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Tomato Fruit Cell Wall Synthesis during Development and Senescence[1]

In Vivo Radiolabeling of Wall Fractions Using [^{14}C]Sucrose

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ABSTRACT

The pedicel of tomato fruit (*Lycopersicon esculentum* Mill., cv 'Rutgers') of different developmental stages from immature-green (IG) to red was injected on the vine with 7 microcuries [^{14}C(U)]sucrose and harvested after 18 hours. Cell walls were isolated from outer pericarp and further fractionated yielding ionically associated pectin, covalently bound pectin, hemicellulosic fraction I, hemicellulosic fraction II, and cellulosic fraction II. The dry weight of the total cell wall and of each cell wall fraction per gram fresh weight of pericarp tissue decreased after the mature-green (MG) stage of development. Incorporation of radiolabeled sugars into each fraction decreased from the IG to MG3 (locules jellied but still green) stage. Incorporation in all fractions increased from MG3 to breaker and turning (T) and then decreased from T to red. Data indicate that cell wall synthesis continues throughout ripening and increases transiently from MG4 (locules jellied and yellow to pink in color) to T, corresponding to the peak in respiration and ethylene synthesis during the climacteric. Synthesis continued at a time when total cell wall fraction dry weight decreased indicating the occurrence of cell wall turnover. Synthesis and insertion of a modified polymer with removal of other polymers may produce a less rigid cell wall and allow softening of the tissue integrity during ripening.

The fruit cell wall has generally been viewed as a static structure which is enzymically degraded during ripening leading to fruit softening. Much emphasis in studies of softening has been placed on the activity of endo-D-galacturonase (EC 3.2.1.15, PG[2]), an enzyme which cleaves α1,4-galacturonosyl linkages in pectin (2–4, 16). Expression of the PG gene increases at the time of ripening in several fruits (2, 16, 22). However, evidence suggests that PG activity is not solely responsible for tissue softening. Strawberries, which contain no detectable PG activity, soften extensively (11) and apples, which contain no endo-PG, soften and show increases in soluble polyuronide during ripening (1) suggesting that softening involves other mechanisms. Using tomato fruit, Gross and Wallner (5) and Gross (6) showed that a net loss of galactosyl residues from the pectic portion of tomato fruit cell wall occurs during ripening. This loss does not result from PG activity (5–7) and strongly suggests that other enzymes or mechanisms, in addition to PG, are involved in cell wall modifications that occur during ripening of tomato fruit.

Little research has addressed the synthesis and turnover of cell wall polysaccharides during fruit development and ripening and their potential role in cell wall modifications that lead to softening (14). Cell expansion ceases at the MG stage of tomato fruit development (9). It has generally been assumed that cell wall synthesis also ceases at this time. However, the radiolabeling studies necessary to measure cell wall synthesis have not been conducted. The objective of this study was to measure synthesis of tomato fruit cell wall components at various stages of development from IG to R using a [^{14}C] sucrose pedicel injection technique developed to radiolabel fruit cell walls *in vivo*.

MATERIALS AND METHODS

Plant Material

Tomato plants (*Lycopersicon esculentum* Mill., cv 'Rutgers') were grown in a greenhouse under natural lighting using standard cultural practices. Flowers were pollinated and tagged at anthesis and fruit were selected at a rapidly growing, IG stage for use in development of the radiolabeling technique.

In Vivo Radiolabeling

To optimize cell wall radiolabeling, 5 μL ddH_2O containing 1 μCi [^{14}C(U)]sucrose (NEN Research Products[3], 671 mCi/mmol) was injected with a microsyringe into the pedicel of the tomato fruit (1–2 cm above calyx) and the puncture was

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[2] Abbreviations: PG, polygalacturonase; CDTA, 1,2-cyclohexanediaminotetraacetic acid; ddH_2O, distilled-deionized water; DPA, days post-anthesis; IG, immature green; MG, mature green; B, breaker; T, turning; P, pink; R, red; HF, hemicellulosic fraction; CF, cellulosic fraction.

