Thinning and Bagging Treatments and the Growing Region Influence Anthocyanin Accumulation in Red-fleshed Apple Fruit

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In common apple cultivars with white flesh and red skin, it is known that fruit overload lowers fruit quality, and that skin anthocyanin concentrations are reduced by bagging treatment and warmer climatic conditions.

In this study, the effects of these factors on anthocyanin accumulation in the flesh of red-fleshed apples were investigated using ‘Geneva’ and ‘Pink Pearl’ apple cultivars. Excess fruiting resulted in decreased anthocyanin concentration in the flesh and the titration acidity of the fruit in both cultivars. Fruit bagging treatments using double-layer paper bags revealed that anthocyanin was synthesized to a certain extent in the flesh under dark conditions in both cultivars. The treatment significantly reduced anthocyanin accumulation in the flesh of bagged ‘Geneva’ apples compared with unbagged fruit, but no inhibitory effect was clear in ‘Pink Pearl’ apples. In both cultivars, the anthocyanin concentration in the flesh of unbagged apples grown in sunlight was higher than that in unbagged apples grown in shade, whereas there was no difference between positions for bagged fruit. In apples grown in either sunlight or shade, no significant difference was found between anthocyanin concentrations in the sun-exposed side and shaded side of fruits. These results indicate that sunlight irradiation partially promoted anthocyanin accumulation in the flesh of unbagged ‘Geneva’ and ‘Pink Pearl’ apples and accelerated its accumulation on both the sun-exposed and the shaded side. A comparison of the fruit quality of ‘Pink Pearl’ apples grown in different climatic regions showed that apples harvested at Suzaka, a warmer region, were lower in firmness, starch index, and titration acidity and higher in soluble solids concentration compared with those from Morioka, a cooler region. The anthocyanin concentration in the flesh of ‘Pink Pearl’ apples from the trees grown at Morioka was more than ten-fold higher than that in apples from the trees grown at Suzaka. Our results suggest that an appropriate fruit load, growth in sunlight, and growth under cooler climatic conditions, may redden the flesh of red-fleshed apples, as is the case for common apples.

Key Words: firmness, soluble solids concentration, sunlight, temperature, titration acidity.

Introduction

Red pigmentation in the flesh of certain apples is attributed mainly to the flavonoid compound cyanidin 3-galactoside, which is commonly found in apple skin (Rupasinghe et al., 2010). Red-fleshed apples are not widely produced because of poor fruit quality (e.g., small size and high acidity) (Faramarzi et al., 2014; Rupasinghe et al., 2010). Therefore, improving the fruit quality of red-fleshed apple cultivars may lead to an increase in their production. In Japan, new red-fleshed apple cultivars with low acidity have recently been registered, including ‘Ruby Sweet’ and ‘Rose Pearl’ (Abe et al., 2013a, b). In particular, ‘Ruby Sweet’ is expected to be consumed as an unprocessed apple due to its high soluble solids concentration (14.5 °Brix) and low titration acidity (0.36 g malic acid/100 mL).

Two different MYB transcription factor (TF) genes that induce anthocyanin accumulation in apple flesh have been identified. The first, MdMYB10, is located on
linkage group (LG) 9 (Chagné et al., 2007; Volz et al., 2013), and the other, *MdMYB110a*, is located on LG 17 (Chagné et al., 2013; Umemura et al., 2013). In apples with *MdMYB10*, designated as type 1, not only the flesh, but also other organs including the skin, flower, leaf, and stem, are red in color. In apples with *MdMYB110a*, designated as type 2, only the flesh is red, and other organs look the same as those of common apple trees with white-fleshed fruit.

Recent studies have shown that the genotype of the MYB TF gene, which is located on LG 9, determines apple skin color (red or yellow) (Ban et al., 2007; Takos et al., 2006). Exogenous factors additionally affect anthocyanin accumulation in the red skin of apples, including light and temperature conditions (Saure, 1990; Ubi, 2004). Sunlight irradiation is essential for the pigmentation of apple skin so in Japan leaves covering the fruit are removed before harvest to improve fruit coloration, which requires considerable labor (Iwanami et al., 2016). Experiments have shown that high temperature conditions before harvest suppress anthocyanin accumulation in apple skin. Lin-Wang et al. (2011) showed that warmer temperatures during the week prior to harvest, created using an orchard-based, on-tree heating system, suppressed anthocyanin synthesis and down-regulated the expression of MYB TF and anthocyanin synthetic genes in the skin of ‘Gala’ apples. Honda et al. (2014) demonstrated that the warmer conditions found in greenhouses, where the mean air temperature was consistently 4°C warmer during the 5 weeks before harvest, suppressed pigmentation in the skin of ‘Misuzu Tsugaru’ apples. However, whether such environmental factors influence anthocyanin synthesis in apple flesh is unknown.

‘Geneva’ is a type 1 apple, with red skin and flesh, whereas ‘Pink Pearl’ is a type 2 apple, with yellow skin and red flesh. They are used in breeding programs that produce red-fleshed apples for both processing and table use. Apples produced for processing use labor-saving cultivation management to a greater extent than do those produced for table use, and the trees are often excessively set. It has been shown that fruit from high-cropping apple trees has a poor red coloration, with a low soluble solids concentration and titration acidity (Kon et al., 2013; Magness, 1928; Wünsche et al., 2000). However, the effect of excess fruit set on anthocyanin accumulation in the flesh of red-fleshed apples is unknown. In this study, we examined the effect of fruit setting on anthocyanin accumulation in the flesh of ‘Geneva’ and ‘Pink Pearl’ apples. In addition, we investigated the effect of light conditions on anthocyanin accumulation in the flesh of both cultivars. Finally, we compared the fruit quality of ‘Pink Pearl’ apples grown in two different climatic regions in Japan to explore whether anthocyanin accumulation in fruit flesh was influenced by the temperature of the growing region, as is true of red-skinned fruit.

