Reduced Cell Size and Cell Wall Components of Apple Softening before Ripening on the Tree

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Abstract. The softening of fruit dramatically reduces its market value, especially when this occurs on the tree before ripening. The causes of fruit softening, before ripening, were examined through anatomical and phytochemical comparative analyses between normal fruit, fruit softened on the tree, and stored fruit. The typical morphological changes that occurred with the fruit included early senescence and decreased firmness. The decrease in firmness of softening fruit was due to smaller cell sizes but larger intercellular spaces. The water and free sugar content of the fruit flesh, as well as the weight and sugar content of the cell walls, were significantly lower in softening fruit. Conversely, uronic acid levels and β -galactosidase activity were slightly higher in the softening compared to normal fruit, but the latter was lower than in stored fruit. The results indicated that reduced cell size and cell wall mass were major changes occurring during fruit softening on the tree before ripening, suggesting a difference in the softening mechanisms in ripening and stored fruit.

Fruit softening before ripening on the tree (FSBRT) is a major physiological disorder that reduces the market value and storage ability of the fruit. Various physiological disorders, such as internal breakdown, water core, browning, and core spots, often accompany FSBRT. Even though FSBRT is a major problem, which reduces the product quality as well as fruit production, little information is available on its causes and processes.

A few reports have indicated that FSBRT is genotype-dependent, with varieties such as Mollie's Delicious, Jonagold, and Hukudobing highly susceptible to the disorder. The occurrence of FSBRT is also influenced by environmental conditions. The degree of FSBRT varies in relation to the cultivation area and climatic conditions (Choi, 1996). Choi et al. (2001) reported that high respiration and low accumulation of carbohydrate due to high night temperature during maturing state are likely associated with the early softening of fruit.

Understanding the causes and processes of the disorder is a prerequisite for its prevention and in the breeding for resistant varieties. In this study, the causes of FSBRT were investigated by comparative analyses of the phytochemical and anatomical characteristics of the flesh of normal and softening fruit.

Materials and Methods

Field conditions. The study was conducted

in a commercial orchard in Iksan, Korea, with 'Hokudo'/'M26' trees planted in 1989. The experimental plot was located at a longitude of 35.5° east, latitude of 127.7° north and sea level altitude of 10 m. The mean yearly temperature, precipitation, and duration of sunshine were 13.0 °C, 1287 mm, and 2105 h·year⁻¹, respectively.

Plant material. The 'Hokudo'/'M26' trees were spaced 3.0 × 5.5 m in east–west rows and trained to slender spindle bushes. Trees received trickle irrigation and standard cultural practices. This experiment was carried out from 1995 to 2002. The optimal harvest season is in early October in most of the years, and the average occurrence rate of FSBRT was about 10% per tree. Since the softening fruit could be visually identified as early as one month before the optimal harvest season, samples of normal and softening fruit were taken between 11 Sept. and 3 Oct. 1995, 1997, 1999, 2001, and 2002. Normal fruit, stored at room temperature for 15 d, were used as the stored fruit samples. Morphological and chemical characteristics were analyzed for the 1995, 1997, and 1999 samples. A preliminary anatomical analysis with the 2001 samples indicated difference in the ultrastructure between the normal and FSBRT samples and detailed anatomical analyses were conducted for the samples collected in 2002.

Ultrastructure of cells. Anatomical analyses were conducted using scanning electron microscope (JSM-5410LV, JEOL, Japan) and transmission electron microscope (JSM-1010, JEOL, Japan). The samples were prepared according to the procedure described by Dawes (1984).

Carbohydrate analyses. The ethanol soluble glucose, fructose and sucrose from fruit tissue were analyzed by gas chromatography (HP6850, Hewlett-Packard). Freeze-dried normal and FSBRT apple samples were homogenized in 60% ethanol and filtered using Miracloth. These filtrates were used for the GC analysis, using allose as an internal standard.

Cell wall fractions were prepared from residues extracted, successively, with water, sodiumdodecylsulfate (SDS), chloroform and methanol, and acetone. Arabinose, rhamnose, xylose, mannose, glucose, and galactose were quantified by GC, using myo-inositol as an internal standard. The concentrations were calculated from the chromatograms as the ratios in relation to the internal standard. Alditol acetate, a monosaccharide, was used to prepare a standard chromatogram (Henry et al., 1983).

