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Ca^{2+} in Cell Walls of Ripening Tomato and Peach

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Additional index words. mesocarp, pericarp, placenta, pectinmethylesterase, *Lycopersicon esculentum, Prunus persica*

Abstract. **Ca^{2+} content in cell wall–middle lamella (CW–ML) areas of outer and inner pericarp, placenta, and gel parenchyma of ripening tomato (*Lycopersicon esculentum* Mill. cvs. Celebrity and Jumbo) and mesocarp of ripening peach [*Prunus persica* (L.) Batsch cv. Georgia Belle] was determined by energy dispersive (EDS) X-ray microanalysis. Ca^{2+} increased from 1.50 to 6.95 $mg \cdot g^{-1}$ dry weight in CW–ML of outer pericarp and 0.98 to 2.60 $mg \cdot g^{-1}$ dry weight in CW–ML of inner pericarp during ripening. Ca^{2+} content remained constant in tomato placenta and peach mesocarp, and was undetectable in tomato gel parenchyma throughout ripening. CW–ML of peach mesocarp had lower Ca^{2+} content than tomato pericarp and placenta at all ripening stages, but total peach uronic acid content was 2.5 times greater. Pectin methylesterase (PME) activity increased in tomato pericarp as fruit ripened, but remained low and unchanged in placenta and gel parenchyma. PME treatment of pericarp increased amounts of CW–ML Ca^{2+} in the breaker stage but not in the green mature stage. The results indicate that increased amounts of Ca^{2+} are bound to CW–ML of tomato pericarp as ripening occurs but not in placenta or peach mesocarp. Pectin deesterification and wall softening during ripening may in part be factors that control the presence and amount of CW–ML Ca^{2+}.**

The importance of Ca^{2+} in the regulation of fruit ripening has been realized for some time. Raising the Ca^{2+} content of many climacteric fruit tissues retards ripening as measured by inhibition of color change, softening, and CO_2 and C_2H_4 production (8). Indeed, many postharvest practices include preharvest Ca^{2+} sprays to prevent senescent disorders of fruit. Most of the Ca^{2+} introduced into fruit tissues apparently accumulates in the cell wall–middle lamella (CW–ML) region, and it is here that Ca^{2+} is thought to have its antisenescent effects (7, 8).

Because Ca^{2+} affects fruit ripening so dramatically, the movement of Ca^{2+} in the CW–ML has been implicated as a factor that may control ripening. Rigney and Wills (21) measured CW–ML Ca^{2+} content in ripening tomato pericarp with an electron microprobe and determined that Ca^{2+} was solubilized during early ripening stages. Decrease of CW–ML Ca^{2+} content was hypothesized as an initiating event prerequisite to C_2H_4 production and polygalacturonase action. As a result of the loss of bound CW–ML Ca^{2+}, soluble or cytoplasmic Ca^{2+} was thought to increase and affect metabolism, which leads to senscence in fruit tissues. Previous to this report, Suwaan and Poovaiah (14, 25) found a shift in Ca^{2+} from bound to soluble forms during ripening, which did not occur in the ripening mutant *rin*.

The release of Ca^{2+} from the CW–ML area recently has been disputed. Brady et al. (4) failed to measure differences in CW–ML Ca^{2+} content between mature green tomatoes and those fruit up to 12 days after the increase of color. Similarly, CW–ML Ca^{2+} content remained constant in apple during ripening (11).

The discrepancy noted may reflect differences in tissue preparation methods and subsequent Ca^{2+} determination. Cell walls are heterogeneous in nature, and distribution of ions depends on the charge density and configuration of the pectic fraction. Furthermore, cell walls behave as effective cation exchangers (13). Because of this, care must be taken to prevent ion movement during tissue preparation for accurate CW–ML Ca^{2+} determinations. Most studies have included conventional solvent extractions, which may result in erroneous CW–ML Ca^{2+} measurements. In this report, Ca^{2+} was determined in ethanol–cryofractured CW–ML areas of ripening tomato and peach by the use of EDS X–ray microanalysis in the scanning electron microscope. In addition, Ca^{2+} content of tomato CW–ML re-

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gions are discussed in relation to pectin methylesterase (PME) (E.C. 3.1.1.11) activity and uronic acid content.

