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Cell Wall Metabolism in Ripening Fruit[1]

IX. Synthesis of Pectic and Hemicellulosic Cell Wall Polymers in the Outer Pericarp of Mature Green Tomatoes (cv XMT-22)

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Discs of outer pericarp were excised from mature green tomato (*Lycopersicon esculentum* Mill.) fruit and kept in sterile tissue culture plates for 4 d, including 2 d of incubation with D-[U-^{13}C]glucose. Cell walls were prepared and the water-soluble, pectic, and hemicellulosic polymers were extracted. Cell wall synthetic capacity was determined by gas chromatography-mass spectrometry analysis of incorporation of the heavy isotope label. The "outer" 2-mm pericarp region, which included the cuticle, had a lower cell wall synthetic capacity than the "inner" 2-mm region immediately below it (closer to the locules), based on the percentage of labeling of the neutral sugars. There were no significant differences in relative abundance of glycosidic linkages in the two tissue regions. Label was incorporated into neutral sugars and linkages typical for each polysaccharide class were identified in the cell wall preparations. Galacturonic acid and glucuronic acid were labeled to an extent similar to that of the neutral sugars in each tissue region.

The softening of fruits during ripening is one of the major limiting factors in the transportation and storage of freshly harvested commodities (Brady, 1987). Technologically, softening is retarded by low temperature and/or modified atmosphere storage, but to maximize the control of this event, we need to understand the physiology and biochemistry that brings it about.

Throughout the years, studies have focused largely on cell wall breakdown as the primary reason for softening (Gross and Wallner, 1979; Grierson and Tucker, 1983; Huber, 1983). This has led to the suggestion that certain hydrolytic enzymes are responsible for the dissolution of the middle lamella as well as for the degradation of the cell wall itself, thereby leading to a general loss of cohesion of the tissue, perceived as softening. This model is supported by anatomical work that demonstrated the breakdown of middle lamella polymers (Ben-Arie et al., 1979), by numerous studies characterizing the ripening-related changes in cell wall polymers (Gross and Wallner, 1979; Ahmed and Labavitch, 1980; Gross, 1984), and by studies that demonstrated increases in the activities of some putative cell wall polysaccharide hydrolases during ripening (Hobson, 1968; Tucker et al., 1982; Brady et al., 1983; Pressey, 1989; for review, see Fischer and Bennett, 1991). However, work involving antisense RNA (Sheehy et al., 1988) and chimeric gene expression (Giovannoni et al., 1989) has demonstrated that pectin degradation by PG is insufficient to explain ripening-related tissue softening. This has led to increased efforts to investigate alternative mechanisms of fruit softening, including aspects of cell wall metabolism other than those that involve polymer hydrolysis.

One such alternative is cell wall synthesis. Baker and Ray (1965) proposed that the wall weakening necessary to allow cell elongation in vegetative tissues may require the synthesis of certain polymers (not specifically identified, either then or subsequently) that, when incorporated into the wall, caused its relaxation. Knee et al. (1977) showed that wall synthesis occurred in strawberry fruits after the onset of ripening and extended this idea with work on apple fruits (Knee, 1978). He proposed but did not demonstrate that the synthesis could contribute to wall component dissociation and fruit softening. Mitcham et al. (1989, 1991) continued the work using [^{14}C]Suc as a precursor to cell wall polysaccharides in tomato (*Lycopersicon esculentum* Mill.) fruits ripening on the vine. They demonstrated incorporation of label into various classes of polysaccharides (defined only by differential extractability) throughout the course of fruit ripening. Greve and Labavitch (1991) used [^{13}C]Glc as a precursor in excised tomato pericarp discs and expanded on the results of Mitcham et al. (1989, 1991) by following label into individual monosaccharides that were identified by GC-MS analysis of hydrolyzed cell wall preparations. In this paper we describe a more detailed investigation of the incorporation of ^{13}C label into specific glycosidic linkages of pectic and hemicellulosic wall fractions extracted from two regions of the outer pericarp of tomato fruit. Only MG fruits were used in this study, since it has been shown that maximal synthesis occurs at the

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Abbreviations: AIS, alcohol and acetone insoluble solids (crude cell wall); amu, atomic mass unit; CDTA, cyclohexane diamine tetraacetic acid; MG, mature green stage of tomato fruit development; PG, endo-polygalacturonase; SIM, selected ion monitoring; TFA, trifluoroacetic acid.

transition between the MG and turning stages of ripening (Mitcham et al., 1989; Greve and Labavitch, 1991) prior to perceptible softening.

MATERIALS AND METHODS

MG tomatoes (*Lycopersicon esculentum* Mill. cv XMT-22) were obtained from a commercial wholesaler and were sorted to eliminate fruits with surface blemishes and those showing signs of color break. An excised pericarp disc system was utilized, based on the method of Campbell et al. (1990). The experimental design consisted of three treatments: an in vivo control, an in vitro control, and an in vitro labeled group. The in vivo control consisted of discs excised and frozen immediately. Analysis of the cell wall components of the in vivo controls provides a reference with which to evaluate the changes observed in the in vitro controls, which were held in culture for 4 d prior to sampling and analysis. All in vitro discs were incubated for 2 d to allow the dissipation of the disc excision (i.e. wound)-related responses we reported previously (Campbell et al., 1990; Greve and Labavitch, 1991). Then, the in vitro labeled discs were given [^{13}C]Glc and incubated for an additional 2 d. The in vitro control discs were given water and incubated for an additional 2 d. There were two tissue types within each treatment: the "outer" 2 mm of pericarp, including the cuticle, epidermis, hypodermis, and some mesocarp; and the 2-mm portion immediately inward from that, consisting entirely of mesocarp, designated as "inner." There were three replicates per treatment and tissue type.