[3] Use of a company or product name by the U.S. Department of Agriculture does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

sealed with Parafilm. Fruit were harvested at various times (0–54 h) after injection and the incubation time which yielded the most highly labeled cell walls was determined to be 18 h (data not shown). For consistency, all fruit were injected at 1400 h with 5 μL ddH_2O containing 7 μCi [^{14}C]sucrose and harvested at 0800 h on the following day.

To determine the efficiency and uniformity of fruit labeling, the pedicels of two IG fruit, 26 DPA, were injected with 7 μCi [^{14}C]sucrose and harvested after 18 h, as described above. The fruit were divided evenly at the equator and each half was quartered, yielding eight sections. The outer pericarp was separated from the inner tissues (locule, columella, and radial pericarp). The other pericarp and inner tissues of each section as well as the pedicel (~8 cm) and calyx were weighed, diced, homogenized in 80% ethanol using a Polytron homogenizer (Brinkmann Instruments), and filtered through Miracloth (Calbiochem). Total radioactivity in the ethanol extract was then determined. Ethanol-soluble radioactivity per mg tissue was determined and used to calculate the percent of total fruit ethanol-soluble radioactivity for each anatomical sample.

Selection of Developmental Stages

Fruit were selected at various stages of maturity for radiolabeling. Selection of IG fruits was based on DPA. Selection of MG, B, T, P, and R fruits was based on visual appearance. Internal appearance at harvest was used as the criterion to determine the stage at injection for MG fruits: MG1, locules not jellied, seeds cut (32 DPA); MG2, locules slightly jellied, few seeds cut (34 DPA); MG3, locule jellied but still green, no seeds cut (36 DPA); MG4, locule jellied and yellow to pink in color, no color in columella (38 DPA). DPA were used to assist in determining internal development of MG fruit before injection of [^{14}C]sucrose; however, internal appearance was the final determining factor. External appearance at injection determined the stage for B, T, P, and R fruits: B, pink color on <10% of blossom end of fruit and in center of columella (40 DPA); T, pink color on 10 to 30% of blossom end (41 DPA); P, pink color on 30 to 60% of fruit surface (43 DPA); R, red color on >90% of fruit surface (46 DPA).

Three fruit were selected at each of 10 stages: IG18 (18 DPA), IG25 (25 DPA), MG1, MG2, MG3, MG4, B, T, P, and R. In other experiments, three fruit were selected at each of seven stages: IG (21–23 DPA), MG1-2 (either MG1 and/or MG2; as above), MG3–4 (either MG3 and/or MG4), B, T, P, and R.

Cell Wall Extraction

Fruits were harvested and weighed. The outer pericarp was excised, weighed, and frozen in two volumes of 80% ethanol at −70°C. For cell wall extraction, pericarp tissue was thawed and macerated in 80% ethanol with a mortar and pestle. The outer skin was removed and the remaining tissue homogenized in 80% ethanol using a Polytron homogenizer and filtered through Miracloth. The 80% ethanol filtrate was retained for determination of radioactivity. The residue was thoroughly rinsed with 3 volumes of 20 mM Hepes-NaOH (pH 6.9), stirred in 2 volumes of phenol:acetic acid:H_2O (2:1:1, v/v/v) for 20 min to inactivate endogenous wall-associated enzymes (12, 17), filtered through Miracloth and transferred to a sintered-glass filter. The residue was then resuspended and washed with 3 volumes of chloroform:methanol (1:1, v/v) followed by 3 volumes of acetone. Cell wall material was dried 3 d or more over P_2O_5 *in vacuo* at 37°C. Total pericarp cell wall dry weight was determined for each fruit.