Materials and Methods

Fruit from field-grown 'Celebrity' tomatoes were picked at four ripening stages based on color. Alternatively, fruit ('Jumbo') were purchased from a local greenhouse operation. Freestone peaches ('Georgia Belle') were obtained from a local orchard. Outer and inner pericarp, placenta, and gel parenchyma sections of tomato and mesocarp sections of peach were removed from the equatorial region of the fresh fruit and prepared for scanning electron microscopy (SEM) and X-ray microanalysis. Both tomato (pericarp and placenta) and peach (mesocarp) tissues were used for pectin analysis, but only tomato tissues for the determination of PME activity.

Tissue sections (1 mm^3) were fixed at 4°C for 2 hr in 2% glutaraldehyde and 100 mM Na^+ cacodylate buffer, pH 7.2. For PME incubations, outer pericarp sections were removed and placed in 50 mM 4-morpholinepropanesulfonic acid (Mops), pH 7.0, and incubated for 1 to 2 hr at 25° in purified PME extract obtained from ripe tomatoes (17). Control tissues were treated with enzyme boiled for 6 min. These sections then were fixed as described. Tissues were postfixed in 1% aqueous OsO_4 for 1 hr at 4°. After dehydrating in ethanol, tissue blocks were frozen quickly in 100% ethanol at liquid-N_2 temperatures and fractured. Tissue pieces were collected, critical-point-dried, and stored in vacuo until use.

Immediately before use, tissues were mounted with carbon adhesive paint to carbon stubs and carbon coated. Tissue blocks then were placed in a Phillips 505T SEM equipped with a Tracor-Northern 5500 EDS X-ray detection and analysis system for viewing and X-ray microanalysis. Spectra were collected over a period of 200 sec with an accelerating voltage of 15 keV. Crystalline CaF_2 was used as a Ca^{2+} standard.

Only CW–ML areas that fractured clearly were used for X-ray microanalysis. In general, CW–ML areas at the junction of three adjacent parenchyma cells were used, assuring that sufficient CW–ML material was present for sampling. Electron beam–specimen interaction was assumed to be $\approx 1\ \mu m^3$.

For pectin determination, tissues were cut into boiling 95% ethanol (10 g fresh weight tissue, 45 ml 95% ethanol), refluxed for 5 min., homogenized, and then centrifuged at 10,000 rpm for 15 min. The alcohol-insoluble substances were extracted in cold H_2O, 0.5% ammonium oxalate, and 0.05 N NaOH (5, 22). The uronic acid content of each pectic fraction was determined by the method of Blumenkrantz and Asboe-Hanson (2) and expressed on a fresh-weight basis.

PME activity was determined in extracts of tomato fruit tissue types. Tissues were homogenized in 1.0 M NaCl (1:1) and then adjusted to pH 6.0. The homogenates were stirred for 20 min at 25°C. After centrifugation at 10,000 rpm for 15 min, the supernatant was collected, adjusted to pH 7.0, and assayed for PME activity (16). Results were calculated as microequivalents of H^+ released per gram fresh weight per min.

Results

The Ca^{2+} content in CW–ML regions of tomato tissue types and peach mesocarp is shown in Fig. 1. The CW–ML regions of green mature (GM) and breaker (BR) tomato outer pericarp ('Celebrity') contained between 1.50 and 1.55 mg Ca^{2+}/g dry weight CW–ML, levels that were not significantly different. As ripening proceeded from BR to RE, Ca^{2+} rose to 6.95 $mg \cdot g^{-1}$ dry weight CW–ML in red (RE) pericarp. Similar rises in CW–ML Ca^{2+} content in 'Jumbo' were observed (data not shown). A rise in Ca^{2+} occurred in CW–ML regions of inner pericarp from the pink (PI) to RE stage. In contrast, CW–ML Ca^{2+} content was low and remained constant in placenta during ripening. Peach mesocarp CW–ML content of Ca^{2+} was very low and remained constant during ripening as well. Ca^{2+} levels in CW–ML areas of clearly fractured gel parenchyma were undetectable throughout ripening (data not shown).