Thirty-six fruits were surface-sterilized in 5% commercial bleach (0.25% sodium hypochlorite) for 2 to 3 min, and 18 pericarp discs (12 mm in diameter) were excised aseptically from the equatorial region of each fruit using a cork borer. Each of the 18 discs per fruit was assigned at random to one of the three replicates within the treatments and tissue types, ensuring that each fruit contributed an equal volume of tissue to each replicate of the tissue types and treatments. The outer discs were trimmed to a thickness of 2 mm, and the inner discs were trimmed to a thickness of 4 mm using a plexiglass block with holes of the correct depth. This approach to analysis of label incorporation into inner pericarp tissues was necessary because discs without a cuticle were not viable over 4 d in culture; they undergo severe browning, particularly in the vascular strands, which may be a response to desiccation in spite of attempts to maintain high humidity in the incubation chamber.

Disc epidermal color was measured on the CIELAB L*a*b* scale, using a reflectance colorimeter (CR-200, Minolta, Tokyo, Japan). The a* value is a measure of hue on the green-red axis, with green being the more negative value. The inner discs of the in vivo control were then halved equatorially in the block, retaining the inner portion and discarding the outer, cuticle-containing portion. At this stage all of the other discs retained the cuticle surface. The in vivo control discs were frozen in liquid nitrogen and stored at −30°C until analysis.

The in vitro discs were placed epidermis side down in sterile multiwell tissue culture plates and kept for 48 h at 20°C in a container continually flushed with humidified air. Disc color was then determined, and a 50-μL water droplet containing 100 μg of D-[U-^{13}C]Glc (99%, Cambridge Isotope Laboratories, Woburn, MA) was added to the uppermost (cuticle-free) surface of each disc in the in vitro-labeled treatment. The in vitro controls received water as a placebo. The plates were returned to the humidified container for an additional 48 h. Color was then measured before the inner discs of both the control and labeled treatments were collected by halving the 4-mm discs equatorially in the plexiglass block and discarding the outer 2-mm portion containing the cuticle. All discs were frozen as before.

Cell Wall Preparation and Fractionation

Frozen discs were diced and boiled in absolute ethanol for 30 min to inactivate autolytic enzymes and then homogenized using a polytron (Brinkmann). After the sample was centrifuged at 2000*g* for 10 min, the supernatant was saved and the pellet was washed twice (by centrifugation) with 95% ethanol and twice with acetone. All washings were added to the ethanol fraction and evaporated under a stream of air. Pellets (crude cell wall) were dried overnight and stored in a vacuum oven. An 80-mg portion of this pellet was stirred in water with 0.02% azide for 12 h and centrifuged at 2000*g* for 15 min. The resultant pellet was washed once in water, yielding a pellet, which was frozen and lyophilized, and a water-soluble supernatant. A 60-mg portion of the water-extracted, lyophilized pellet was stirred sequentially (15 h each) in 50 mM CDTA/50 mM sodium acetate (pH 6.5) and then 50 mM Na_2CO_3. Residues were washed once in deionized water after each extraction and the water from the washings was combined with the appropriate extract and filtered through Miracloth (Calbiochem). The filtered extracts were dialyzed (1000 molecular-weight cutoff) against deionized water at 4°C, as were the water-soluble supernatant and remaining pellet. The dialysates were frozen and lyophilized, and the pellets were extracted for 6 h with 4 N KOH containing 1% (w/v) $NaBH_4$. The KOH extract was filtered off through Miracloth, neutralized with addition of glacial acetic acid, and dialyzed as before, prior to lyophilization.

Cell Wall Analysis

The ethanol/acetone supernatant and water-soluble, pectic (combined chelator- and carbonate-soluble materials), and hemicellulosic (KOH-soluble) fractions were analyzed for neutral sugar composition by hydrolysis in 2 N TFA (1 h at 121°C; Albersheim et al., 1967) and preparation of alditol acetate derivatives (Blakeney et al., 1983). These were injected onto a 30-m × 0.25-mm i.d. DB-225 capillary column (J & W Scientific) in a Perkin-Elmer 8320 gas chromatograph run isothermally at 210°C, using H_2 as the carrier gas. Peaks were quantified against a *myo*-inositol internal standard by a Perkin-Elmer (Sigma 10) data system. The same preparations were also analyzed by GC-MS to determine the proportion of ^{13}C label in each neutral sugar (Greve and Labavitch, 1991). Methylation analysis

(Blakeney and Stone, 1985) by GC-MS was conducted on the pectic and hemicellulosic fractions to determine glycosidic linkage compositions and the percentage of label in specific linkages.

Methanolysis was performed on the pectic fraction (Bhat et al., 1991) using GC-MS to determine incorporation of label into GalUA and GlcUA. Methanolysis involves solvolytic cleavage of polysaccharides (including GalUA and GlcUA) with methanolic HCl to yield the corresponding monomeric methyl glycosides and uronosyl residues with methylesterified carboxyl groups. The esterified carboxyl groups are then reduced to the corresponding alcohols using NaB^2H_4. Hydrolysis with 2 N TFA (which removes the methyl glycoside) is followed by reduction in $NaBH_4$ and acetylation with acetic anhydride in the presence of 1-methylimidazole (Blakeney et al., 1983). The NaB^2H_4 imparts an additional 2 amu to the Gal/Glc derived from GalUA/GlcUA, but does not affect the native Gal or Glc (in which C_6 is already in the alcohol form). MS analysis of the uronic acid-derived alditol acetates is therefore conducted using ions of 189 and 193 amu (for ^{12}C- and ^{13}C-derived sugars, respectively) instead of the corresponding 187 and 191 amu from native Gal and Glc (Fig. 1).