The uronic acid (UA) in the cell walls was analyzed using the cell wall hydrolysates according to the procedure of Ben-Arie and coworkers (1979). The UA content was measured by carbazole colorimetric analysis (Bitter and Muir, 1962), using a standard equation prepared with galacturonic acid.

β -Galactosidase activity assay. An enzyme assay was conducted according to the methods of Moshrefi and Luh (1984) and Pressey (1983). The β -galactosidase activity was assayed after filtration of the homogenate through G25-Sephadex columns. One unit of β -galactosidase activity was defined as one nmole of p-nitrophenol released for one hour at 30 °C in 0.1 M sodium acetate, pH 4.0, using phenyl- β -galactopyruronide as a substrate and stopped reaction with 0.2 M Na₂CO₃.

Experimental data were obtained 3 repeats of three samples each year and analyzed using the SAS system for windows, release 6.12 (SAS Institute Inc, Cary, N.C.).

Results

Morphological and anatomical changes. The occurrence of FSBRT was noticed in some fruit, and was initiated in the early stage of fruit development. The softening fruit could

Fig. 1. Normal (upper right) and softening (upper left) fruit on a tree in the middle of September, and vertical sections of normal (lower right) and softening (lower left) fruit of 'Hokudo' apple.

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Table 1. Morphological and anatomical characteristics of normal and softening fruit at different sampling dates for 3 years (1995, 1997, 1999).

		Fruit size			Firmness		Soluble	Cell	Intercellular
Sampling date	Condition	Length (mm)	Width (mm)	Wt (g)	kg/φ 5 cm)		solids (°Brix)	size (μm)	space (μm)
11 Sept.	Normal	87.0 c ^z	71.5 b	395 a	3.0 a	2.1 a	12.1 a	14.9 bc	6.9 d
	Soften	89.2 bc	72.5 b	370 bc	2.7 b	1.9 bc	11.3 c	12.8 d	7.9 ab
3 Oct.	Normal	93.4 ab	73.1 b	408 a	2.6 b	1.9 b	13.2 a	16.2 a	7.2 cd
	Soften	96.2 a	82.1 a	365 c	2.4 c	1.8 c	12.7 ab	13.9 c	8.1 a
18 Oct.	Stored	93.0 ab	72.0 b	390 ab	2.5 bc	1.8 bc	12.8 ab	15.5 ab	7.5 bc

^zValues with same letters within each column are not significantly different at $p \leq 0.05$.

be visually identified as early as one month before the optimal harvest season. The peels of the softening fruit turned yellow and reddish-yellow before ripening on the trees (Fig. 1). The fruit flesh became spongy, and the growth ceased after the softening had started, with most of the softened fruit falling from the trees earlier.

The weights of the softening fruit were reduced by 25 to 43 g compared with those of normal fruit. There was a little difference between the sizes of the normal and softening fruit. The softening fruit were a little longer on 11 Sept. and a little wider on 3 Oct. than the normal fruit, while the firmness and soluble solid content were significantly lower in the softening fruit (Table 1).

Since the changes in the fruit size and firmness of the softening fruit were indicated by changes in the fruit flesh, the anatomical characteristics of the fruit were examined. As expected, the cell size was smaller, but the intracellular spaces were larger, in the softening fruit (Table 1, Figs. 2 and 3). The cells were compact and regularly distributed in the normal fruit, but shrunken and irregularly distributed in the softening fruit. Collapse of the cell walls was also indicated in the softening fruit. The separation and displacement of adjacent cell walls, and the separation of the plasma membrane from the cell wall, were prominent in the softening fruit, resulting in larger apoplastic spaces (Figs. 2 and 3).

Therefore, the smaller cells and larger intercellular spaces in the softening fruit were thought to be the major anatomical factors causing the reduced weight and firmness but unchanged volume of the softening fruit.

Changes in water and sugar contents. The water content of the softening fruit flesh was lower than in the normal fruit but slightly higher than in the stored fruit (Table 2). The levels of free sugars, such as glucose, fructose, and sucrose, increased with ripening and were lower in the softening fruit than in the normal fruit (Table 2). The fruit in the ripening stage were enlarged as the assimilation products in the leaves were transported to the cytoplasm of the flesh cells, resulting in a reduced osmotic potential. Thus, water moves into the cells, generating the driving force for cell enlargement. Therefore, the reduced free sugar and water content in the softening fruit could be positively related to the smaller cells and larger intercellular spaces.

