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Cell wall modifications lead to cultivar differences in apple (*Malus domestica*) fruit mealiness

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Abstract

Recently, four quantitative trait loci linked to flesh mealiness in apples were identified, with one associated with the MdPG1 allele. Hence, this study analyzed cell wall changes in two mealy (Orin and Akane) and three non-mealy (Kiou, Kitaro, Fuji) apple cultivars during ripening. The fruits were harvested for each cultivar at optimum maturity and stored at 20°C for 20 days. The flesh firmness of 'Kitaro' and 'Fuji' fruit did not change strikingly over the 20 days, whereas that of the other three cultivars, especially 'Akane' and 'Orin', gradually decreased during ripening. Between the two cultivars with a mealy texture, 'Akane' fruit produced extremely low levels of ethylene, whereas 'Orin' fruit produced high levels. The water-soluble polyuronide (WSP) contents of 'Kiou' and 'Fuji' fruit did not change clearly. In contrast, the WSP contents of the other three cultivars, especially 'Akane' and 'Orin', increased during ripening. In 'Kiou', 'Kitaro', and 'Fuji' fruit, the molecular-mass distributions of WSPs did not change during ripening. Conversely, the molecular-mass distribution of WSPs in 'Akane' and 'Orin' fruit exhibited downshifts during ripening. These results indicate that solubilization and depolymerization of pectic polyuronides occur during ripening in mealy 'Akane' and 'Orin' fruit, and that ethylene may not be involved in these changes.

INTRODUCTION

In fruit, including apples, flesh texture is an important trait for consumers, who generally prefer crisp apples. Fruit texture, however, changes gradually after harvest. Mealiness is considered a significant deterioration of fruit flesh texture, as it is commonly associated with a dry texture, a soft, unpleasant taste in the mouth, and a lack of juiciness and aroma (Bowen et al., 2019; Hunter et al., 2021). Mealiness has been widely investigated in numerous fruit during the storage and softening process, such as in apples (Arefi et al., 2017), pears (Cronje, 2019; Dong et al., 2018; Dong et al., 2019), squash fruit (Sameshima et al., 2018), tomatoes (Hunter et al., 2021), and peaches (Nilo-Poyanco et al., 2019; Tatsuki et al., 2021). In apples, the level of mealiness relies greatly on growing or storage conditions, size of fruit, date of harvesting, and cultivar type (Mollazade and Arefi, 2017). Iwanami et al. (2005) and Vanoli et al. (2018) demonstrated that mealiness develops after a plateau of flesh softening is reached or in more advanced softening although softening can occur without mealiness.

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Mealiness is correlated with pectin dissolution in the middle lamella and modification of cell wall components, leading to the destruction of cell wall and loss of adhesion cells (Segonne et al., 2014). The degree of cell separation in mealy tissue is higher than in non-mealy tissue, and it appears that mealiness can cause decreased cell adhesion (Buergy et al., 2021). Levels of galacturonic acid, a water-soluble polyuronide (WSP), are higher in mealy apples than in non-mealy apples during storage (Billy et al., 2020).

Several studies have also found associations between enzyme activities or genes and the development of mealiness. In tomatoes, mealiness was found to be closely related to decreased levels of polygalacturonase (PG) (Buccheri and Cantwell, 2014). PG is an enzyme that acts in hydrolysis of alpha-1,4-glycosidic bonds between galacturonic acid residues and is responsible for the process of fruit tissue softening (Moya-Leon et al., 2019; Zhang et al., 2019). In apples, MdPG1 is a genetic determinant of flesh texture (Poles et al., 2020). Recently, Moriya et al. (2017) identified four quantitative trait loci (QTL) linked to the development of apple mealiness during storage at 20°C. One of them was closely associated with MdPG1 and was identified using QTL analysis and genome wide association study (GWAS). In Moriya's study, the grouping of mealy and non-mealy apples was also reported. 'Kitaro', 'Fuji', and 'Kiou' are classified in the non-mealy group, while 'Akane' and 'Orin' are classified in the mealy group. Hence, this study would analyze cell wall changes in both mealy and non-mealy apple cultivars during softening.