GC-MS analysis utilized a gas chromatograph (Hewlett-Packard model 5890) with a 20-m × 0.18-mm i.d. DB-225

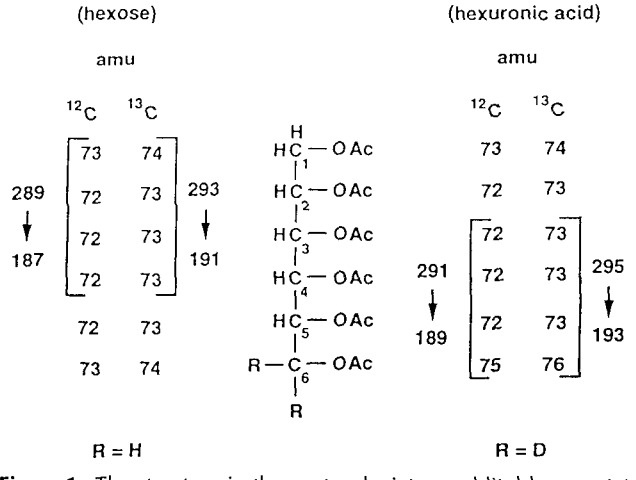

Figure 1. The structure in the center depicts an alditol hexaacetate derived from a neutral hexose (R = [^{1}H] because postmethanolysis reduction using NaB^2H_4 does not affect the protons at C_6) or a hexuronic acid (R = ^{2}H), which is reduced at C_6 following methanolysis. The brackets indicate the points at which the [^{12}C]- or [^{13}C]-derived alditol hexaacetates are fragmented in the GC-MS ion source to produce ions that are used for quantitation of incorporation. Primary fragment ions of 289 and 293 amu from hexoses can be produced by initial fragmentation between C 2 and 3 or 4 and 5 of the hexose backbone. Primary fragment ions of 291 and 295 amu from ([^{2}H]-reduced hexuronic acids) can be derived only by fragmentation of the hexose backbone between C 2 and 3. In either case, these primary fragments lose a 102-amu fragment (from sequential loss of acetate and ketene) to give secondary fragment ions of amu 187 and 191 (hexose-derived) and 189 and 193 (hexuronic acid-derived). The primary fragment ions (231 and 235 amu) used for analysis of the 6-deoxyhexoses (Rha and Fuc) can only be derived from fragmentation of the hexose backbone between C 2 and 3 (not shown). OAc, O-Linked acetyl group.

capillary column (J & W Scientific) interfaced to a mass detector (Hewlett-Packard model 5970) using He as the carrier gas. Alditol acetates were separated using temperature programming from 160 to 210°C at 5°C/min, holding at that temperature for 25 min. Partially methylated alditol acetates were separated using temperature programming from 100°C at 10°C/min to 160°C and then 0.5°C/min to 205°C.

The percentage of each residue or glycosidic linkage that was labeled was determined by GC-MS-SIM analysis of ^{12}C- and ^{13}C-labeled homologous ions. The MS was used in the scan mode (100–350 amu) for identification of peaks in permethylated samples, and in the SIM mode for determination of percentage of label in specific linkages, neutral sugars, and uronic acids. Ion abundance thresholds were set at 100 for SIM runs and 500 for scan runs. Threshold values were kept constant for all runs for which comparisons of ion abundance were used to determine relative incorporation of [^{13}C]Glc precursor. Secondary ions of 187 amu (from the ^{12}C precursor) and 191 amu (from the ^{13}C precursor) from the alditol acetates for Ara, Xyl, Man, Gal, and Glc were used for relative quantitation, and the primary ions of 231 and 235 amu were used for Rha and Fuc (Greve and Labavitch, 1991). In glycosidic linkage analysis partially methylated alditol acetates were monitored based on abundant three- or four-C backbone fragments selected from a full-scan spectrum of the peak. The probability of the selected mass arising through incorporation of naturally occurring ^{13}C rather than from the tracer [^{13}C]Glc is calculated by the following formula:

$$[(m!)/(n!p!)](q)^n(r)^p,$$

where m is the number of C atoms in the fragment (including backbone-, acetyl-, and methyl-carbons), n is the number of ^{13}C atoms, p is the number of ^{12}C atoms, q is 0.011 (natural abundance of ^{13}C), and r is 0.989 (natural abundance of ^{12}C). Thus, for a 1,3-*O*-acetyl-2,4-*O*-methyl fragment derived from a hexose or pentose (pyranose), the ions of 234 (^{12}C) and 238 (^{13}C) amu may be selected, with a probability of 0.0003% that the 238-amu ion has arisen by incorporation of a naturally occurring precursor present in the tissue prior to addition of tracer Glc.

RESULTS AND DISCUSSION

The tomatoes used for disc preparation were halved equatorially after disc excision and inspected for final determination of the stage of maturity. All 36 fruits were rated as either MG 3 (locules filled with jelly, no internal or external red color) or MG 4 (signs of internal red color apparent in the columella region, no external red color) (Grierson and Kader, 1986). Fruits from the same shipment ripened normally in ambient conditions over 7 to 10 d. During the 4-d incubation period disc color did not change significantly, ranging in a* value from −4 to −6 at both times (SDs for treatments were approximately 2).