Cell Wall Fractionation

The dried cell walls from three fruit at each stage were combined for fractionation, as previously described (6). Briefly, walls were incubated in 50 mM Na-acetate (pH 6.5) (100 mL/g cell wall), containing 50 mM CDTA, at 25°C for 6 h with constant stirring. The suspension was filtered through a sintered glass filter. The filtrate was dialyzed for 72 h at 4°C against ddH_2O, and lyophilized (ionically associated pectin). The residue was suspended in 50 mM Na_2CO_3 (100 mL/g cell wall), containing 2 mM CDTA, and incubated with constant stirring for 20 h at 4°C, then for 1 h at 25°C. The suspension was filtered and the filtrate brought to pH 6.5 using gradual addition of glacial acetic acid (temperature kept <35°C). Samples were then dialyzed as described above and lyophilized (covalently bound pectin). The residue was suspended in 4 N KOH (100 mL/g cell wall), containing 100 mM $NaBH_4$, and incubated with constant stirring for 1 h at 25°C. The suspension was filtered and the filtrate brought to pH 6.5, dialyzed, and lyophilized as described above (HFI). The residue was rinsed with ddH_2O and lyophilized (CFI). CFI was then suspended in 8 N KOH (100 mL/g cell wall), containing 100 mM $NaBH_4$, and incubated with constant stirring for 3 h at 25°C. The suspension was filtered and the filtrate adjusted to pH 6.5, dialyzed as described above, and lyophilized (HFII). The residue was rinsed with ddH_2O and lyophilized (CFII). Total dry weight of each fraction was determined after being completely dried *in vacuo* over P_2O_5.

Scintillation Counting

Two 20 mg aliquots of cell wall material and 1 to 8 mg of each cell wall fraction were counted. Samples were placed in 15 mL of scintillation cocktail (Aquasol-2, NEN Research Products) and counted using a scintillation counter (Beckman LS6800) with quench correction. Unlabeled cell walls were counted as a control. Using total dry weight, total radioactivity was determined.

RESULTS

As determined by total ethanol-soluble dpm, 20% of the total fruit radioactivity was detected in the outer pericarp tissue (Table I). Sixty-two percent of the total outer pericarp radioactivity was detected in the top half of the fruit and the amount of radioactivity in each quarter of pericarp tissue varied as much as 2-fold. Thirty-one percent of the total fruit radioactivity was detected in the inner tissues (locule, columella, and radial pericarp). The pedicel and calyx retained 48% of the detected radioactivity.

The dry weight (mg) of cell wall material per gram fresh

Table I. *Distribution of Ethanol-Soluble Radioactivity after Injection*

Two fruit (26 DPA) were labeled with 7 μCi [^{14}C]sucrose, harvested after 18 h and sectioned into top and bottom, and quartered. Total ethanol-soluble radioactivity was determined in each tissue section after homogenization using a Polytron. Data represent percent of total ethanol-soluble radioactivity. Inner tissue = locule, columella and radial pericarp.

Tissue	Quarter				Total
	1	2	3	4	
					%
Pericarp					
Top	2.2	3.6	4.7	2.2	12.7
Bottom	1.2	2.2	3.2	1.3	7.9
Inner tissue					31.2
Calyx					8.6
Pedicel					39.6
					100.0

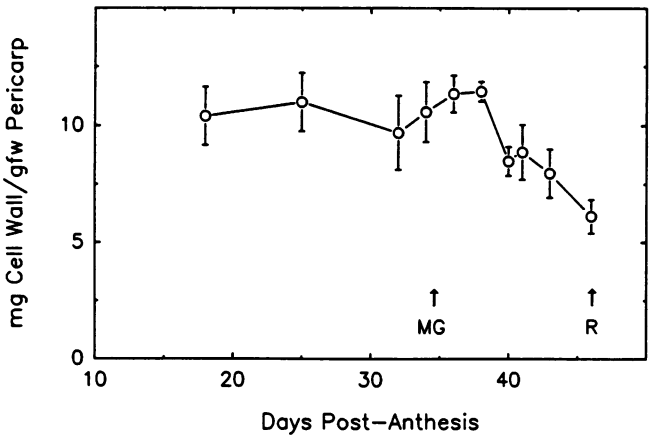

Figure 1. Mean cell wall dry weights (mg) per gram fresh weight (gfw) outer pericarp tissue. Cell walls were extracted from the outer pericarp of two to eight fruit per developmental stage. Vertical bars = SD. See text for other developmental stages which correspond to DPA.

