglucan network stabilized by strong interactions between these two polymers (14). Lacking cellulose, adapted cells secrete the xyloglucan to the medium, a result which also suggests the interesting possibility that much of the xyloglucan is not further linked to other polymers within the wall.

These studies, as well as other recent studies with cells adapted to growth in NaCl (16-19), point out the remarkable flexibility of plant cells to tolerate induced changes in cell wall composition, and further show that the study of such induced changes can shed light on normal wall structure. The work presented here extends our analyses of the walls of DCB-adapted cells and addresses the following issues: 1) A demonstration that adapted tomato cells, growing on DCB possess a normal capacity for

cellulose synthesis immediately upon removal of DCB, and that the pathway has retained its sensitivity to DCB; 2) A comparison of altered wall composition between two dicot cell lines (tomato and tobacco) and a monocot grass (barley endosperm). These analyses were undertaken because the grasses are known to have markedly different primary walls from those of the dicots, possessing reduced levels of galacturonic acid-rich polymers, xyloglucan and HRGP's, elevated glucurono-arabinolyxan (GAX), and a mixed-linked glucan which is lacking in dicot walls (2). Thus, it seemed possible that the compensatory changes induced to provide wall strength to walls with reduced cellulose might be quite different between these cell types, and that an understanding of such changes might shed light on how the polymers of these different walls interact to provide tensile strength; and, 3) A comparison of the tensile strength and porosity of dicot and monocot walls from cells grown in the presence and absence of DCB.

MATERIALS AND METHODS

Cell Cultures

Cell suspensions of tomato (Lycopersicon esculentum VF 36) were cultured and adapted to DCB as described previously (28); however, the original adapted line used in that work died, and a new line was adapted for these studies. Tobacco cells (Nicotiana tabacum Wisc. 38) were generated from pith and grown as for tomato except that the concentration of 2,4-D was raised 1.5 X and of isopentyladenine was lowered 10 X. Barley (Hordeum bulbosum L.) cultures, generated from endosperm tissue, were the kind gift of Adina Berman, Tel Aviv University; this line was cultured as for tomato except that isopentyladenine was omitted from the growth medium. All cells were grown at 26°C on a rotary shaker; tobacco lines were grown under low intensity fluorescent illumination. We found that it was not advisable to dissolve DCB stock solutions in ethanol or DMSO, as long-term growth of cells was affected with even low concentrations of these solvents. Therefore, DCB was added directly to medium in screw-capped bottles and dissolved by autoclaving; bottles were capped immediately after autoclaving. Since DCB is somewhat volatile, some losses may have occurred during autoclaving and long-term growth on shakers; the concentrations given refer to the initial concentrations calculated during media preparation.

In Vivo Labelling Studies

NA and A12 tomato cultures in the mid-log phase of growth were harvested and washed with fresh growth medium lacking glucose using a scintered glass filter. Cells were resuspended (about 5 mg fresh weight cells/ml) in medium lacking glucose with or without 25 μ M DCB and

aliquoted to 2 ml aliquots in glass scintillation vials. These cultures were incubated 15 min with shaking at 25 °C, then U-¹⁴C-glucose (5.0 uCi/umol) was added to a final concentration of 1.0 mM. At times 0, 1, and 2 hrs, three replicate vials were harvested from each treatment by addition of 10 mL 66% (v/v) ethanol, filtration on glass fiber filters, and washing 4 times with 66% ethanol and then once with water. Filters with cells were immediately weighed for fresh weight determinations, and then they were placed in 5 mL of acetic-nitric reagent (29) and digested for 1 hr at 100 °C to solubilize all polymers except cellulose. These incubations were again filtered, the supernatants collected, and the residues washed extensively with water and radioactivity in the supernatants and residues (cellulose) determined by scintillation counting.

Preparation and Extraction of Cell Walls

All procedures were carried out at 4°C. Cells at the mid-log phase of growth were harvested by filtration on a coarse sintered glass filter. The cells were washed with 20 mM Tris-HCl, pH 8.0, containing 2 mM fresh DTT, their fresh weight determined, and then frozen in liquid N₂ and stored at -20°C. Frozen cells were suspended in a solution (10 ml/g fresh weight) of 20 mM Tris-HCl, pH 8.0, containing 3 mM EDTA, 250 mM sucrose, and 2 mM fresh DTT and disrupted in a Parr Disruption Bomb at 1000 PSI (about 70 bars). The suspension was then centrifuged at 4,000 g for 5 min. Cell wall pellets were then washed twice by similar centrifugation and resuspension in 5 mM potassium phosphate buffer, pH 7.2, containing 2 mM DTT, followed by 5-10 washes with 2 mM DTT. For each cell type, the speed of centrifugation during washing was adjusted

(range between 20-500 g) in order to obtain the optimal speed to pellet the walls and leave starch in the supernatant, as judged by microscopic examination.

Wall preparations were extracted with 20 mM Tris-HCl, pH 8.0, containing 0.5 M LiCl and 2 mM DTT (approximately 1 ml/original g fresh weight of cells) at room temperature for 1 hr, followed by a second extraction with the same volume for 15 min. Walls were then washed 5-10 times with dH₂O. For all walls except those of tomato, these procedures effectively removed starch; however, a wash with 20% (v/v) methanol followed by α -amylase treatment (100 units/ml in 50 mM potassium phosphate buffer pH 7.2 and 0.05% (w/v) sodium azide; 37°C; 18 hrs with shaking followed by several water washes) was necessary to remove the last traces of starch from NA and A12 walls of tomato. Clean, starch-free, LiCl-extracted walls were suspended in a known volume of dH₂O, and, while stirring, aliquots were removed and lyophilized for dry weight determinations. Walls were stored frozen at -20°C.