Neutral Sugar Compositions

There were no significant differences in neutral sugar composition of the ethanol/acetone supernatants within tissue type (inner and outer) among the three treatments. The Gal and Glc contents of the in vivo control were slightly higher (not shown), suggesting that there was little net change in the size of precursor sugar pools over the 4-d incubation period. Glc made up between 93 and 97% of the total, with the inner discs containing 90 to 120% more Glc than the outer discs (on a disc basis). These values include free Glc and Glc released from Suc during the 2 N TFA hydrolysis. ^{13}C must also enter Fru pools, which can be appreciable in cultivated tomatoes (Yelle et al., 1988). However, Fru (including that released from Suc) is largely destroyed (>98% loss, data not shown) by the TFA hydrolysis used in this analysis, with the remainder being converted equally to the alditol acetates for Man and Glc. Of the recovered Glc, 3.3% (outer) and 0.7% (inner) was labeled. We presume that the proportions of ^{13}C in the Fru and Suc pools, which are essentially metabolically interchangeable with Glc, would have been similar to that in Glc. Of the 100 μg of label added per disc, 18.5% (outer) and 8.9% (inner) was recovered in this fraction. Most of the Fru in this fraction would have been lost, so an additional amount of the ^{13}C provided as Glc would have been in this fraction and not accounted for. Based on the data of Yelle et al. (1988) for three tomato cultivars (none of them being cv XMT-22), the amount of label lost when Fru was destroyed would have been similar to that recovered in Glc. Greve and Labavitch (1991) added twice as much label to discs of twice the volume (4 mm thick) and recovered more than 80% of the [^{13}C]Glc in the ethanol supernatant after a 24-h incubation. Added label made up 12.3% (outer) and 6.9% (inner) of the ethanol/acetone-soluble Glc in the tissue at the time of excision, compared with the 4% added by Greve and Labavitch (1991) to cv Castlemart discs.

In the water-soluble fraction, only the Gal content of the in vivo control was significantly higher than in the two in vitro treatments (not shown). Within tissue types, Gal and Glc contents of the inner discs were higher than in the outer discs. In the CDTA/Na_2CO_3-soluble (combined pectic) fraction, the only significant difference between treatments was a higher Gal content in the in vivo control (not shown). Within tissue type, the outer discs had significantly more Rha than inner discs.

In the water-soluble fraction, only the Gal content of the in vivo control (Table I) was significantly higher (about 50%) than in the two in vitro treatments (not shown). Within tissue types, Gal and Glc contents of the inner discs were higher than in the outer. In the CDTA/Na_2CO_3-soluble (combined pectic) fraction, the only significant difference between treatments was a higher Gal (about 35%) content in the in vivo control (Table I). The lower Gal contents of the water- and CDTA/Na_2CO_3-soluble fractions from the in vitro treatments undoubtedly reflect the ripening-related loss of wall galactan reported for all tomatoes studied, including cv Castlemart (Gross and Wallner, 1979; Campbell et al., 1990). Within tissue type, the outer discs had significantly more Rha than the inner discs. There were no significant differences between treatments or tissue types in the neutral sugar compositions of the 4 N KOH extracts. These compositions suggest a complement of hemicellulosic polymers including xyloglucan, glucomannan, and xylan (Table I for in vivo control), consistent with the report of Tong and Gross (1988) for cv Rutgers tomatoes.

^{13}C Label Incorporation into Neutral Sugars

The percentages of ^{13}C label in the neutral sugars of the water-, combined pectic-, and 4 N KOH-soluble fractions are shown in Figure 2A, B and C, respectively. The inner discs exhibited a greater capacity for conversion and incorporation of labeled Glc into the major noncellulosic neutral sugars associated with the tomato cell wall. Despite the fact that the outer discs were, in effect, given almost twice as much label (calculated as a percentage of the endogenous Glc/Suc pool that dilutes the added label), the inner discs consistently had higher levels of incorporation. Differences in dry weight between the tissue types would not affect

Table I. *Neutral sugar compositions of the water-soluble, combined CDTA- and Na_2CO_3-soluble, and 4 N KOH-soluble fractions of cell walls prepared from outer and inner in vivo discs of cv XMT-22 tomato pericarp*

Values presented are μg/mg of cell wall, with the percentage of the total neutral sugars in the extract represented by each sugar presented in parentheses. Data are the means of duplicate analyses.

Sugar	Extract					
	Water-soluble fraction		CDTA-/Na_2CO_3-soluble fraction		4 N KOH-soluble fraction	
	Outer	Inner	Outer	Inner	Outer	Inner
	μg/mg cell wall					
Rha	0.98 (7.8)	0.75 (5.3)	3.62 (9.6)	3.12 (7.0)	0.11 (0.2)	0.25 (0.5)
Fuc	0.02 (0.2)	nd[a]	0.26 (0.3)	0.26 (0.3)	nd	nd
Ara	2.20 (17.6)	2.24 (15.7)	7.21 (19.1)	7.65 (17.1)	2.70 (5.2)	3.32 (6.0)
Xyl	1.52 (12.1)	1.39 (14.2)	1.20 (3.2)	1.29 (2.9)	16.42 (31.4)	17.21 (31.3)
Man	1.01 (8.1)	1.40 (9.8)	0.51 (1.4)	0.45 (1.0)	8.31 (15.9)	7.63 (13.9)
Gal	5.49 (43.8)	6.58 (46.1)	22.33 (59.2)	30.15 (67.6)	4.74 (9.1)	5.66 (10.3)
Glc	1.31 (10.5)	1.92 (13.5)	2.71 (7.2)	1.84 (4.1)	20.01 (38.3)	20.93 (38.1)

[a] nd, Not determined.