MATERIALS AND METHODS

Plant materials

Fruit of five apple cultivars (*Malus domestica* Borkh)—'Akane', 'Orin', 'Kiou', 'Kitaro' and 'Fuji' were harvested at optimal maturity for each cultivar from an orchard of the Division of Apple Research, Institute of Fruit Tree and Tea Science, National Agriculture and Food Research Organization (NARO, Morioka, Iwate Prefecture, Japan) and then transported to the Faculty of Agriculture, Yamagata University (Tsuruoka, Yamagata Prefecture, Japan). 'Akane' and 'Orin' fruit easily developed a mealy texture after harvest, whereas 'Kiou', 'Kitaro', and 'Fuji' fruit did not. For each cultivar, 30 fruits without any damage or visual defects were selected on the day after harvest and stored at 20 °C. Five fruits of each cultivar were sampled every five days.

Analysis of ethylene production and flesh firmness

Individual fruits were placed in a 1.5 L desiccator flushed with air and sealed for 1 h while maintaining a temperature of 20°C. Gas samples were collected using a syringe (MS-GLL100; Ito Co., Shizuoka, Japan) and directly injected into the gas chromatograph (GC-8A; Shimadzu Co., Kyoto, Japan) equipped with an activated alumina column and a flame ionization detector (FID).

Flesh firmness was determined by a rheometer (Model CR-200D; Sun Scientific Co., Tokyo, Japan) equipped with an 8 mm diameter cylindrical stainless probe at a speed of 60 $mm.min^{-1}$. Measurement was conducted twice at opposite points along the equator of individual fruit.

Extraction, fractionation, and characterization of structural cell wall polysaccharides

The apples were peeled and cut into dice shapes. The flesh from an individual apple fruit (approximately 20 g) was freeze-dried and crushed into a powder. The powder was suspended in 80% (v/v) of ethanol (20 ml), heated (at 80°C, 20 min) using an aluminum block and centrifuged. The filtrate was removed while the residues were re-suspended three times in 80% of ethanol. After being filtered, the residues were suspended successively in 100% of ethanol, and 100% of acetone. Last, the residues were washed with 100% of acetone and dried at room temperature overnight.

Alcohol-insoluble residues (50 mg) were extracted with distilled water (20 ml), shaken overnight on a mechanical shaker (20°C), and centrifuged. Then, the residues were re-suspended in distilled water (20 ml), shaken for 1 h and re-centrifuged. The supernatants were pooled and determined as a fraction containing WSPs. The residues were re-extracted in two repetitions with 20 ml of 50 mM ethylenediaminetetraacetic acid (pH 6.5) at 20°C. The supernatants were pooled and determined as a fraction containing chelator-soluble polyuronides (CSPs). The residues were re-extracted in two repetitions with 20 ml of 50 mM sodium carbonate (Na_2CO_3) which had been mixed with 20 mM sodium borohydride ($NaBH_4$) at 20°C. The supernatants were pooled, neutralized with acetic acid solution,

and determined as a fraction containing alkali-soluble polyuronides (ASPs). The amount of uronic acid content in all fractions was analyzed using the m-hydroxy diphenyl method. A 0.5 mL sample was added to 0.0125 M $Na_2B_4O_7$. 10 H_2O/ conc. H_2SO_4, then boiled at 100°C for 5 min. The sample was re-added using 0.15% m-hydroxydiphenyl/0.5% NaOH, then measured in optical density at 520 nm (Blumenkrantz and Asboe-Hansen, 1973).

After extraction of the ASP fraction, the residues were used for further extraction of the hemicellulose polysaccharides. The residues were first suspended in 50 mM Na-acetate buffer with pH 6.5 (2 ml) and boiled for 1 min. After ice-cooling, 2 ml of amylase solution was added to the sample and shaken at 37°C for 3 h using a water bath shaker. After being filtered, the residues were then suspended in 4 M potassium hydroxide (20 ml) which had been mixed with 20 mM sodium borohydride and centrifuged. The residues were re-suspended in 4 M potassium hydroxide solution (20 ml) and shaken for 1 h. After being filtered, the supernatants were pooled, neutralized with glacial acetic acid solution, and determined as a fraction containing hemicellulosic polysaccharides. The total sugar content of the solution was analyzed using the phenol sulfuric acid method. A 0.5 mL sample was added to a 5% phenol solution and 2.5 mL of sulfuric acid, and then measured in optical density at 490 nm (Dubois et al., 1956).