CDTA extraction of these walls was carried by stirring them overnight at room temperature in 50 mM CDTA, pH 7.6 (1ml/10 mg walls). Following centrifugation at 9,680 g for 10 min, the residues were again extracted with the same volume of CDTA for 15 min, then washed with a small volume of dH₂O. Supernatants of these extractions were combined, filtered through a coarse sintered glass filter to remove remnants of walls and dialyzed for 24 hrs against 100 mM ammonium acetate, pH 5.2 (23) and then extensively (4 days) with dH₂O. Wall residues after CDTA extraction were washed once with 100 mM ammonium acetate, pH 5.2, followed by 3 washes with dH₂O.

Cell Wall Analyses

Cellulose was quantified by the method of Updegraff (29), uronic acids by the method of Blumenkrantz and Asboe-Hansen (6), total neutral sugars by the method of Dubois et al. (9), and xyloglucan by the method of Kooiman (19). Xyloglucans were obtained by sequential extraction of walls in CDTA, 4% (w/v) NaOH/0.1% NaBH₄, and finally, the xyloglucans were extracted from these residues in 24% KOH/0.1% borohydride. Extractions were carried out twice for 30 min each at 25°C in a sonic bath. For quantitation of protein, walls were hydrolyzed in 6 N HCl for 18 hrs at 120°C under vacuum, and the hydrolyzate analyzed for amino acids by HPLC separation of Pico-tag derivatives. Individual neutral sugars were quantified as the alditol acetates (4) by GLC separation using a 15 m DB-225 capillary column run isothermally at 220°C. Methylation of polysaccharides (still in progress) ^{was performed} ~~were prepared~~ as described by Blakeney and Stone (5). Partially methylated, partially acetylated derivatives were analyzed by GC-MS as described by Iraki et al. (7). Treatment of barley walls with porcine esterase (2.5 units/ml reaction; obtained from Sigma) or lichenase (1 unit/ml reaction of endo-1,3-1,4- β -glucan hydrolase from B. subtilis, obtained from Megazyme (Aust) Pty. Ltd.) was carried out in 50 mM Hepes/KOH, pH 7.3 at 37°C for 24 hrs with shaking.

Phenolic material was extracted from CDTA-extracted barley walls (25 mg) by overnight incubation under argon in the dark at room temperature with 5 ml of 0.1 M NaOH. The suspension was centrifuged 10 min at 9,680 g, the supernatant was acidified to pH 2.2 with phosphoric acid, and phenolic material was extracted into ethyl acetate. This was

concentrated 5X and analyzed by TLC in the dark on Silica Gel G plates using toluene:acetic acid (9:1) as solvent. Compounds separated were detected by UV light.

Cell wall peroxidases were eluted from whole cells by extraction with 50 mM CaCl_2 . Equal quantities of extracted protein were separated by native PAGE either by the procedure of Laemmli (20) at pH 8.3 (SDS omitted) or by the procedure of Reisfeld et al. (25) at pH 3.8. Peroxidase activities in the gels were detected as described by Bruce and West (7).

Protoplast Formation

Endopolygalacturonase was extracted from cells of an E. coli clone (HB101/pAKC::Tn5-2) expressing a peh gene from Erwinia carotovora (a gift of A. Mort, Oklahoma State University) according to the procedure of Maness and Mort (21). The enzyme was purified on a cross-linked polypectate column as described by Maness and Mort (21) and yielded an essentially pure protein of 45 kD as judged by SDS-PAGE.

NA and A12 cells of tobacco at the mid-log phase of growth were filtered on a sintered glass funnel, washed with growth medium, and then suspended in a 24-well plate in a total volume of 0.5 ml of growth medium containing 3% (w/v) sucrose, 400 mM sorbitol, and 100 mM glucose. Protoplasting conditions tested included: 1) 50 mM CDTA; 2) 0.03 units of purified endopolygalacturonase; 3) a combination of (1) and (2); and 4) a control with no additions. Samples were incubated at 25°C and examined under the microscope for wall and cell integrity at time intervals from 15 min to 5 hrs, and photographs were taken with a Leitz light microscope. Protoplasts were visible in treatments (2) and (3) after

about 1-3 hrs.

For wall regeneration, protoplasts were generated from A12 tobacco cells as described above for treatment 3, except that the procedure was performed aseptically. Protoplasts were then isolated on a ficoll gradient and cultured for wall regeneration by the method of Shea et al. (27) at 25°C with shaking at 150 rpm.

Tensile Strength and Porosity Measurements

Determinations of tensile strength were made similarly to the method described by Carpita (8) and Iraki et al. (16). Cells were harvested at the mid-log phase of growth, washed twice and resuspended in aliquots in medium containing 50 mM Mes-KOH, pH 5.7. Cells were equilibrated 15 min and broken at various pressures in a Parr Disruption Bomb using nitrogen gas. Percent broken cells was estimated by light microscopy, and plots were made of percent cells broken as a function of breaking pressure.

Porosities were measured by the technique of Baron-Epel et al. (3), except that cells were not plasmolyzed. Instead, movement of the dye to the cell interior was monitored on cells which had been treated with 0.1% Triton-X-100 to destroy membrane integrity.

RESULTS AND DISCUSSION

General Characteristics of DCB-adapted Cell Lines

All three cell lines (tomato, tobacco, barley) were adapted by gradual increases in the level of DCB in the medium, starting with a concentration of 1 μ M DCB. After about every 6 subcultures, the concentration was elevated in steps until cell lines were obtained which could grow on 12 μ M DCB (referred to hereafter as A12 cells), a concentration which is highly toxic to non-adapted (NA) cells. During growth on DCB, all three types of A12 cell lines grew as clumps of cells, as opposed to NA cells which existed as single cells or small clumps. In general, for A12 cells growing on DCB, lag times following subculture were longer than for NA cells, and A12 cells could not be cultured to as high a density without deleterious effects. However, during the optimal "window" for logarithmic growth, the rates of growth (as measured by increase in packed cell volume) for A12 tomato and tobacco were not markedly different than for NA cells (see ref.28 for growth curve for tomato), but were considerably slower for barley.