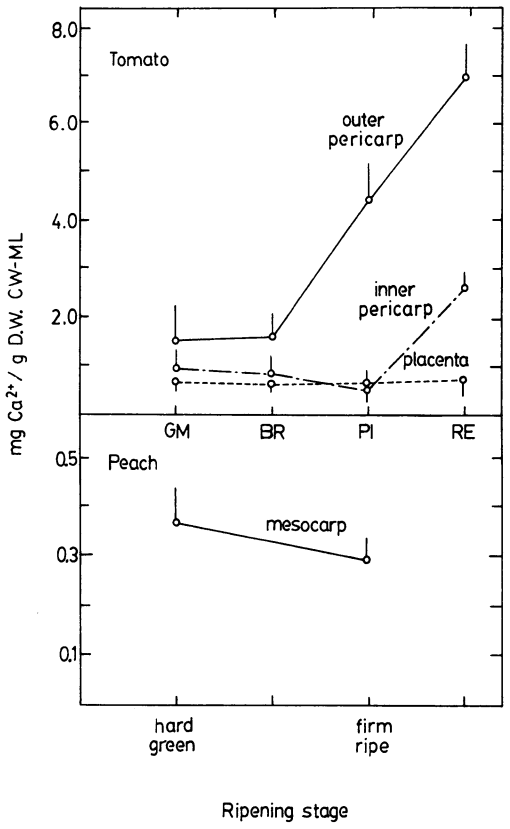

Fig. 1. CW–ML Ca^{2+} content of 'Celebrity' tomato fruit at four ripening stages and 'Georgia Belle' peach at two ripening stages. Data presented are the means ± SE.

During ripening, total uronic acid content increased slightly in tomato outer pericarp or decreased slightly in inner pericarp and placenta (Table 1). Tomato had only 40% of the quantity of total uronides found in peach, and differences existed between pectic fractions in the two fruits. The H_2O-soluble pectic component was 86% of the total uronides present in ripe peach, whereas 40% of the total uronides were H_2O-soluble in RE tomato pericarp. Furthermore, chelate-soluble pectin was 2.5% of the total in ripe peach and 23–27% in ripe tomato pericarp. Placenta H_2O-soluble uronides remained constant and chelate-soluble uronides rose only slightly during ripening.

PME activity in tomato pericarp increased during ripening (Fig. 2). The placenta had low PME activities, which remained constant throughout ripening. Gel parenchyma had very low PME activities (<0.5 μeq H^+ released/g fresh weight per min), which did not change as senescence proceeded (data not shown).

The activity of PME increased only slightly as ripening progressed in tomato pericarp, yet a large increase in CW–ML Ca^{2+} was seen at later ripening stages. Thus, the amount or activity of the enzyme could be altered at early ripening stages, or steric hindrance could prevent interaction of substrate and

Table 1. Uronic acid content of tomato and peach fruit tissue during ripening. Values presented are the means ± SE.

Tissue type	Uronic acids ($mg \cdot g^{-1}$ fresh wt) H_2O-soluble	Chelate-soluble	NaOH-soluble	Total
		Tomato		
Outer Pericarp				
GM	0.26 ± 0.03[z]	0.23 ± 0.03	0.97 ± 0.10	1.46 ± 0.11
BR	0.29 ± 0.02	0.34 ± 0.02	0.87 ± 0.08	1.50 ± 0.10
PI	0.64 ± 0.02	0.42 ± 0.04	0.67 ± 0.08	1.73 ± 0.08
RE	0.72 ± 1.10	0.45 ± 0.02	0.53 ± 0.12	1.70 ± 0.12
Inner Pericarp				
GM	0.30 ± 0.02	0.09 ± 0.01	1.32 ± 0.05	1.71 ± 0.14
BR	0.35 ± 0.05	0.25 ± 0.03	1.00 ± 0.10	1.60 ± 0.09
PI	0.47 ± 0.02	0.22 ± 0.01	0.81 ± 0.10	1.50 ± 0.10
RE	0.46 ± 0.03	0.26 ± 0.01	0.41 ± 0.05	1.13 ± 0.11
Placenta				
GM	0.30 ± 0.02	0.10 ± 0.01	1.05 ± 0.11	1.48 ± 0.17
BR	0.24 ± 0.01	0.15 ± 0.03	1.00 ± 0.08	1.39 ± 0.10
PI	0.31 ± 0.01	0.20 ± 0.02	0.72 ± 0.12	1.23 ± 0.12
RE	0.27 ± 0.02	0.19 ± 0.04	0.77 ± 0.09	1.23 ± 0.09
		Peach		
Mesocarp				
Hard green	1.03 ± 0.11	0.01 ± 0.001	3.39 ± 0.11	4.52 ± 0.13
Firm ripe	3.48 ± 0.09	0.10 ± 0.01	0.48 ± 0.06	4.06 ± 0.10