Molecular mass analysis of WSPs

The WSP fraction was lyophilized using a freeze dryer. The residue was dissolved in 2 ml of 50 mM sodium phosphate buffer (pH 7.2), which was then filtered using a 0.45 µm membrane (JHWP01300; Merck Millipore, Darmstadt, Germany). The aliquot was directly injected onto a high-performance liquid chromatography (HPLC) system (Nexera XR; Shimadzu Co.) fitted with a gel filtration column (TSK-Gel G5000 PWXL, 8 mm × 30 cm; Tosoh, Tokyo, Japan). The sample was eluted with 50 mM sodium phosphate buffer (pH 7.2) and the flow rate for sample loading was 0.4 $ml.min^{-1}$.

Statistical analysis

The data were analyzed using SPSS software and displayed as mean ± standard error. One-way ANOVA was performed to compare ethylene production and flesh firmness among cultivars at different storage time-points (1, 5, 10, 15, and 20 days of storage). Tukey's honest significance test was applied as a post-hoc test with statistical significance at $P < 0.05$. Independent t-tests were used to assess whether the average amount of cell wall polysaccharides in the fruit of each cultivar differed from 1 to 20 days of storage.

RESULTS AND DISCUSSION

Ethylene Production and Flesh Firmness

Based on patterns of ethylene production, the fruit was split into three groups (Figure 1A). In 'Kitaro' and 'Kiou' fruit, the ethylene levels increased immediately after harvest, peaked at 5 days, and then gradually decreased during ripening at 20 °C. In 'Orin' fruit, ethylene levels also increased just after harvest and then remained high after 5 days. By contrast, ethylene levels were very low throughout the experimental period in 'Akane' and 'Fuji' fruit, although the levels increased slightly after 5 days in 'Fuji' fruit.

Flesh firmness in 'Kitaro' and 'Fuji' fruit did not change over 20 days (Figure 1B). Conversely, flesh firmness in the other three cultivars, especially 'Akane' and 'Orin', decreased gradually during ripening at 20 °C.

Cell Wall Polysaccharides

Pectic and hemicellulosic polysaccharides were measured on day 1 and 20 in the five apple cultivars. The WSP contents of 'Kiou' and 'Akane' fruit on 1 day were 17.12 and 21.74 $mg.kg^{-1}$, respectively (Figure. 2A), which were lower than those of the other three cultivars. The WSP contents of 'Kiou', 'Kitaro' and 'Fuji' fruit did not change during ripening, whereas those of 'Akane' and 'Orin' fruit increased during ripening.

The CSP content of 'Kiou' fruit was 11.99 $mg.kg^{-1}$ on day 1, markedly lower than those of the other four cultivars (Figure 2B). In all cultivars, the CSP contents did not change between day 1 and 20. The ASP contents also did not change during ripening although a decreasing trend was observed for 'Kiou', 'Akane', and 'Orin' fruit (Figure 2C). The total polyuronide content of 'Kiou' fruit on day 1 was 141.7 $mg.kg^{-1}$, lower than those of the other four cultivars (Figure 2D). The total polyuronide contents did not significantly change between day 1 and 20 in all cultivars.

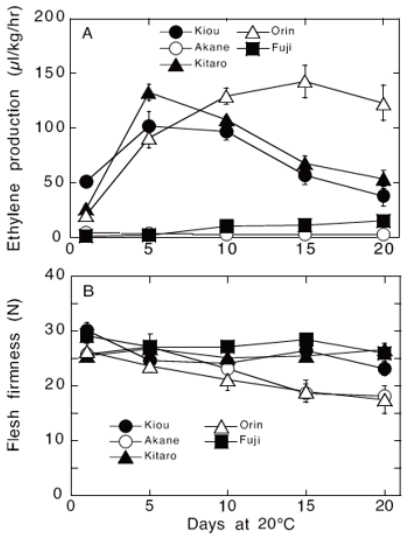

Figure 1. Changes in ethylene production and flesh firmness after harvest in five apple cultivars. Values are presented as the means ± standard errors (SEs, n = 5).