One notable observation is that we have found it impossible to plasmolyze cells of all A12 lines growing on DCB. Neither 1.5 M mannitol nor 4 M NaCl was able to effect separation of the wall from the plasma membrane. However, we did find that some plasmolysis did occur in the presence of 50 mM CDTA, and we could estimate that turgor pressures were similar to those of NA lines. Such a result suggests the interesting possibility that there exist enhanced wall-membrane connections of the type formed in animals by integrins (15) which may be stabilized by divalent cations. A similar, but less dramatic, enhancement of wall membrane connections has been found

in tobacco cells adapted to growth on NaCl (N. Carpita, personal communication).

Capacity for Cellulose Synthesis in DCB-adapted Tomato Cells

From our previous work (28), we deduced that the primary mode of adaptation of tomato cells to growth on DCB rested upon their ability to survive with little or no cellulose in their walls. However, we did not assess the capacity for cellulose synthesis in such cells. The data of Figure 1 represent measurements of the rate of cellulose synthesis in vivo in tomato cells pulse-labelled with ^{14}C -glucose for 3 hrs; following incubation, the cells were harvested and incorporation of label into cellulose was determined. The data show that, in the absence of DCB during labelling, adapted cells which had grown for many generations on 12 μM DCB (A12) and which were washed briefly and immediately placed in medium lacking DCB for labelling, display rates of cellulose synthesis which are quite similar to those of NA cells grown in the absence of DCB. This is also true for adapted cells which had been grown initially for long periods of time in DCB and then grown for 30 generations in the absence of DCB (A0). As might be predicted from the reduced levels of cellulose found in the walls, the pathway in adapted cells is still equally sensitive to DCB as evidenced by its ability to inhibit cellulose synthesis in these short-term experiments. Incorporation of label into non-cellulosic polymers was inhibited by DCB only 10-20% in these experiments, and no differences were seen among the cell lines (not shown). In sum, these results indicate that the cellulose synthesis pathway exists, but is inhibited, in A12 cells growing on DCB and resumes activity as soon as cells are placed on medium lacking DCB. Therefore,

the mechanism of adaptation does not result from a change either in the level of the enzymes required for cellulose synthesis, nor from a change in sensitivity of the pathway to DCB. Experiments with A12 lines of tobacco and barley indicate a similar situation occurs with these cells as well (not shown). These results are also supported by our observation that A12 tomato cells transferred to medium lacking DCB gradually with time revert to having walls with a normal level of cellulose (28).

Cellulose Content in Adapted Cells

Table I shows compositional analyses of the walls of tomato, tobacco and barley. Cellulose content is markedly reduced in the walls derived from A12 cells of all three cell types. Tomato A12 walls have about 12% the normal level of cellulose compared to NA walls; this value is somewhat higher than we reported for our previously-adapted tomato cell line (about 3% of normal), but this line had grown for several years on DCB when analyses were performed (28). The current line has only been adapted for about 6 months. Tobacco A12 walls have only about 6% the normal level of cellulose, whereas those of barley have about 30% of the amount found in their NA counterpart.

Figure 2 shows the birefringence in polarized light of walls of tomato NA cells grown without DCB (NA) and A12 cells grown on 12 μ M DCB. Compared to control cells, which have normal levels of cellulose and show clear birefringence indicative of cellulose in the walls, no birefringence is observed in the tomato A12 walls (compare Figs. 1C and 1D). We have observed that the walls of cells of A12 tobacco show a very weak birefringence, whereas those of A12 barley show birefringence almost as strong as their NA counterpart (results not shown). Apparently A12

barley walls possess sufficient residual cellulose, and/or more ordering of the microfibrils (or some other highly ordered polymer) to produce such birefringence.

Compositional Analyses of Other Wall Components

Uronic Acid-Rich Polymers. Our previous work with tomato (28) indicated that walls of A12 cells were enriched for uronic acid-containing pectic polymers. In that study, only a small portion of these polymers was extracted by EDTA, but a high percentage was solubilized by mild alkali treatment. From this, we speculated that these polymers may be cross-linked in the wall via ester linkages. However, in more recent studies using the chelator CDTA, which is generally more effective than EDTA in solubilizing calcium-bridged pectates (26), we have observed that a much higher proportion of the tomato A12 wall is solubilized. Table I shows results of compositional analyses of NA and A12 walls from all three types of plants and also shows what percent of each component (total non-cellulosic neutral sugars, uronic acids, protein, cellulose) can be extracted from the walls by CDTA. A12 walls of both of the dicot species are enriched in uronic acids and protein. Furthermore, compared to NA walls, a much higher proportion of the uronic acids can be solubilized from A12 dicot walls by treatment with CDTA. In contrast, barley walls contain much less uronic acid, and very little material is solubilized from either NA or A12 barley walls, which is consistent with data from the literature indicating that the monocot grass walls are "pectin-poor" (2). Thus, these results suggest that a major load-bearing network in A12 walls of dicots may be composed of calcium-bridged pectic polymers. In contrast, the barley does not elevate levels of such

polymers in response to DCB, suggesting that other polymers provide wall strength under conditions of reduced cellulose content.