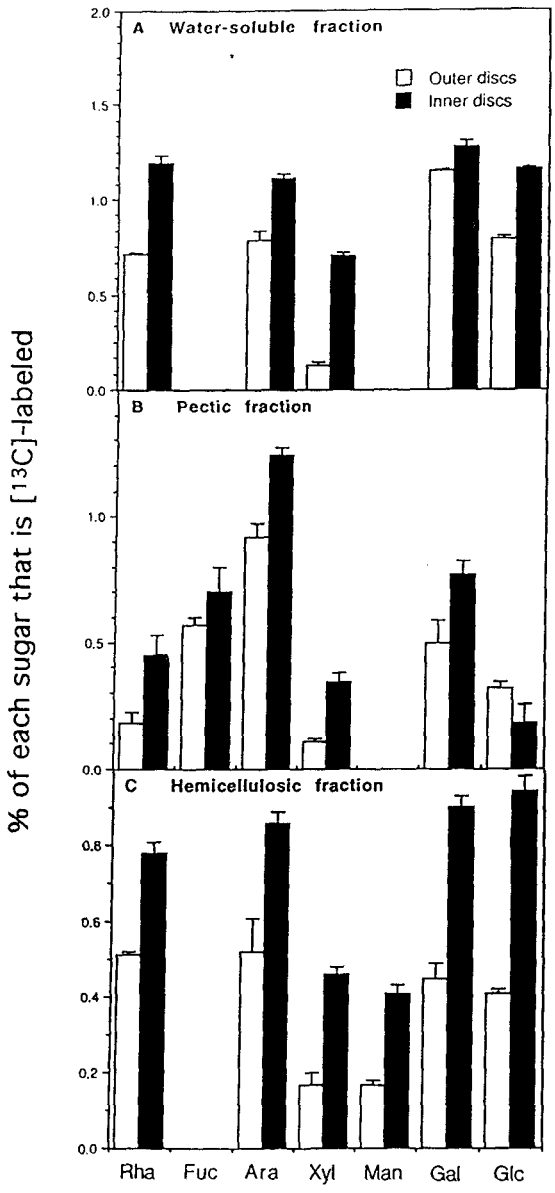

Figure 2. Incorporation of [^{13}C]Glc into the neutral sugars of the (A) water-soluble, (B) combined pectic (CDTA- and Na_2CO_3-soluble), and (C) hemicellulosic (4 N KOH-soluble) polymers prepared from the crude cell walls of inner and outer discs of MG tomato pericarp that had been incubated in vitro for 4 d prior to sampling, the last 2 d in the presence of [^{13}C]Glc. Data indicate the percentage of each sugar that has been labeled during the 2-d treatment with [^{13}C]Glc. SDs are based on analysis of three replicates per treatment per tissue type.

this calculation, as data are expressed as a percentage of each sugar that contains ^{13}C rather than as absolute amounts of labeled sugars. Compared with the results of Greve and Labavitch (1991), levels of incorporation are much higher. This is probably due to the longer incubation period. Furthermore, because we have observed differences in the ripening of discs cut from the same variety of tomato but harvested at different times of the year, this factor, too, could contribute to differences in uptake and incorporation.

Incorporation of precursor into cell wall polymers is relatively substantial at the MG stage of development. The amount of labeled Glc provided to discs represented between 9 and 18% of Glc present in total (ethanol- and acetone-soluble) pools. At the end of the 4-d incubation period (2 d with label), labeled Glc represented between 3.3 and 0.7% of the total Glc in these pools. While this could indicate a selective utilization of the labeled species (from cellular pools destined for use in synthesis or respiration), we think it more likely that hydrolysis of starch (predominantly ^{12}C) has led to the label dilution. We presume, therefore, that the soluble, labeled Glc we have measured represents the distribution of label in pools destined for synthesis of UDP-Glc (and other UDP-sugars) and subsequent incorporation into cell wall polysaccharides. If, therefore, the average penetrance of [^{13}C]Glc into these pools is near 10% (an estimate based on the values at the start and at the end of the incubation period), then the presence of 0.5% ^{13}C in a given sugar residue (as seen for several wall residues, Fig. 2) would indicate that 5% of the polysaccharides containing that residue (on average, since several polymer types may contain a given sugar) had been synthesized in the 2 d during which labeled precursor was provided; 90% of this coming from unlabeled Glc in cellular pools and the remainder from the ^{13}C-tracer. A better estimate could be developed from data for relative ^{13}C levels in the various UDP-sugar pools, but we do not have these data. It is difficult to predict what such a level of incorporation could mean in relation to overall wall structure and integrity.