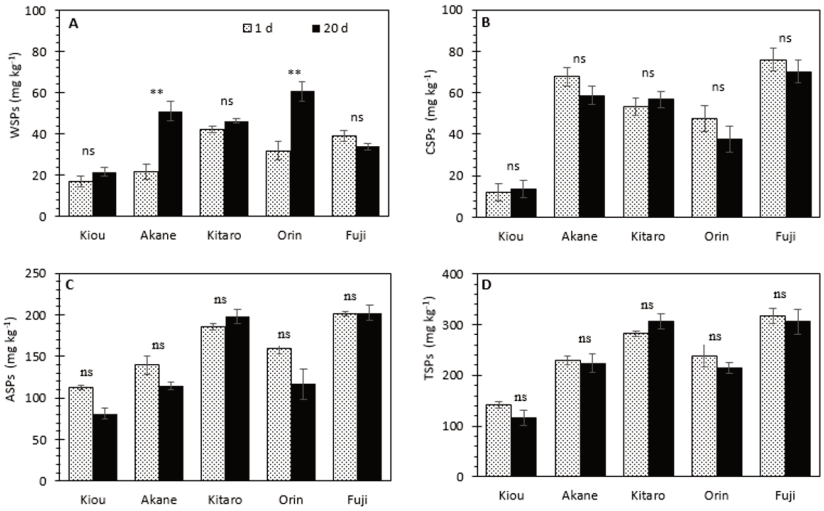

Figure 2. Changes in water-soluble polyuronides (WSPs) (a), chelator-soluble polyuronides (CSPs) (b), alkali-soluble polyuronides (ASPs) (c), and total soluble polyuronides (TSPs) (d) after harvest in five apple cultivars. Values are presented as the means ± SEs (n = 5). Independent t-tests were performed to compare the WSPs, CSPs, ASPs, and TSPs content in a given cultivar between 1 and 20 d after harvest. * P <0.05; ** P <0.01; *** P <0.001; ns, not significant (P >0.05).

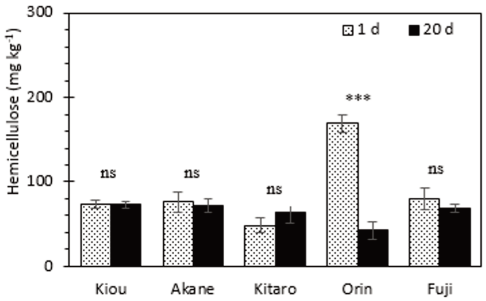

Figure 3. Changes in hemicellulose polysaccharides after harvest in five apple cultivars. Values are presented as the means ± SEs (n = 5). Independent t-tests were performed to compare the hemicellulose content in a given cultivar between 1 and 20 d after harvest. *** P <0.001; ns, not significant (P >0.05).