Hemicellulosic Polymers. Our previous work (28) indicated that xyloglucan was synthesized in A12 cells, and most (but not all) was secreted to the medium, apparently because it lacked cellulose with which to interact. Results in Table II, showing neutral sugar compositions of cell walls, indicate that A12 walls of the other dicot tobacco also contain less xylose suggesting that it also has reduced xyloglucan in the walls. This was confirmed by direct analysis of the material extracted from these walls by strong alkali (classically the "xyloglucan-rich" fraction from dicot primary walls; see ref. 14). Thus, both tomato and tobacco A12 walls have a much reduced level of xyloglucan compared to their NA counterparts (Table II),

In contrast, barley NA and A12 walls show a neutral sugar composition indicative of the presence of GAX (low levels of uronic acid and high levels of arabinose and xylose) and the non-cellulosic 1,3-1,4- β -glucan characteristic of monocot grasses. As opposed to the situation for xyloglucan, there is no indication that the level of these polymers is reduced under conditions where cellulose content is reduced by at least 75%. In fact, the mole percent non-cellulosic glucose (indicative of the mixed-linked glucan) is elevated in A12 walls. That this is, in fact, due to an increase specifically in the mixed-linked glucan, was confirmed by monitoring the amount of glucose released from barley walls following overnight treatment with a specific, purified endo-1,3-1,4- β -glucanase. These results, also shown in Table II, indicate that the level of this glucan is elevated about 4-fold in the A12 walls, a result

in marked contrast to the situation for xyloglucan in dicot walls. In sum, these results suggest that neither GAX nor the mixed-linked glucan play a role comparable, at least in magnitude, to that of xyloglucan in dicot walls in terms of interaction with cellulose. To confirm these concepts, methylation analyses for assessment of sugar linkages in both walls and secreted polymers are in progress and should be completed within another month.

Protein. Amino acid analyses indicate that NA walls of the two dicots have a total protein content with a relatively high mole percent of hyp (NA tomato: 23 mol% hyp; NA tobacco: 23 mol% hyp), indicative of the presence of HRGP's which remain bound to the wall even after LiCl extraction which removes precursors to insolubilized HRGP's. As reported previously (28), we found that the mol% hyp declines in A12 walls of tomato to about 7%; similarly, recent analyses with tobacco A12 walls indicate a marked decline in A12 walls to 11 mol% hyp. There is, however, a notable increase in the total level of protein found in the A12 cells of both tomato and tobacco (Table I). The results with tomato are in contrast to our previously published finding that there was no substantial increase in protein in A12 tomato walls (28). The only explanation we can offer is that the data presented here for tomato was obtained from a new line adapted independantly from that used in our previous work. Since the total protein is much elevated in these A12 dicot walls, this indicates that there are actually more HGRP's in these walls even though the mol% hyp has declined; however, it must also mean that a larger quantity of other hyp-poor proteins are also deposited in these walls. Since CDTA is not effective in releasing much protein from

the A12 dicot walls, but releases a substantial portion of the uronic acid-containing polymers (see Table I), the residues of the walls after these extractions are quite enriched in protein. One of our future goals is to determine how this protein may be linked to the residual polymers in these walls. Preliminary evidence indicates that protease treatment of such residues can release a significant amount of additional uronic acid, suggesting some possible connections between residual pectic-type polymers and protein (A. Marcus, M. Shmuel, and D. Delmer, unpublished).

As expected for monocot grasses, both NA and A12 walls of barley have a low mol% hyp (<1%), and we know very little about the nature of the protein components of these walls, nor about how they might be linked to other polymers. However, our analyses suggest that major alterations in the protein components of barley do not occur in response to growth on DCB, since we see little change either in the ^{total} content (see Table I) or in the content of individual amino acids as analyzed by HPLC.

Protoplasts Can be Prepared from A12 Cells Simply by Treatment with CDTA and a Purified Polygalacturonase

The hypothesis that a major load-bearing network in A12 cells of dicots consists of calcium-bridged pectates was further supported by our rather surprising observation that A12 cells of both dicots could be lysed by overnight treatment with 50 mM CDTA (Fig. 3 A,B). By contrast, the walls of A12 cells of barley (Fig. 3C) and NA cells of all three cell types (not shown) remained intact after this treatment.

These results suggested that protoplasts might be produced simply by treatment of A12 dicot cells with 50 mM CDTA in the presence of an osmotic protectant such as sorbitol. Using A12 cells of tobacco, we

observed that under these conditions, one could see protoplasts, but they were still embedded in a gelled matrix; careful inspection of these cells showed that thin walls were also still present (see arrows, Fig. 4D). We also tried treatment with a purified endo-polygalacturonase; this resulted in release of a few protoplasts (Fig. 4E). However, the combination of CDTA and the polygalacturonase resulted in release of large quantities of protoplasts (Fig. 4F). Similar treatments of NA cells released no protoplasts (Fig. 4A-C).

We are not sure why the combination CDTA and polygalacturonase is much more effective than either alone. CDTA alone definitely breaks the strong connections between the cell wall and the plasma membrane and solubilizes pectic material, since this treatment alone results in protoplast formation, but the protoplasts remain trapped in a pectic gel; addition of polygalacturonase then apparently hydrolyzes enough of this gel to allow release of the protoplasts. With enzyme alone, the load-bearing matrix may be hydrolyzed, but the connections between the residual wall and the plasma membrane may not be severed, and this prevents true protoplast formation from the majority of cells. Furthermore, the solubilization of the pectic polymers by CDTA may also increase accessibility for hydrolysis by the enzyme.

This may be an oversimplification, since we also see a very thin wall separated from the protoplast remaining after treatment with CDTA alone; it would seem that a portion of the uronic acid-rich polymers may be held in covalent linkage and must also be cleaved by the enzyme in order to effect release of protoplasts. The fact that CDTA alone does lyse whole cells in medium lacking osmoticum might be explained by the

fact that the osmotic shock which occurs during pectin solubilization is sufficient to disperse the pectic gel and break this thin residual wall. Nevertheless, it is remarkable that these two agents can effect protoplast formation, and indicates that Ca^{2+} -bridged pectates form a critical load-bearing network in the walls of A12 dicot cells.

Previous work (12,22) indicates that DCB is very effective in preventing wall regeneration; protoplasts remain alive, but regenerate no wall until DCB is removed from the medium. It was therefore of interest to note that protoplasts prepared under aseptic conditions using CDTA and endopolygalacturonase, and subsequently isolated on a ficoll gradient and placed in regeneration medium, regenerate walls within several days. This occurs at an approximately equal rate in medium lacking or containing 5 μM DCB (Fig. 5). After a few more days, cell divisions were apparent in the cultures (Fig. 5C), and the walls of all protoplasts showed a strong fluorescence in the presence of Tinopal (Fig. 5B,D).