^{13}C Label Incorporation into Uronides

GalUA accounted for most of the uronic acid in the combined pectic fraction, although the GalUA:GlcUA ratios differed between outer and inner tissues (68 [7.5]:1 versus 44 [2.0]:1, respectively [SDs in brackets]). The GalUA:GlcUA ratio for the total cell wall was less (approximately 18:1 for both outer and inner discs). This may reflect a greater GlcUA content in the hemicelluloses; for example, an acidic xylan (tentatively identified as glucuronoxylan) has been identified in alkaline tomato cell wall extracts (J.M. Labavitch and C.J. Brady, unpublished data). [^{13}C]Glc was incorporated into GlcUA and GalUA, with the specific activity in GlcUA being considerably higher (Table II), probably reflecting differences in initial UDP-sugar pool sizes and the shorter pathway for [^{13}C]Glc incorporation into UDP-GlcUA.

As for incorporation into neutral sugars, a larger percentage of the uronides was labeled in the inner discs than in outer discs. For GalUA the proportion of ^{13}C in inner discs is about three times as great as in the outer, whereas for GlcUA the factor is only 1.1, indicating that the increased incorporation into uronide largely represents pectic polymer synthesis. Greve and Labavitch (1991), using the same methodology, failed to detect label in uronides from ripening cv Castlemart tomato discs, and subsequent work by L.C. Greve (unpublished data) similarly failed to detect label in uronides from discs from actively growing, immature cv Castlemart tomatoes supplied with 400 μg of

Table II. *Incorporation of ^{13}C from [^{13}C]Glc into GalUA and GlcUA in the combined pectic fraction prepared from outer and inner discs of mature green cv XMT-22 tomato pericarp*

Data given indicate the percentages of GalUA and GlcUA in the combined CDTA- and Na_2CO_3-soluble fractions of crude cell walls that were labeled during a 2-d incubation of pericarp discs with [^{13}C]Glc. Quantitation of the percentage of labeling was performed by GC-MS-SIM analysis of ions of 291 and 295 amu in the GC peaks representing galactitol and glucitol hexaacetates after samples were subjected to methanolysis, reduction with NaB^2H_4, hydrolysis with 2 N TFA, reduction with $NaBH_4$, and acetylation. SDs are based on analysis of three replicates per treatment per tissue type.

Uronic Acid	Outer		Inner	
	% ^{13}C	SD	% ^{13}C	SD
GalUA	0.26	0.01	0.75	0.02
GlcUA	1.12	0.05	1.24	0.01

[^{13}C]Glc. Thus, the incorporation of label into uronides and, possibly, into neutral sugars may be dependent on the variety of tomato.

Glycosyl Linkage Composition and Labeling

The glycosidic linkage compositions of the combined pectic and hemicellulosic fractions, together with the percentage incorporation of label into certain linkages, are given in Tables III and IV, respectively. In both fractions, no significant differences were found in the relative abundances of the glycosidic linkages between treatments or tissue types, and, consequently, the results are given as the average over treatments and tissue types.

In the combined pectic fraction (Table III), linkages identified as typical for tomato pectic polysaccharides (based on the report of Pressey [1983] and the thorough analysis of Seymour et al. [1990]) comprised more than 90% of the total identified linkages. The most abundant linkages were 1,5-arabinosyl (furanosyl) residues (23% of the total), 1,4-galactosyl residues (18% of the total), and terminal galactosyl, and 1,2- and/or 1,4-xylosyl residues (not separable under the chromatographic conditions employed [15% of the total]). This indicates the presence of typical pectic arabinan and galactan side chains in these pectin extracts and also that the capacity to synthesize them continues past physiological maturity. It is therefore not surprising that the capacity for synthesis of galacturonan backbones also persists.

Inner discs had a higher degree of incorporation of label into most of the linkages, as would be expected based on the incorporation into the individual neutral sugars of this fraction (Fig. 2B). A possible exception was incorporation into 1,6-galactosyl linkages, in which it appears as if the outer discs had a higher incorporation, although the higher level in outer discs was not large. Among the linkages for a given neutral sugar, degree of labeling varied substan-

Table III. *Glycosidic linkage composition and relative ^{13}C abundance in different glycosidic linkages identified in the combined pectic fraction of cell walls prepared from outer and inner discs of mature green cv XMT-22 tomato pericarp*

Data for relative abundance are based on integration of the GC peaks from temperature-programmed runs of permethylated alditol acetates produced from the combined CDTA- and Na_2CO_3-soluble fractions of crude cell walls that were labeled during a 2-d incubation of pericarp discs with [^{13}C]Glc. The sum of the areas for all peaks identified as permethylated alditol acetates was 100%. Individual peaks were identified by retention time and comparison of their mass spectra with standards. The percentage of each linkage that was labeled was determined by GC-MS-SIM analysis. SDs are based on analysis of three replicates per treatment per tissue type.

Sugar and Linkage	Relative Abundance		Label Incorporation			
			Outer		Inner	
	%	SD	% ^{13}C	SD	% ^{13}C	SD
t-Ara *(f)*	1.5	0.89	0.6	0.09	1.1	0.10
t-Deoxyhexose	0.2	0.11				
t-Ara *(p)*	0.7	0.18				
t-Xyl	1.1	0.25				
1,2-Rha	5.2	1.28	0.2	0.01	0.5	0.10
1,3-Rha	1.5	0.33				
1,5-Ara	22.5	5.59	2.0	0.03	2.6	0.08
t-Gal/1,2-Xyl/1,4-Xyl	15.0	2.99				
1,2,4-Rha	3.0	1.21	1.2	0.21	1.9	0.02
1,3-Gal/1,4-Man	6.2	0.48				
1,4-Gal	18.1	2.72	0.5	0.08	0.8	0.06
1,4-Glc	5.7	0.93				
1,6-Gal	2.3	0.61	1.8	0.03	1.5	0.13
1,3,4-Hexose	1.8	0.21				
1,3,4-Hexose	1.7	0.30				
1,4,6-Gal	1.9	0.62				
1,3,6-Gal	3.4	0.92	2.7	0.15	3.1	0.09
Other	8.2					

Table IV. *Glycosidic linkage composition and relative ^{13}C abundance in different glycosidic linkages identified in the hemicellulosic fraction of cell walls prepared from inner and outer discs of mature green cv XMT-22 tomato pericarp*

The analysis is of the 4 N KOH-soluble fraction of cell walls that had been previously extracted with CDTA and Na_2CO_3 to remove pectic polysaccharides. Details of the analytical protocol are described in the legend for Table III. SDs are based on analysis of three replicates per treatment per tissue type.