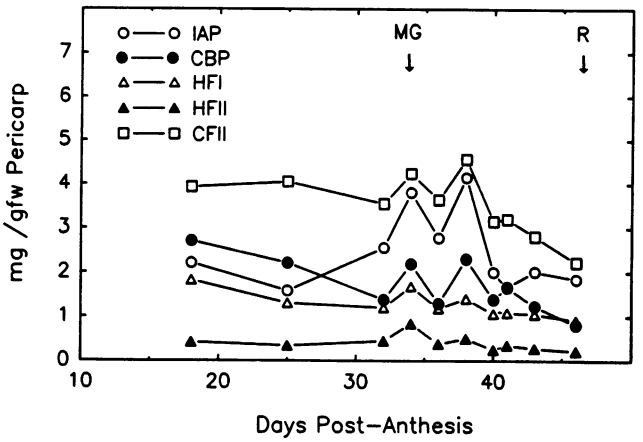

Figure 2. Dry weights of each fraction per gram fresh weight (gfw) outer pericarp tissue. Cell walls from three fruit at each developmental stage were combined and fractionated. IAP = ionically associated pectin, CBP = covalently bound pectin.

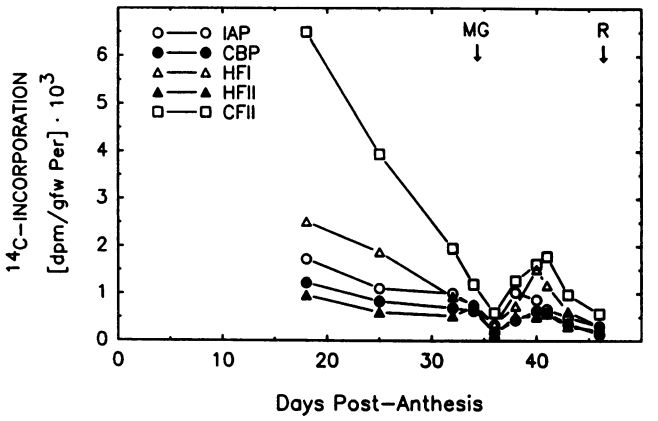

Figure 3. ^{14}C Incorporation into cell wall fractions. Cell walls from three fruit at each developmental stage were combined and fractionated. The total radioactivity in each fraction is divided by the gram fresh weight (gfw) of outer pericarp tissue from which the fraction was extracted. Per = pericarp; IAP = ionically associated pectin; CBP = covalently bound pectin.

weight of pericarp tissue decreased significantly from MG4 to B stage and continued to decrease to the R stage (Fig. 1). The dry matter content, as a percent of fruit fresh weight does not change after 20 DPA (8). The dry weight of cellulosic fraction II per gram fresh weight of pericarp, which comprised 36% of the cell wall dry weight, followed a pattern similar to the total cell wall dry weight showing a 51% decrease beginning after the MG4 stage (Fig. 2). The dry weight for the remaining cell wall fractions also decreased after the MG4 stage. The variability in dry weight per gram fresh weight pericarp increased during the MG stages (Fig. 2).

The degree of total outer pericarp radiolabeling (soluble and insoluble components) varied from fruit to fruit within and between stages. This may be due to factors which affect the amount of ^{14}C entering the pericarp tissue, *i.e.* exact location of injection within the pedicel, and the amount of [^{14}C]sucrose which moves into the fruit and into the pericarp tissue. The total radioactivity in each cell wall fraction estimates the relative net synthesis of the fraction and is adjusted for differences in the quantity of pericarp tissue labeled (Fig. 3). The percent fresh weight of the pericarp tissue did not change significantly during development and ripening (data not shown).

^{14}C Incorporation into all fractions decreased from IG18 to MG3, increased transiently beginning at MG4 to the B or T stage, and decreased to R (Fig. 3). Although relative synthesis decreased at the R stage, incorporation of ^{14}C into the cell wall continued at a significant rate. Total fraction radioactivity at R stage were 39,500 (ionically associated pectin), 18,000 (covalently bound pectin), 40,000 (HFI), 23,000 (HFII), and 64,000 dpm (CFII).