The ability to regenerate walls in the presence of DCB may relate to our observation that adapted cells retain some "memory", in the sense that they can be cultured for long periods of time in the absence of DCB and still survive and grow when transferred back to medium containing DCB (23). In terms of gross composition, the walls of A12 cells transferred to, and grown in, medium lacking DCB appear to gradually return to a composition resembling NA cells. Nevertheless, there seems to be some relatively stable change in the strategy of wall deposition which allows rapid adjustment back to growth on DCB. The fact that A12 protoplasts can regenerate a wall in the presence of DCB indicates that this "memory" seems not to require the presence of a preformed wall to be expressed.

Load-bearing Polymers in the Walls of the Monocot Barley

The results with CDTA treatments indicate that Ca^{2+} -bridged pectates are not major contributors to wall strength in A12 walls of barley. Since the barley walls apparently contain normal or even elevated levels of GAX and mixed-linked glucan, we have considered that differential cross-linking of these polymers might occur to compensate for loss of strength when cellulose levels are reduced. It is well-known that the cereals, unlike most dicot primary walls, contain substantial amounts of ferulic acid in ester linkage which can serve as a potential cross-link between polysaccharides (2,10,11). This ferulic acid imparts a characteristic green autofluorescence in NH_3 to the walls (13). Figure 6A shows that the walls of NA barley cells show such fluorescence. However, walls of A12 cells show a characteristic yellow fluorescence, suggestive of altered phenolic composition (Fig. 6B). This material is apparently not classic lignin, as these walls do not stain positive with phloroglucinol (not shown).

To examine this issue further, CDTA-extracted NA and A12 barley walls were treated with mild alkali to cleave ester linkages, and the released phenolic material was extracted at pH 2.2 into ethyl acetate. Figure 7 shows a TLC profile of these phenolic materials. Whereas NA walls clearly contain ferulic acid and a compound which migrates with di-ferulic acid, the levels of these compounds is much reduced in the extracts from A12 cells, and the level of coumaric acid is increased. In addition, extracts from A12 walls contain somewhat more UV-absorbing material which remains at the origin in TLC. Figure 7 also shows that a comparison of the overall UV spectra of extracted material indicatives

compositional differences between the two extracts, although the total quantity of UV absorbing material released per mg dry weight of walls was similar. Sufficient material for ^{13}C -NMR spectroscopy is now being prepared for future structural analyses of the phenolic fraction of the wall.

Another indication of potential differences in cross-linking between polymers comes from experiments in which purified barley walls were treated overnight with porcine esterase. Whereas such treatment released 0.4% of the non-cellulosic carbohydrate from NA walls, 3.7% of such carbohydrate was released from A12 walls. Such a result does indicate that more carbohydrate may be insolubilized through ester linkages in the A12 walls.

Analyses of isozyme patterns of basic peroxidases which were salt-extracted from intact cells also indicate that A12 cells may have altered cross-linking capabilities. Figure 8 shows the results of native gel electrophoresis at low (left panel) or high (right panel) pH followed by activity staining from equal amounts of salt extracted protein from both tomato and barley NA and A12 cells. For tomato, only minor quantitative differences were observed in the profiles (similar results were observed for tobacco which are not shown). However, in the barley, a distinct, more basic peroxidase activity was eluted from the A12 cell walls, coupled with a reduction in level of the other basic forms (left panel). No qualitative differences were noted in isozyme profiles for the barley acidic peroxidases (right panel). Since basic, rather than acidic, peroxidases are believed to function in phenolic cross-linking and lignification within the wall (1,7), the appearance of a new basic

peroxidase isozyme in barley is of interest, and may relate to the altered phenolic composition of these walls. We note that Bruce and West (7) also observed the appearance of two new forms of basic peroxidases upon elicitation of lignin synthesis by pectic fragments in cultures of castor bean.

Tensile Strength and Porosity of Walls

We have attempted to compare the tensile strength of NA and A12 walls using the method of Carpita (8,16) which involves determining the pressure required for 50% wall breakage using a nitrogen-bomb cell disruption technique. Coupled with measurements of cell size, wall thickness, and turgor pressures, one can then calculate a tensile strength according to the equation:

$$\text{Tensile strength} = \frac{P \times r}{2t}$$

where P = turgor pressure + breaking pressure, r = average cell radius, and t = wall thickness.

We encountered some technical problems in attempting these analyses. First, because cells could not be plasmolyzed, we estimated turgor pressures ^{by} plasmolysis in the presence of 50 mM CDTA which does break wall-membrane connections (see Fig. 4D); this seems satisfactory, as results for NA cells were similar to those obtained in the absence of CDTA. It was also more difficult to assess the percentage of broken A12 cells, since these cells grow as clumps ^{which} ~~which~~ contain many cells, and we were not able to effect cell separation by chromic acid treatment as was done by Carpita for cell counting (8) because this and other treatments attempted all resulted in wall disintegration of A12 cells. Thus, we

relied on many replicate determinations, and have obtained ^{replicate} plots of percent cell breakage vs. breaking pressure for either NA or A12 cells which did not vary in slope by more than 10-15%. The data of Table III summarize these results for tobacco and barley. Results with the dicot tobacco indicate that A12 walls are significantly weaker than their NA counterpart, indicating that the cellulose-xyloglucan network is, not unexpectedly, a major contributor to wall strength in dicots, and that a wall relying heavily on Ca^{2+} -bridged pectates is substantially weaker. We note that Iraki et al. (16) also found about a 4-fold decrease in tensile strength when tobacco was adapted to growth on high concentrations of NaCl, and that these walls also had altered composition, including a decrease in content of cellulose. Their absolute numbers for adapted and non-adapted wall strengths were about 10-fold higher than ours, a result which can be attributed to their much lower values for wall thickness. We are now obtaining more accurate numbers by EM for wall thickness, and this may change the final values for tensile strengths, but it already is clear even from light microscopy and the few EM results we have that wall thickness is about 1.5-2 times greater in all A12 cells compared to their NA counterpart, and that the isolated walls are more highly hydrated. It would seem that the values of Iraki et al., must have erred on the low side which could be due to dehydration during preparation for EM.