Sugar and Linkage	Relative Abundance		Label Incorporation			
			Outer		Inner	
	%	SD	% ^{13}C	SD	% ^{13}C	SD
t-Ara (f)	0.1	0.03	Trace[a]		Trace	
t-Xyl	0.2	0.05	1.2	0.10	2.1	0.03
t-Man	1.4	0.04	0.2	0.17	0.7	0.08
1,5-Ara (f)	0.9	0.34	2.6	0.11	2.6	0.05
t-Gal/1,2-Xyl/1,4-Xyl	17.8	1.20				
1,4-Man	31.8	1.49	0.1	0.00	0.3	0.00
1,2,6-Hexose 1,4-Gal	2.6	0.33				
1,2-Gal	3.9	0.35	0.4	0.11	0.9	0.11
1,4-Glc	24.3	0.69	0.3	0.02	0.6	0.01
1,4,6-Man	10.3	0.28	0.5	0.01	0.8	0.04
1,4,6-Glc	3.5	0.44	0.5	0.03	1.0	0.04
Other	3.2					

[a] Trace, Incorporation of <0.05%.

tially (e.g. incorporation by outer discs into 1,4-, 1,6-, and 1,3,6-galactosyl residues was 0.5%, 1.8%, and 2.7%, respectively). The incorporation into 1,2,4-rhamnosyl residues was 3- to 6-fold greater than into 1,2-rhamnosyl residues, suggesting a preference for synthesis of branched rhamnogalacturonans in MG fruit. Seymour et al. (1990) analyzed the pectins from tomato cv Sonata that had been extracted into four fractions, and their results cannot be used for direct comparison. It does appear, however, that there is broad similarity in the abundances of the major linkages in their work and those reported here.

Based on previous cell wall descriptions of nonfruit tissues (for review, see McNeil et al., 1984) and our own analysis of tomato polymers (L.C. Greve and M. Huysamer, unpublished data), we feel that the [^{13}C]-galactosyl residues identified by GC-MS (Table III) indicate the synthesis of pectic β-1,4-linked galactan and branched arabinogalactan(s). If the incorporation into 1,6- and 1,3,6-linked galactosyl residues described in Table III is indicative of synthesis of arabinogalactan, and if we assume that [^{13}C]Glc represents 10% of soluble precursor pools (estimate based on the relative proportion of [^{13}C]Glc in soluble pools at the beginning and end of the 2-d incubation period, discussed above), then the incorporation of [^{13}C] into 1,6-Gal and 1,3,6-Gal residues (1.8 and 2.7%, respectively; on average, approximately 2%) suggest that approximately 20% of the arabinogalactan measured in the outer pericarp was synthesized during the 2-d treatment with labeled sugar. Arabinogalactan proteins may play important roles in cell-cell recognition and plant differentiation (Kreuger and van Holst, 1996). Therefore, this relatively substantial amount of synthesis could have important implications regarding the control of tissue softening.

The linkages identified in the 4 N KOH-soluble fraction (Table IV) are typical of tomato hemicellulose (based on Tong and Gross, 1988; Seymour et al., 1990) and comprised 96% of the total linkages identified on a relative basis. Most abundant were 1,4-mannosyl residues (32% of the total), 1,4-glucosyl residues (24% of the total), and terminal galactosyl, and 1,2- and 1,4-xylosyl residues (18% of the total). Tong and Gross (1988) proposed that there was continuing synthesis of glucomannan during tomato fruit ripening, based on a relative increase in 1,4- and 1,4,6-mannosyl and 1,4-glucosyl residues in hemicellulose extracts of cell walls from ripe tomatoes, and our data are in agreement with this. Once again, levels of incorporation were substantially higher in inner discs except in the case of 1,5-arabinosyl (furanosyl) residues, in which there was no difference. Xyloglucan presence is indicated by the 1,4- and 1,4,6-glucosyl, 1,2- and terminal xylosyl, and terminal galactosyl residues identified. (Tomato fruit xyloglucan lacks terminal-fucosyl residues [Maclachlan and Brady, 1994].) The relatively low amount of 1,4,6-glucosyl residues measured suggests that most of the 1,4-glucosyl residues measured are in a polymer other than xyloglucan. This may indicate that some cellulosic glucan was solubilized during the base treatment, but is most likely indicative of the glucomannan synthesis discussed above.