[z] ± SE.

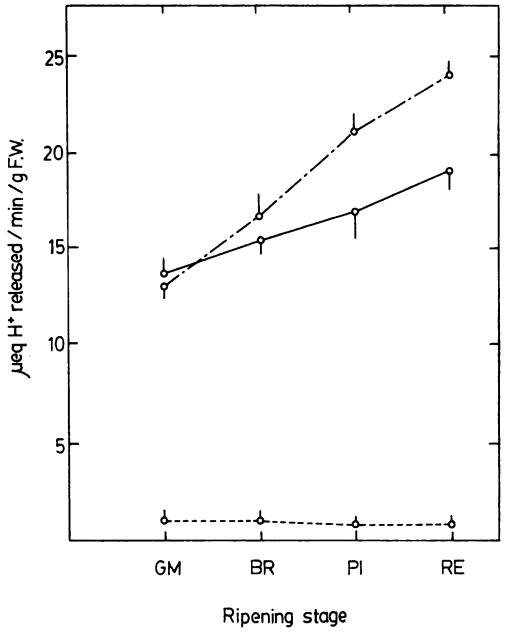

Fig. 2. PME activity in extracts of outer pericarp (————), inner pericarp (— — —), and placental (-----) tissues of tomato fruit at four ripening stages. Data presented are the means ± SE.

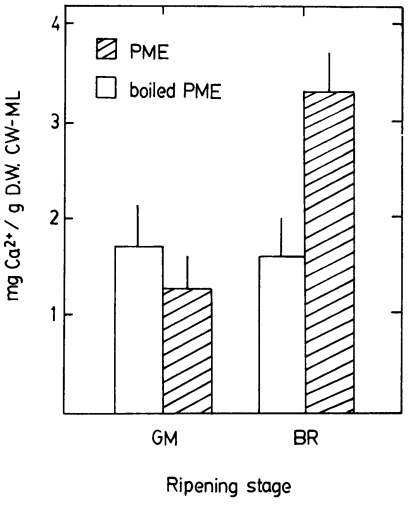

Fig. 3. Ca^{2+} content in cell walls of 'Jumbo' tomato pericarp after PME treatment. Data presented are the means ± SE.

enzyme or movement of Ca^{2+} into CW–ML regions. To determine the effect of PME, we added it to tissue pieces and then measured CW–ML Ca^{2+} content. Treatment of GM outer pericarp with exogenous PME from ripe fruit did not affect Ca^{2+} content; however, treatment of BR pericarp with the enzyme was effective in enhancing the amount of CW–ML Ca^{2+} (Fig. 3).

Discussion

We have shown that the different tissue types of tomato undergo changes in CW–ML Ca^{2+} content during ripening that are distinct. Pericarp CW–ML Ca^{2+} content increased as ripening ensued, whereas placenta CW–ML Ca^{2+} content remained unchanged. Evidence has accumulated that suggests that cell wall components are unevenly distributed throughout the wall (24). Middle lamella pectic areas are highly esterified with relatively few side chains, whereas primary cell wall pectic areas are thought to contain an abundance of side chains along the rhamnogalacturonan backbone. The degree of esterification is also reduced in the primary cell wall pectic regions. Thus, al-

though CW–ML areas chosen for analysis were consistently regions between adjacent parenchyma cells, the areas of analysis were probably rich in middle lamella. Because deesterification occurs during tomato fruit ripening, Ca^{2+} accumulate would increase in the middle lamella. Therefore, the data may not adequately represent the overall change in CW–ML Ca^{2+} levels.