Rather surprisingly, we have found that the A12 walls of barley are even stronger than the parental NA cell line. This would indicate either that cellulose is not a major contributor to wall strength in barley (which seems unlikely to us), or, more likely, that barley A12

cells make compensatory changes to provide wall strength. On the basis of our analyses to date, it would seem that alterations in the phenolic composition and/or cross-linking patterns may be one such compensatory change.

We also encountered technical difficulties in assessing porosity of walls using the technique of Baron-Epel et al.(3). This technique involves assessing the movement of fluorescent-labelled dextrans of different sizes through the walls of plasmolyzed cells. Since we were not able to plasmolyze A12 cells except with CDTA which certainly alters wall porosity, we eventually found that we could assess movement by disrupting membranes with the 0.1% Triton-X-100, and thus create wall ghosts which were used for measurements. As a control, we have ascertained that similar porosities were observed in NA cells when measured either by plasmolysis or by detergent treatment. Table IV shows the results of these studies for NA cells. For all three NA cell types, dextrans with radii of up to, and including, 4.3 nm can pass through the walls, whereas dextrans of radius 5.8 nm and greater are excluded. It would seem that 4.3 nm is approaching the limit of porosity, since it takes more than an hour for this dextran to penetrate the walls, whereas those of smaller radii penetrate within just a few minutes. Unfortunately, this report was due before we could complete the analyses with the adapted walls; analyses of these are just beginning, and results will be presented in the completed MS.

LITERATURE CITED

1. Abeles FB, Biles CL (1991) Characterization of peroxidases in lignifying peach fruit endocarp. *Plant Physiol* **95**: 269-273
2. Bacic A, Harris PJ, Stone BA (1988) Structure and function of plant cell walls. In J Preiss, ed, *The Biochemistry of Plants*, Vol. 14, Academic Press, San Diego, pp 297-371
3. Baron-Epel O, Gharyal PK, Schindler M (1988) Pectins as mediators of wall porosity in soybean cells. *Planta* **175**: 389-395
4. Blakeney AB, Harris PJ, Henry RJ, Stone BA (1983) Simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr Res* **113**: 291-299
5. Blakeney AB, Stone BA (1985) Methylation of carbohydrates with lithium methylsulphonyl carbanion. *Carbohydr Res* **140**: 319-325
6. Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. *Anal Biochem* **54**: 484-489
7. Bruce RJ, West CA (1989) Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. *Plant Physiol* **91**: 889-897
8. Carpita N (1985) Tensile strength of cell walls of living cells. *Plant Physiol* **79**: 485-488
9. DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**: 350-356
10. Fry SC (1983) Feruloylated pectins from the primary cell wall: their structure and possible functions. *Planta* **157**: 111-123
11. Fry SC (1986) Cross-linking of matrix polymers in the growing

cell walls of angiosperms. *Annu Rev Plant Physiol* 37: 165-186

12. **Galbraith DW and Shields BA** (1982) The effects of inhibitors of cell wall synthesis on tobacco protoplast development. *Physiol Plant* 55: 25-30
13. **Harris PJ, Hartley RD** (1976) Detection of bound ferulic acid in cell walls of the Gramineae by ultraviolet fluorescence microscopy. *Nature* 259: 508-510
14. **Hayashi T** (1989) Xyloglucans in the primary cell wall. *Annu Rev Plant Physiol* 40: 139-168
15. **Hynes RO** (1987) Integrins: a family of cell surface receptors. *Cell* 48: 549-554
16. **Iraki NM, Bressan RA, Hasegawa PM, Carpita NC** (1989) Alteration of the physical and chemical structure of the primary cell wall of growth-limited plant cells adapted to osmotic stress. *Plant Physiol* 91: 39-47
17. **Iraki NM, Bressan RA, Carpita NC** (1989) Cell walls of tobacco cells and changes in composition associated with reduced growth upon adaptation to water and saline stress. *Plant Physiol* 91: 48-53
18. **Iraki NM, Bressan RA, Carpita NC** (1989) Extracellular polysaccharides and proteins of tobacco cell cultures and changes in composition associated with growth-limiting adaptation to water and saline stress. *Plant Physiol* 91: 54-61
19. **Kooiman P** (1960) A method for the determination of amyloid in plant seeds. *Recl Trav Chim Pays-Bas Belf* 79: 675-678

20. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685
21. Maness NO, Mort AJ (1989) Separation and quantitation of galacturonic acid oligomers from 3 to over 25 residues in length by anion-exchange high-performance liquid chromatography. *Analyt Biochem* **178**: 248-254
22. Meyer Y, Herth W (1978) Chemical inhibition of cell wall formation and cytokinesis, but not of nuclear division, in protoplasts of Nicotiana tabacum L. cultivated in vitro. *Planta* **142**: 253-262
23. Mort AJ, Moerschbacher BM, Pierce ML, Maness NO (1991) Problems encountered during the extraction, purification, and chromatography of pectic fragments, and some solutions to them. *Carbohydr Res* **215**: 219-227
24. Peters R (1986) Fluorescence microphotolysis to measure nucleocytoplasmic transport and intracellular mobility. *Biochim Biophys Acta* **864**: 305-359
25. Reisfeld RA, Lewis UJ, Williams DE (1962) Disk electrophoresis basic proteins and peptides on a polyacrylamide gels. *Nature* **195**: 281-283
26. Selvandran RR, O'Neill MA (1987) Isolation and analysis of cell walls from plant material. *Methods Biochem Anal* **32**: 25-153
27. Shea EM, Gibeaut DM, Carpita NC (1989) Structural analysis of the cell walls regenerated by carrot protoplasts. *Planta* **179**: 293-308