It is difficult to quantify the glycosidic linkages on a basis other than by relative abundance, due to inherent problems with undermethylation and differential loss of some residues. Therefore, it is not possible to explain the differences in percentage of labeling of each individual neutral sugar (in Fig. 2, B and C) and its individual linkages (Tables III and IV, respectively). This problem is compounded by the difficulty in separating some of the linkages chromatographically in order to determine levels of incorporation and relative abundances. Nevertheless, evidence has been presented to support previous work (Mitcham et al., 1989; Greve and Labavitch, 1991) that demonstrated the capacity for cell wall synthesis in ripening tomato pericarp, particularly at the MG-breaker transition. Our study has also

shown that the newly synthesized (i.e. labeled) polymeric material has the neutral sugar composition and glycosidic linkage profiles typical of tomato cell wall pectic and hemicellulosic polysaccharides. Our use of pericarp discs raises the concern that the [^{13}C]Glc incorporation we have described is a consequence of the wounding caused by disc excision. We have described two aspects of wound metabolism, an increase in ethylene synthesis (Campbell et al., 1990), and incorporation into callose-like glucan (Greve and Labavitch, 1991) by freshly cut discs from cv Castlemart tomatoes, but these responses are transient, ending within 36 h of disc preparation. We did not provide labeled Glc until 48 h after excision. There was no incorporation of tracer into 1,3-linked glucosyl residues in the cv XMT-22 discs (Tables III and IV). Whether other wound-induced responses continue beyond 48 h is unclear, but discs in culture display many of the physiological and biochemical changes described for ripening, intact tomato fruits, including several aspects of cell wall metabolism (Campbell et al., 1990). Furthermore, the wall synthetic events we have detailed here are consistent with the pattern of incorporation of [^{14}C]Suc provided to intact, ripening tomatoes reported by Mitcham et al. (1991).

The data in Tables III and IV indicate that ^{13}C was not incorporated into some linkage types. When relative abundance is low (e.g. t-deoxyhexose at 0.2% as reported in Table III), instrument sensitivity would make detection of incorporation at the 1.0% level (i.e. relatively high) difficult. For more prevalent linkage types (e.g. the unresolved t-galactosyl/1,2-xylosyl/1,4-xylosyl residues reported in Tables III and IV), the absence of ^{13}C-incorporation must be taken as an indication that polymers containing these linkages are not being synthesized at a signicant level. The fact that other Gal (e.g. 1,4-linked; Table III) and Xyl (e.g. terminal; Table IV) residues are labeled indicates that UDP-Gal and UDP-Xyl pools are penetrated by label.

Our work has also demonstrated a difference in the synthetic capacities of two regions of the outer pericarp, a tissue that has traditionally been treated as homogeneous in its cell wall composition. In a separate report (Huysamer et al., 1997), distinct differences in cell type and cell wall composition for these regions in the tomato cv Jackpot were described. In cv XMT-22, however, compositional differences were not as distinct, and there were no significant differences in the relative abundances of the glycosidic linkages in these regions in the pectic and hemicellulosic fractions. These observations demonstrate that problems may arise if results from one tomato variety are presumed to apply to others. Thus, generalizations about the relationship of ripening-related cell wall metabolism and fruit softening should not be made without supporting data.

The significantly greater cell wall synthetic capacity of the inner tissue as compared with the outer may be even more pronounced than shown in this work. The inner tissue was, in effect, supplied with a lower concentration of label because of its inherently higher free Glc/Suc (and, presumably, Fru) content, and also because of our manipulation of the disc labeling system. Because discs without a cuticle are not viable over 4 d in culture, we were compelled to label a 4-mm-thick disc, which included the cuticle, and discard the outer 2-mm section (containing [^{13}C]Glc that had moved past the inner tissues) at the time of harvest, to obtain a 2-mm-thick labeled disc representing only inner tissue.

^{13}C from the fed precursor has been found in all of the neutral sugars commonly associated with the cell wall (this work; Greve and Labavitch, 1991), and it does not appear as if there is preferential synthesis of new, ripening-associated polysaccharides. We may, in fact, be investigating a homeostatic response of the tissue (Knee and Bartley, 1981), in which the same material already present in the wall is being synthesized in an effort to maintain cell wall structure at a time when digestion of cell wall components is increasing. Not all of the polysaccharides of the MG tomato outer pericarp are being synthesized, however. Redgwell (1996) used ^{14}C-containing precursors (both CO_2 and Glc) to label cell wall components in ripening kiwifruit and also concluded that incorporation did not show preference for a particular polymer type. This does not rule out the possibility that some of the newly synthesized polymers (e.g. branched rhamnogalacturonans or arabinogalactans) may play a direct role in the onset and/or progress of ripening-associated softening.

In this regard, it may prove valuable to conduct similar studies on the ripening mutants *rin*, *nor*, and *Nr*, which soften at much slower rates (Mitcham et al., 1991), and studies utilizing fruits from all developmental stages. However, based on the fact that the outer pericarp includes at least two tissue areas containing distinct cell types (Wilson and Sterling, 1976; Ho and Hewitt, 1986) with different cell wall compositions and biosynthetic capacities (Huysamer et al., 1997), greater selectivity of the tissue to be studied needs to be made to avoid problems of cell wall heterogeneity. In this way it may be possible to determine changes in the patterns of synthesis, which might allow identification of polymers with synthesis restricted to the period of tissue softening. Variety-dependent differences must be distinguished in all cases.

Future work should include time-course analyses of the movement of density label through the activated sugar pools to determine pathways of interconversion of the sugars starting from the Glc precursor. Quantifying the endogenous levels of NDP-sugars would also aid in the interpretation of the varying degrees of labeling of each neutral sugar, as this directly affects the amount of label incorporated into individual polymers and is therefore just as likely to influence the extent of label incorporation as is the inherent synthetic capacity (i.e. polymer synthase activity) of the tissue.

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