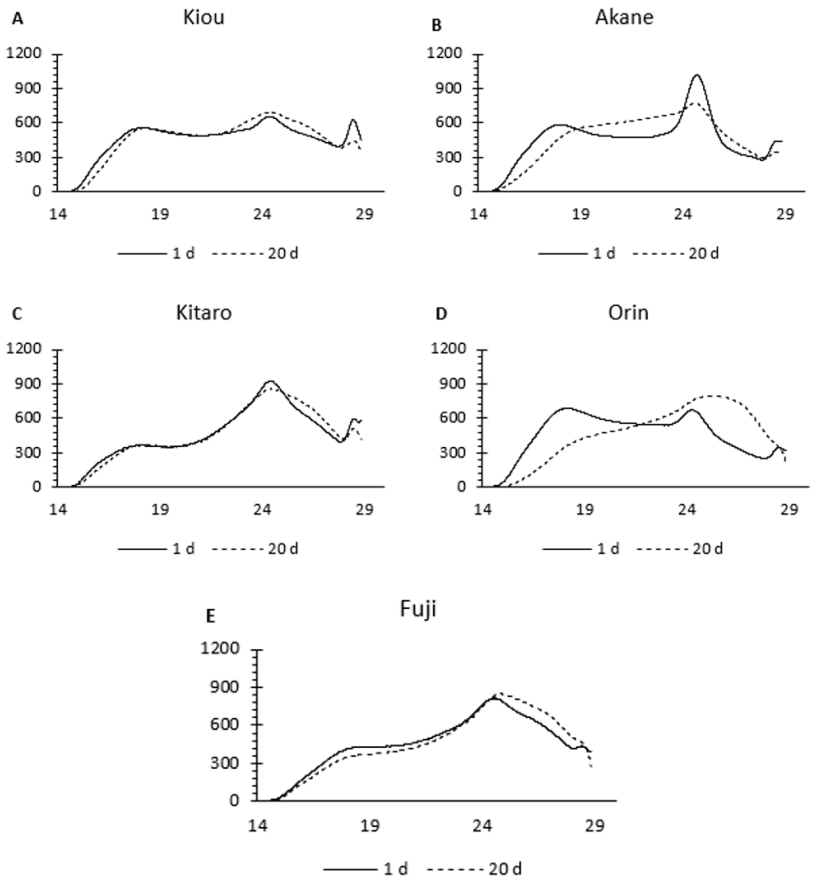

Figure 4. Molecular-weight distribution profiles of WSPs on 1 and 20 d of storage in five apple cultivars. The y-axis and x-axis represent the corrected intensity and retention time, respectively.

The hemicellulosic polysaccharide content of 'Orin' fruit on day 1 was higher than those of the other four cultivars, which then decreased markedly during ripening (Figure 3). On day 20, 'Orin' fruit had the lowest hemicellulosic polysaccharide content among all cultivars. Excluding 'Orin', no changes in hemicellulosic polysaccharide content were observed for the other cultivars during ripening.

Molecular-Mass Distributions of WSPs

Prominent changes in WSPs were observed during ripening. In addition, these changes occurred in tandem with the development of a mealy texture. Thus, an analysis of WSP molecular masses was performed.

In 'Kiou', 'Kitaro', and 'Fuji' fruit, the WSP molecular-mass distributions did not change during ripening (Figure. 4), but downshifts in the molecular-mass distributions of 'Akane' and 'Orin' fruit were observed. The downshift was particularly striking and extensive for 'Orin' fruit.

In the present study, the flesh firmness of mealy 'Akane' and 'Orin' apples decreased gradually during ripening. In the three non-mealy cultivars, flesh firmness only decreased gradually in 'Kiou' fruit, whereas it did not change over 20 days in 'Kitaro' and 'Fuji' fruit. These results are identical to those of Iwanami et al. (2005), who reported that 'Akane' and 'Orin' fruit softened faster than did 'Fuji' and 'Kitaro' fruit, which remained hard during storage. Iwanami et al. (2005) also differentiated types of mealy cultivars, with the fruit of one type softening with a mealy texture, and the fruit of the other type softening without developing mealiness. 'Akane' and 'Orin' may belong to the former type due to sensory evaluation. In addition, after 20 days of storage, the texture of 'Akane' and 'Orin' became softer and drier, and had a crumbly feeling on the tongue indicating mealy properties. 'Fuji' and 'Kitaro' cultivars still had a juicy and crispy texture after 20 days of storage, while 'Kiou' showed intermediate of both properties.

In climacteric fruit, ethylene plays a main role in

controlling fruit ripening (Fenn and Giovannoni, 2021). In this study, ethylene production levels in 'Akane' and 'Fuji' fruit were very low throughout the experimental period. The results obtained for 'Fuji' apples make sense, as this cultivar maintains its quality well in storage. Unexpectedly, 'Akane' fruit softened gradually during ripening despite their low rates of ethylene production. On the opposite, 'Kiou' and 'Kitaro' fruit did not soften strikingly throughout the experimental period despite high levels of ethylene production. Moreover, in the two cultivars associated with a mealy texture in this study, the ethylene production level was low in 'Akane' fruit and high in 'Orin' fruit. This indicates that the development of a mealy texture is not solely controlled by ethylene production.