DISCUSSION

Injection of [^{14}C]sucrose into the pedicel of tomato fruit resulted in the distribution of radioactive metabolites throughout the fruit (Table I). The inner tissues (locule, columella, and radial pericarp) contained a similar amount of radioactivity per gram fresh weight as the outer pericarp tissue. There

was sufficient uniformity in the distribution of ^{14}C in the different sections of outer pericarp to justify use of the entire pericarp tissue in cell wall analysis.

During fruit growth, cell wall synthesis accompanies cell expansion (18). In tomato fruit, cell expansion ceases at the MG stage; therefore, the total amount of cell wall material probably does not increase as no secondary thickening occurs. The amount of cell wall material per gram fresh weight pericarp tissue decreased after the MG stages (Fig. 1). Evidence indicates that the cell wall may undergo turnover, *i.e.* degradation of cell wall polysaccharides coincident with synthesis and insertion of replacement polymers (13–15). Turnover may continue after expansion has ceased, allowing for modification of the cell wall and possibly the release of biologically active fragments which may trigger developmental changes such as ripening (19, 20).

Several observations in the present study indicate that turnover of cell wall polymers occurs. First, synthesis occurred after cell expansion had ceased and while the net amount of cell wall material decreased. There was an increase in synthesis between MG3 and T while there was a decrease in the dry weight of each cell wall fraction. An indication of synthesis while total material dry weight was decreasing suggests that turnover occurs with degradation exceeding synthesis resulting in a net decrease in total dry weight.

All cell wall fractions were tested for starch using iodine staining. Only CFII contained residual starch. Starch was detected in CFII samples from IG to MG4. No starch was detected after the MG4 stage. The presence of potentially radiolabeled starch at the IG stages may account for a portion of the dpm in CFII at these early stages. Because starch synthesis in tomatoes occurs early in development, any starch present in cell wall preparations from fruit labeled after the IG stages would not be radiolabeled. Starch would not contribute to the dpm detected in cell wall fractions at these stages.

HFI and HFII had relatively high specific activities. Although they comprised a small percentage of cell wall dry weight (HFI 10–15%; HFII 3–5%), they contained a significant amount of radioactivity (HFI 17–22%; HFII 10–26%). Huber (10) reported a shift to a lower average mol wt of tomato hemicelluloses between the MG and T stages. Using glycosyl linkage analysis, Tong and Gross (21) have shown that this mol wt shift involves *de novo* synthesis of polymers or sidechains containing mannosyl and glucosyl residues.

Incorporation of ^{14}C into cell wall fractions demonstrates that synthesis of each fraction continued through the R stage of ripening although the rate of synthesis was low at MG3 and R. It is unknown why cell wall synthesis decreases at the MG3 stage. Perhaps this represents a transition period between development and ripening. It had previously been suggested that the net loss of galactosyl residues during tomato fruit ripening may involve a loss of *de novo* synthesis (15). Our current data clearly show that cell wall synthesis continues at a relatively high rate throughout ripening (Fig. 3). The net loss of galactosyl residues may result from a specific decrease in synthesis of galactose-containing cell wall polymers.

The radioactivity detected in a fraction after harvest of the fruit is equal to the amount incorporated into the fraction minus any ^{14}C lost by turnover of cell wall polymers. The extent of turnover or changes in extractability during the 18 h incubation in each fraction or in polymers within each fraction is unknown. Experiments involving changes in specific radioactivity of the monosaccharide components of each fraction are in progress to address this question. It is important to study synthesis and turnover of individual polymers to understand ripening-related cell wall metabolism. The composition and structure of the various polymers synthesized in each fraction, at each stage of development, must be determined to ascertain if a modified type of polymer is synthesized during ripening. Either *de novo* synthesis of a modified type of cell wall polymer(s) or modification of an existing cell wall polymer(s) may contribute to softening of the tissue during ripening as opposed to a general degradation of existing polymers.

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