After tissue fixation at 4°C, the tissue was cryofractured in 100% ethanol at liquid N_2 temperatures to expose cell wall surfaces not in contact with the external ionic milieu. The newly fractured CW–ML surfaces were analyzed for the presence of Ca^{2+}. CW–ML surfaces therefore had little or no opportunity of gaining ionic contaminants from the external medium. The question remains whether ionic redistribution occurs within tissues not cut and exposed to the external medium during fixation at low temperatures. With this in mind, CW–ML areas prepared in this manner exhibited an increase in Ca^{2+} as ripening proceeded from GM to RE in tomato pericarp. This is in contrast to work that has shown a decrease in bound Ca^{2+} as ripening ensues (14, 21, 25), or no apparent change in tomato (4) or apple (11). The dissimilarity in CW–ML Ca^{2+} content most likely reflects differences in extraction and determination, which are the primary sources of error in CW–ML Ca^{2+} analysis.

Cell walls of higher plants are heterogenous in nature, and those in tomato undergo dramatic changes as the fruit senesce. Polygalacturonase is the enzyme responsible for the majority of softening that occurs in tomato pericarp (18). More relevant to Ca^{2+} binding, however, is the action of PME on pectin. When fruit ripen, the activity of PME results in the appearance of charged carboxyl groups. As the degree of esterification decreases and the charge density along the pectic chain increases, the affinity for Ca^{2+} binding is enhanced, and its binding is thought to be cooperative in nature (12, 13, 20). Accepting this, it is reasonable to believe that the amount of Ca^{2+} increases in pericarp CW–ML areas as tomato fruit ripen. The increase in substantial quantities of Ca^{2+} from the BR to RE may be due to cooperative binding of Ca^{2+} along the polyelectrolyte. The degree of esterification is high and changes only slightly in peach CW–ML regions during ripening (19). No change in CW–ML Ca^{2+} was observed in this tissue.

The lack of CW–ML Ca^{2+} in peach also is reflected in the amount of chelate-soluble pectin liberated from the fruit upon ripening (Table 1) and very low PME activities (15, 18). Although tomatoes contain much more polygalacturonase than peach (15, 18), peaches release a preponderance of H_2O-soluble pectin upon ripening and very low amounts of chelate-soluble pectin. The Ca^{2+} present in the CW–ML region of tomato pericarp may function to retain pectic polymers in the CW–ML structure, whereas most peach pectin is lost during ripening. A major difference in softening and texture between tomatoes and peach could be a result of the difference in CW–ML Ca^{2+} content and, hence, pectin retention. Whether Ca^{2+} is directly involved in softening of fruit is still a matter of debate (4); however, the presence of wall Ca^{2+} deters their degradation by polygalacturonase (4, 6) and undoubtedly contributes to differences in peach and tomato in terms of flesh texture.

It is well established that the activity of PME slightly increases in pericarp of tomato during ripening (3, 16, 23, 26), but its importance in overall softening is thought to be minimal. Likewise, we have measured an increase in PME during ripening in outer and inner pericarp. The rise in PME activity may not be entirely responsible for the 4- to 5-fold increase in CW–ML Ca^{2+} seen in tomato pericarp. The action of PME in providing binding sites for Ca^{2+} is no doubt important for the concentration of the ion in the CW–ML, but the action of other CW–ML-degrading enzymes such as polygalacturonase may also be important. In tomato, polygalacturonase cleaves high-molecular-weight polymers from the CW–ML area and results in fruit softening. The mobility of molecules and ions can be affected by the relative stiffness of the wall. Removal of the pectins from cell walls of grapefruit segments enhances the enzymatic hydrolysis of cellulose and hemicellulose (1). If the degree of esterification decreases as a result of PME treatment in GM pericarp, then steric hindrance may play an important role in Ca^{2+} binding to CW–ML areas. The importance of steric hindrance in the ability of Ca^{2+} to bind to the CW–ML area may be demonstrated by the lack of Ca^{2+} binding to GM CW–ML areas upon addition of tomato PME. The low PME activity (Fig. 3) and low polygalacturonase activity in tomato placenta (27) may further indicate the importance of charge density and steric hindrance in the presence of Ca^{2+} in the wall.