Changes in the cell wall. The cell wall weight per unit fruit weight decreased in both the normal and softening fruit as they ripened. However, the weight was significantly lower

Fig. 2. Changes in the cell shape and density of normal and softening fruit during storage. N1 = normal fruit at harvest; N2 = normal fruit after 1 week of storage; N3 = normal fruit after 2 weeks of storage; S1 = softening fruit at harvest; S2 = softening fruit after 1 week of storage; S3 = softening fruit after 2 weeks of storage. SEM (×5,000).

Fig. 3. Changes in the placement of the cell walls and plasma membranes in the normal and softening fruit. (A) Normal fruit cells, the cell walls are bound close to each other (TEM, ×2,500). (B) Softening fruit cells, the cell walls are separated from each other (TEM, ×2,500). (C and D) Softening fruit cells, the plasma membranes are separated from the cell walls, resulting in larger apoplast spaces (C, TEM, ×8,000; D, TEM, ×10,000); CW = cell wall, PM = plasma membrane, E = empty space without cell materials.

in the softening fruit (Table 3). The major cell wall cellulose content and the constituents of cellulose, such as glucose and galactose, were concomitantly decreased as the fruit ripened but were consistently lower in the softening fruit. The arabinose, rhamnose, and mannose contents were also lower in the softening fruit in the later ripening stage but were variable in the earlier ripening stage.

Interestingly, however, the xylose and uronic acid contents increased as the fruit ripened. The xylose content was significantly higher in the softening fruit, but that of the uronic acid was not significantly different. Uronic acid is a constituent of pectin, which is another major cell wall component, and has been related to fruit softening (Robinson, 1977); therefore, slight increases in uronic acid might indicate a higher cell wall degradation

activity, which might lead to the accelerated early senescence in the softening fruit.

β-Galactosidase activity. β-Galactosidase is known as a key enzyme responsible for the release of galactose from the cell wall components during fruit ripening. The activity of this enzyme was increased as the fruit ripened, and was significantly higher in the softening than the normal fruit in September but not in October. However, its activity in the softening fruit was lower than the highest activity—6.7 units—observed in the stored fruit (Fig. 4).

Discussion

Our data indicated that FSBRT is a physiological disorder that is accompanied by morphological, phytochemical and anatomical changes in the tissues and cells of fruit.

Table 2. The average water and sugar contents in the flesh of normal and softening fruit at different sampling dates in 3 years (1995, 1997, and 1999).

Sampling date	Condition	Water (%)	Content (mg·g ⁻¹ fresh wt)		
			Glucose	Sucrose	Fructose
11 Sept.	Normal	84.1 bc ^a	6.56 b	11.87 cd	15.66 b
	Soften	83.5 c	6.72 b	10.13 d	14.56 b
3 Oct.	Normal	86.5 a	8.39 a	17.09 a	21.99 a
	Soften	84.9 bc	5.54 c	14.87 ab	19.30 a
18 Oct.	Stored	84.4 bc	6.33 b	13.45 bc	17.56 b

^aValues with same letters within each column are not significantly different at $p \leq 0.05$.

Table 3. Cell wall monosaccharide content of normal and softening fruit for 3 years (1995, 1997, and 1999).

Sampling date	Condition	Cell wall wt (mg·g ⁻¹ dry wt)	Content (mg·g ⁻¹ cell wall)							Concn (%)	
			Arabinos	Rhamnos	Xylose	Manno	Glucose	Galactose	Cellulose	Uronic acid	
11 Sept.	Normal	134 a ^a	25.0 b	186.8 b	96.0 d	19.4 b	200.0 a	161.0 a	50 a	17 a	
	Soften	78 bc	46.6 a	189.6 b	120.0 c	25.4 a	78.4 cd	90.8 d	37 b	18 a	
3 Oct.	Normal	90 b	40.4 b	246.0 a	162.4 a	26.6 a	153.6 b	132.0 b	30 bc	19 a	
	Soften	72 cd	33.6 c	172.6 c	146.0 b	22.4 ab	71.8 d	79.0 e	27 c	20 a	
18 Oct.	Stored	60 d	37.8 bc	160.0 d	168.8 a	21.0 b	100.4 c	104.4 c	25 c	20 a	

^aValues with same letters within each column are not significantly different at $p \leq 0.05$.