Fruit softening occurs due to the breakdown of cell wall materials such as pectic and hemicellulosic and cellulose. Pectic polysaccharides are the major polymers regulating cell adhesion and are found in the middle lamella (Zamil and Geitmann, 2017; Voiniciuc et al., 2018). Previous studies showed that flesh firmness decreased concomitantly along with an increase in WSPs in apples (Li et al., 2020; Win et al., 2019; Win et al., 2021). In this study, WSPs increased markedly during ripening in mealy 'Akane' and 'Orin' apples, but did not change in non-mealy 'Kiou' and 'Fuji' apples. Taken together, the increase in WSPs may be indicative of pectin solubilization during softening.

In 'Akane' and 'Orin' apples, extensive softening occurred concomitantly with the development of a mealy texture. Therefore, it was difficult to ascertain whether the increase in pectic polysaccharides during ripening was linked to fruit softening, the development of a mealy texture, or both. Non-mealy 'Kiou' apples exhibited a slight decrease in flesh firmness and a no-change in WSP content during ripening. This implies that an increase in WSP content affects the development of mealy texture.

Other pectic polysaccharides, CSPs, ASPs, and total polyuronides, did not change during ripening although ASPs tended to decrease in mealy 'Akane' and 'Orin' fruit. The increase in WSPs might have resulted from conversion from ASPs during fruit softening. Chen et al. (2016) and Sun et al. (2013) explained that the solubilization of cell wall polysaccharides is involved in the displacement of polymers from one fraction by another fraction caused by their structural changes. In addition, it can be seen from the data in this study that the cultivar with the largest ASPs fraction ('Kitaro' and 'Fuji') experienced no change in ASP content during storage and there was no change in fruit firmness, while for other cultivars, ASP levels decreased along with the decrease in fruit firmness. Thus, the largest ASP fraction in apples might also play an important role for cell integrity and mechanics.

The hemicellulose content in 'Orin' fruit was notably high on day 1 compared to the other four cultivars and decreased during ripening. The contents of hemicellulose reportedly decreased during softening in 'Summer King' and 'Green Ball' apples (Win et al., 2019). The hemicellulose contents in the other four cultivars did not change during ripening. This is not surprising because their flesh firmness also did not change over the same time period. In 'Akane' fruit, although the hemicellulose content did not change, the fruit softened during ripening.

As mentioned earlier, prominent changes in WSPs were observed during ripening. In addition, these changes occurred in tandem with the development of a mealy texture. WSP molecular masses were also analyzed. In 'Kiou', 'Kitaro', and 'Fuji' fruit, the WSP molecular-mass distributions did not change during ripening. In contrast, downshifts in molecular-mass distributions were observed for 'Akane' and 'Orin' fruit, with the downshift in 'Orin' fruit being particularly striking and extensive. Gwanpua et al. (2014) also reported downshifts in the pectin molecular weight distributions, a component of the WSP fraction, in 'Jonagold' apples. Moriya et al. (2017) identified four QTLs linked to the development of apple mealiness during storage at 20°C. One of them was associated with MdPG1 and identified using QTL analysis and a GWAS. The depolymerization of WSP may mainly result from enzymatic cleavage of alpha-1,4 bonds in polygalacturonan by PG. This may explain the downshifts in WSP molecular-mass distributions observed during ripening in mealy 'Akane' and 'Orin' fruit.

CONCLUSIONS

This study confirmed that each cultivar exhibited different cell wall modification behavior during softening that was independent,whether the cultivar was mealy or non-mealy. WSPs were was found to contribute to fruit softening and development of a mealy texture. Flesh firmness in 'Akane' and 'Orin' fruit

decreased more sharply than in the other cultivars, and the decrease was followed by a great increase in WSPs during softening, implying more broken-down cell walls in the mealy tissues. The molecular weight distributions of pectin in 'Akane' and 'Orin' fruit were indicative of depolymerization, which may be involved in the development of the mealy texture of apples.

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