28. Shedletzky E, Shmuel M, Delmer DP, Lamport DTA (1990) Adaptation and growth of tomato cells on the herbicide 2,6-dichlorobenzonitrile leads to production of unique cell walls virtually lacking a cellulose-xyloglucan network. *Plant Physiol* **94**: 908-987
29. Updegraff DM (1969) Semi-micro determination of cellulose in biological materials. *Anal Biochem* **32**: 420-424

Table I. Compositional Analyses of Total Cell Walls

	Cellulose	Uronic Acids	Neutral Sugars ^a	Protein
<hr/>				
	(ug/mg Dry Weight Walls)			
Tomato				
NA	268 (0%) ^b	299 (35%)	406 (14%)	65 (15%)
A12	32 (0%)	364 (73%)	182 (54%)	411 (9%)
<hr/>				
Tobacco				
NA	306 (0%)	266 (38%)	140 (21%)	76 (9%)
A12	23 (0%)	424 (62%)	160 (40%)	219 (7%)
<hr/>				
Barley				
NA	263 (0%)	35 (3%)	346 (0.3%)	109 (6%)
A12	78 (0%)	42 (3%)	670 (0.3%)	107 (3%)

^aCorrected to exclude cellulose and uronic acids; ^bNumbers in parenthesis indicate the percent of each component solubilized by CDTA treatment.

Table II. Neutral Sugar and Hemicellulose Composition of Total Cell Walls.

Monosaccharide	Tomato		Tobacco		Barley	
	NA	A12	NA	A12	NA	A12
(mol %)						
Rhamnose	12	16	7	9	<1	<1
Arabinose	24	18	46	49	30	25
Xylose	21	15	14	5	47	47
Mannose	4	3	4	<1	1	<1
Galactose	24	23	22	27	13	11
Glucose ^a	15 ^b	27 ^b	7	8	9	17
(ug/mg cell wall)						
Xyloglucan ^c	41	8	50	16	N.D. ^e	N.D.
ML-glucan ^d	N.D.	N.D.	N.D.	N.D.	105	24

^aNon-cellulosic; ^bWalls were not treated to remove starch; analyses of starch-free walls is in progress; ^cDetermined by method of Kooman (19) on components solubilized from walls by 24% KOH; ^dML-glucan: mixed 1,3-1,4- β -glucan; content determined by amount of glucose released from walls by treatment with purified endo-1,3-1,4-glucanase; ^eN.D.: not determined.

Table III. Analyses of Tensile Strength of Cell Walls.

Cell Line	Turgor Pressure ^a	Breaking Pressure ^b	Cell Radius ^c	Wall Thickness ^d	Tensile Strength ^e
	bar	bar	um	um	bar
Tobacco					
NA ^f	5	17	43	1.0	473
A12	4	3	47	1.5	110
Barley					
NA	7	12	29	0.8	344
A12	3	28	32	1.2	411

^aCalculated by plasmolysis in the presence of 50 mM CDTA and varying concentrations of mannitol; ^bPressure in excess of turgor pressure required to break 50% of cells; ^cMeasured by microscopy of living cells; except for NA tobacco, most cells were roughly spherical; only spherical cells were monitored for NA tobacco; ^dEstimated by fluorescence, polarization and conventional light microscopy; these numbers are tentative, and we are waiting for results of photographs from EM; ^eCalculated from equation $(P \times r)/2t$ (see text), where P is the sum of the turgor pressure plus the breaking pressure.

Table IV. Comparison of Porosities of Walls of NA and A12 Cells.

Mol Wt of Fl-dextrans	Stokes radii nm	Ability to pass through walls of			
		Tobacco		Barley	
		NA	A12	NA	A12
9.0	2.7	+ ^a , + ^b , + ^c		+, +, +	
17.9	3.3	+, +, +		+, +, +	
35.6	4.3	-, +, +		-, +, +	
71.0	5.8	-, -, -		-, -, -	

^aFirst measurement, at 0.5 hr; ^bSecond measurement, at 1.0 hr; ^cThird measurement, at 2.0 hr

FIGURE LEGENDS

Figure 1. Capacity for cellulose synthesis and sensitivity to DCB in tomato suspension cultures. Cells were harvested, washed in medium lacking DCB, and then pulse-labelled for 3 hr in ^{14}C -glucose in the presence or absence of 25 μM DCB, and incorporation into cellulose was quantified. NA: non-adapted cells grown without DCB; A12: adapted cells grown in the presence of 12 μM DCB prior to labelling; A0: adapted cells which had been grown for 30 generations in the absence of DCB prior to labelling.

Figure 2. Comparison of birefringence under polarized light the cell walls of NA and A12 cells of tomato. A,C: NA cells; B,D: A12 cells; A,B: light microscopy; C,D: dark-field microscopy with polarized light.

Figure 3. Effect of overnight treatment of intact A12 cells with 50 mM CDTA. A12 cells of tomato (A), and tobacco (B) were completely lysed by this treatment, whereas those of barley (C) were not. NA cells of all three cell types were also unaffected (not shown).

Figure 4. Generation of protoplasts from A12 tobacco cells by treatment with CDTA and a purified endopolygalacturonase. A-C: NA tobacco cells; D-F: A12 tobacco cells. A,D: treatment with 50 mM CDTA; B,D: treatment with endopolygalacturonase; C,F: treatment with both CDTA and endopolygalacturonase. Arrows in D indicate thin cell wall which remains after treatment with CDTA only.

Figure 5. Autofluorescence of NA and A12 walls of barley. When viewed by fluorescence microscopy in the presence of dilute ammonia, the walls of NA cells (A) show a green autofluorescence characteristic of the presence of ferulic acid; A12 cells (B) show instead a distinct yellow fluorescence indicative of altered phenolic composition.