The most prominent morphological changes were the reduced weight and firmness, with a little change in the fruit size. These morphological changes were manifested as anatomical changes in the flesh. The cell size was reduced, but the intercellular space enlarged, in the softening fruit.

Since the development of apple fruit is the result of assimilates accumulation and water absorption after cell divisions (Archhold, 1992; Lakso et al., 1989; Pavel and Dejong, 1995), the limited accumulation of assimilates and water can reduce the cell size. This notion was supported by the observed decrease in the free sugars and water contents in the softening fruit (Table 2).

Another possible reason for the smaller cells in the softening fruit could be the increased consumption of assimilates due to increased respiration. The possible involvement of increased respiration in fruit softening has been indicated by the higher occurrence of softening fruit with increases in the night temperature (Choi et al., 2001). The decreased free sugar content in the softening fruit might be the expected result of higher respiration due to higher temperature. Increased water evaporation, and the resulting decreases in the water content and cellular activity under higher temperatures can also inhibit cell growth (Morris and Sistrunk, 1991). Thus, the morphological and cellular characteristics of the softening fruit suggest that the reduced transport and increased consumption of the assimilates required for cellular enlargement might be the significant physiological causes for FSBRT.

Conversely, no significant anatomical signs of cell wall degradation or thinning, which are the typical characteristics of softening during storage, were observed in the fruit that softened before ripening (Figs 2 and 3). Rather, the decreases in the cell wall weight and cellulose content, together with the decreases in most of the monosaccharide constituents of the cell wall components (Table 3) strongly suggest that the reduced cell wall synthesis was positively related to the smaller cell size and increased intercellular space in the softening fruit. Since

no cellulase is expressed by the apple tissues, hydrolysis of cellulose can only occur by microorganism infections. Therefore, with no disease symptoms, the reduced cellulose content may indicate the reduced synthesis of cellulose. Furthermore, a reduced cellulose production has been reported in softening fruit (Goodwin and Mercer, 1983; Wareing and Phillips, 1981). Decreases in cellulose and hemicellulose should result in decreases in the proportion of the molecules in the cell walls, which was observed. Consequently, the decreases in galactose, a cellulose constituent, and rhamnose, a hemicellulose constituent, also indicate insufficient synthesis of the cell wall in softening fruit. Thus, it is highly possible that a reduced cellulose synthesis is a reason for the reduction of cell wall weight in softening fruit.

The increases in the uronic acid content in the cell wall and the β -galactosidase activity are

indicative of the cell wall degradation observed in softening fruit. However, the uronic acid content in the softening fruit was a little higher than that in the normal fruit. The genotype difference in the occurrence of fruit softening has been positively related to the activities of the cell wall degradation enzymes (Byun et al., 1993; Fischer and Bennete, 1991; Huber, 1983; John and Dey, 1985). Knee and Bartley (1981) reported that the β -galactosidase activity was increased in softening apple fruit, and thus, the galactose content was decreased in the cell wall. A possible role of the enzyme in fruit softening is also indicated by the gradual increase in the enzymic activity during ripening. However, β -galactosidase activity in the softening fruit was a little higher in the softening fruit but was lower than that in the stored fruit. Therefore, cell wall degradation is not likely to be a major cause of FSBRT.

In conclusion, it is highly likely that FSBRT

Fig. 4. β -Galactosidase activities of normal, softening, and stored fruit at different sampling times for 3 years (1995, 1997, and 1999). ^aValues with different letters within each column are significantly different at $p \leq 0.05$.

could be the result of the reduced supply of sugars that can be used in the synthesis of the cell wall and stored in the cell, resulting in smaller cells but enlarged intercellular spaces. The reduced supply of sugars during the fruit ripening, due to either reduced assimilation, increased respiration, or both, could limit the cell wall synthesis and sugar accumulation in the cells, consequently resulting in the smaller cells, but enlarged intercellular spaces, of the fruit softening on the tree prior to their harvest.

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