The methods employed to determine CW–ML Ca^{2+} content often involved conventional extractions in dilute or strong salt solutions, followed by Ca^{2+} analysis by atomic absorption spectrometry (4, 14, 25). Although atomic absorption spectrometry is a convenient and sensitive method of analysis, liberation of Ca^{2+} from the extracellular space for its quantification is highly dependent on solvent choice (4, 9). Ion determination and localization within plant tissues by the use of X-ray microanalysis is a useful technique for Ca^{2+} determination and is extremely reliable, providing that steps are taken to prevent ion migration and redistribution during tissue preparation (10). Since we have measured an increase in tomato pericarp CW–ML Ca^{2+} levels, our results are in contrast to previous reports. Furthermore, peach, exhibits no change in CW–ML Ca^{2+} content during ripening. In light of these findings, it is apparent that the movement of Ca^{2+} out of the CW–ML area as an initiating event of fruit ripening should be reevaluated.

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Effects of Sequential Low-oxygen and Standard Controlled Atmosphere Storage Regimens on Apple Quality

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Additional index words. *Malus domestica*, titratable acids, firmness, senescent disorders, low-oxygen injury

Abstract. **Storing 'McIntosh' apples (*Malus domestica* Borkh.) initially in 1.0% or 1.5% CO_2 plus 1.0% O_2 at 3°C (LO) was more effective in retarding firmness loss than initial storage in 4.5% or 5.0% CO_2 plus 3.0% O_2 at 3° (SCA), indicating that early exposure to LO suppressed the subsequent rate of softening in SCA. 'McIntosh' firmness retention, determined 1 day after storage and after 7 days at 20°, decreased with fruit maturity for all LO and SCA storage regimens, but mature fruit tended to soften more rapidly in SCA after being exposed initially to LO. Initial storage in SCA for >75 days with subsequent storage in LO (in 1984) did not result in firmer apples than those stored continuously in SCA. The initial exposure period in LO needed to induce retention of apple firmness depended on cultivar and temperature. For 'McIntosh', 'Spartan', and 'Golden Delicious' cultivars, respectively, maximum poststorage fruit firmness was reached after 7.5, 4.5, and 1.5 months of initial exposure to LO at 0° and after 4.5, 3.0, and 3.0 months at 3°. Poststorage evolution of CO_2 and C_2H_4 and the severity of senescent disorders generally decreased with continuing storage in LO, but the incidence of low-O_2 injury increased. Poststorage ethanol evolution increased with exposure to 1.0% O_2 in 'McIntosh' apples stored at 0°, and 'Spartan' apples held at either 0° or 3°. Chemical name used: butanedioic acid mono(2,2-dimethylhydrazide) (daminozide).**

Ethanol evolution from 'McIntosh' apples increased during storage in 5.0% CO_2 + 3.0% O_2 at 3°C (SCA) at a greater rate than in 1.5% CO_2 + 1.0% O_2 at 3° (LO) (12), indicating that 1.0% O_2 may not stimulate ethanol production in 'McIntosh' apples. Several cultural factors and storage management practices predispose apples to produce ethanol during storage (5, 11). The resultant low-O_2 atmosphere injures the stored apples when acute levels of 120 mg ethanol/100 g are exceeded or if chronic exposure exceeds 20 to 40 mg ethanol/100 g (5, 16). Those factors affecting ethanol accumulation and the resultant fruit injury in storage atmospheres of 0 .5% to 1.0% O_2 are not fully understood, so that recommendations for proper fruit handling are inconclusive.

Little et al. (15) and Lidster et al. (13) attempted to minimize the incidence of low-O_2 injury in 'Granny Smith' and 'McIntosh' and 'Spartan' apples, respectively, by limiting the du-

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