Figure 6. Cell wall regeneration by tobacco A12 protoplasts. Protoplasts were prepared by treatment with CDTA and endopolygalacturonase, then isolated and cultured for 3 days in protoplasting medium in absence (A,B) or presence (C,D) of 5 μ M DCB. A,C: light microscopy; B,D: fluorescence microscopy in the presence of Tinopal.

Figure 7. Analyses of phenolic components released from NA and A12 barley walls by treatment with mild alkali. The upper panel compares the absorption spectra of such phenolic material after extraction at pH 2.2 into ethyl acetate. The lower panel shows TLC separation and detection by UV light of individual phenolic components present in the ethyl acetate extracts. STDS: standards; 1) caffeic acid; 2) di-ferulic acid (standard not available; R_f taken from literature); 3) coumaric acid; 4) sinapic acid; 5) ferulic acid; 6) cinnamic acid. Because plates were developed in the dark, some derivatives ran as doublets of their cis- and trans-isomers.

Figure 8. Peroxidase isozymes salt-eluted from intact cells of tomato and barley. Equal amounts of salt-eluted proteins were separated by

native PAGE at low pH (basic peroxidases; left panel) or high pH (acidic peroxidases; right panel) and assayed in situ for peroxidase activity.

Tom 12

Tom 12
Y1000

Tom 12
Y1000

A - I think have the negative
info in folder II or Notebook I (in the same)

A - B x250

C - F x625

A11 X1200

in color photo.

← green

← yellow

Peroxidase Isozymes in DCB-Adapted Cells

Tom		Bar	
NA	A	NA	A

Tom		Bar	
NA	A	NA	A

Debbie Delmer - best
wishes from Keith

Structures at the plant cell surface

Copyright

K. Roberts

APPENDIX III

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Current Opinion in Cell Biology 1990, 2:920-928

Introduction

The plant cell surface has always been rather a fuzzy zone, not just when viewed under the electron microscope, but in the minds of many plant scientists. I now have the impression that things are sharpening up a little, at least in focus, if not always in fact. This subtle shift in emphasis, seen in the papers that I have chosen to review, is partly due to the belated recognition that if the cell wall (or extracellular matrix; ECM) has something to do with the cell, it is equally true that the cell has something to do with the wall. Thus, while many papers in the last year continued to explore successfully the increasingly byzantine complexity of ECM chemistry (for example, York *et al.*, *Carbohydr Res* 1990, 200:9-31; Kiefer *et al.*, *Carbohydr Res* 1990, 197:139-158; Thomas *et al.*, *Carbohydr Res* 1989, 185:261-277; 185:279-305) others begin to show more concern with specifically cell biological aspects of the surface. The ECM interacts with extrinsic and intrinsic proteins of the plasma membrane, and these in turn interact directly or indirectly with the actin filaments and microtubules of the cortical cytoskeleton. All these elements together make up the cell surface and it is this cell surface that is at the heart of many key events in plant growth and development. In this context, our past emphasis (still vitally important) on understanding the common elements that belong to all primary cell walls is now beginning to be overlaid with new concerns: to understand how the cell surface acquires cell- and tissue-specific character, how the wall changes across its width, how the matrix mediates cell interactions, and how the cell can organize different regions of its ECM in both a temporal and spatial sense. These are, of course, the same concerns that animal cell biologists have had for years, but then it is only recently that the apparent uniqueness of the plant ECM has been shaken by a recognition that the similarities in many cases are more interesting than the differences (Roberts, *Curr Opin Cell Biol* 1989, 1:1020-1027).

The papers that I have highlighted then are those that examine the plant cell in relation to its ECM rather than the ECM itself in isolation. I have, however, excluded the burgeoning literature on the ECM in relation to pathogenesis (elicitors, signalling, pathogenesis-related proteins and

the like) and on the ECM and cell elongation. Both are vitally important areas but the confused and often contradictory literature seems to have made no major leaps or conceptual advances this year. It will be interesting to see if the early reports of a putative oligosaccharin receptor for the hepta- β -glucoside (Cheong and Hahn, *Plant Physiol* 1990, 93:91) and of a 10 kD protein involved in cell wall extensibility (McQueen-Mason *et al.*, *Plant Physiol* 1990, 93:75) rejuvenate these fields.

Pectin is not just jelly

There still persists a view that pectic polysaccharides form some kind of stiff, amorphous and ubiquitous glue, a sticky jelly-like matrix found predominantly in the middle lamella. There are three characterized pectic polysaccharides that have been isolated from plant primary cell walls, polygalacturonic acid (PGA), rhamnogalacturonan (RG)-I, and RG-II, and it is increasingly clear that at least PGA and RG-I are spatially restricted in their distribution, both within a single wall and between different cell types, and that the relative abundance of each varies widely between dicotyledons, monocotyledons and gymnosperms (O'Neill *et al.*, In *Methods in Plant Biochemistry* edited by Day PM and Harbourne JB. Academic Press 1990, 2:415-441). The distribution and functions of pectin are no longer compatible with the idea that it is simply a gel.

A major new contribution to this field is the discovery that tomato cells in culture can become adapted to the presence of 2,6-dichlorobenzonitrile, a potent inhibitor of cellulose synthesis (Shedletzky *et al.*, *Plant Physiol* 1990, in press). Remarkably, these adapted cells still form coherent walls, but these contain virtually no cellulose and hemicellulose, and also have a much reduced protein content. Instead, the walls appear to consist solely of a crosslinked meshwork of the three classes of pectin. This system is likely to prove valuable in understanding pectin crosslinks, but it also provides strong support for the notion of a pectin network in the wall that is separate from the cellulose-hemicellulose network. It is also clear, however, that other pectic polysaccharides can be very tightly

Abbreviations

ECM—extracellular matrix; GRP—glycine-rich protein; HRGP—hydroxyproline-rich glycoprotein; IS—intercellular space; PGA—polygalacturonic acid (a homogalacturonan); RG—rhamnogalacturonan; RPRP—repetitive proline-rich proteins.