### Materials and Methods

#### Plant materials

Trees of two red-fleshed apple cultivars, ‘Geneva’ and ‘Pink Pearl’, were grown at the Apple Research Station, NARO Institute of Fruit Tree and Tea Science, Morioka, Japan (39°8’N, 141°1’E, 193 m altitude). The trees grafted onto M.9 rootstock at Morioka were 6 years of age in 2013 (Soejima et al., 2010). In 2013, 2014, and 2015, the full bloom days at Morioka were 20, 11, and 2 May, respectively. In the experiment comparing fruit quality in two different climatic regions, ‘Pink Pearl’ apples harvested at Suzaka (36°7’N, 138°3’E, 363 m altitude) were transported to Morioka at 4°C and subjected to fruit quality evaluation. The trees grafted onto M.9 rootstock at Suzaka were 12 years of age in 2014. In 2014 and 2015, the full bloom days at Suzaka were 1 May and 26 April, respectively. The annual mean air temperatures were 11.9°C at Suzaka and 10.2°C at Morioka. Except for thinning treatments, the ‘Geneva’ and ‘Pink Pearl’ trees were grown using conventional orchard practices.

#### Fruit thinning treatment

Thinning treatments were carried out in 2014. Two levels of thinning were applied to the ‘Geneva’ and ‘Pink Pearl’ trees: every fourth cluster that had a single fruitlet (control) or every cluster that had a single fruitlet (bearing excess fruit). The leaf/fruit ratios in the control trees and the trees bearing excess fruit were approximately 60 and 15, respectively. Thinning was finished by 20 June (40 days after full bloom, DAFB). The ‘Geneva’ and ‘Pink Pearl’ apples were harvested on 2 and 9 September (114 and 121 DAFB), respectively. Treatments were performed using one tree from each cultivar, and all fruit were harvested for analyses. Three to six representative apples in each weight class from each cultivar were used for further analyses.

#### Fruit bagging treatment

The ‘Geneva’ and ‘Pink Pearl’ apples were bagged in double-layer paper bags (Hirosaki Joto Seitai, Aomori, Japan). The shading rate of the bag was nearly 100% (K. Yoshimura, personal communication). The fruit of three trees was randomly bagged on 16 July 2013 (57 DAFB) and again on 26 June 2015 (55 DAFB). The bagged ‘Geneva’ apples were harvested on 17 September 2013 (120 DAFB) and 25 August 2015 (115 DAFB). The bagged ‘Pink Pearl’ apples were harvested on 19 September 2013 (122 DAFB) and 3 September 2015 (124 DAFB). In 2015, the unbagged and bagged apples were harvested after fruit position (sunlight or shade) and the sun-exposed sides were marked. The apples were removed from the bags just prior to anthocyanin extraction.
Fruit traits
To measure anthocyanin concentrations, pieces of the flesh from the equatorial part of each fruit were cut in columnar form (0.51 cm^2 per piece) using a cork borer. In the 2013 and 2014 experiments, four pieces were cut to make them more representative of the color of the whole flesh of each fruit. In the 2015 experiment, four pieces were cut from the side (sun-exposed or shaded side) of each fruit (eight pieces per fruit in total). The pieces were then soaked in 10 mL hydrochloric acid/methanol (1:99 v/v) at 4°C overnight. The absorbance of each extract was measured at 530, 620, and 650 nm with a spectrometer (UVmini-1240; Shimadzu, Kyoto, Japan). The anthocyanin concentration was calculated following the method of Siegelman and Hendricks (1958). Fruit firmness was measured on opposite sides of the fruit with a fruit pressure tester (FT327; FACCHINI srl, Alfosine, Italy). To rate the starch index, apples were cut at the equator and stained with an iodine solution, and then the starch pattern and intensity were estimated on a scale of 0–5 (0 = completely white to 5 = completely black). The soluble solids concentration in the juice was assessed by refraction (°Brix) using a refractometer (PR-100; ATAGO, Tokyo, Japan). The titration acidity in the juice was determined by titration with 0.1 N NaOH (AUT-701; TOA-DKK, Tokyo, Japan) and is expressed as g malic acid per 100 mL juice.

Statistical analysis
Analysis of variance (ANOVA) using the PRC GLM (SAS Institute, Inc, Cary, NC, USA) procedure was used to test for differences between groups. Means were compared using Tukey–Kramer’s tests or t-tests.

Results
Effect of thinning treatment on anthocyanin accumulation in flesh
In both cultivars, the trees bearing excess fruit generally bore smaller apples than the control trees (Fig. 1). The modal weights of ‘Geneva’ apples were 225 and 150 g in the control tree and the tree bearing excess fruit, respectively. The modal weights of ‘Pink Pearl’ apples were 225 and 175 g in the control tree and the tree bearing excess fruit, respectively.

In the control trees of both cultivars, there were no significant differences in anthocyanin concentration in the fruit flesh among the weight classes (Fig. 2A, B). In the ‘Geneva’ tree bearing excess fruit, there was no significant difference in anthocyanin concentration in the flesh among apple weight classes (Fig. 2C), but the concentration in ‘Pink Pearl’ apples ≥ 226 g was higher than that in smaller classes (Fig. 2D). Excess fruit set significantly reduced anthocyanin accumulation in the flesh of both cultivars compared with the same weight classes, except for the ‘Pink Pearl’ apples ≥ 226 g in the trees bearing excess fruit (t-test, P < 0.05). The anthocyanin concentrations in ‘Geneva’ apples ≤ 200 g and 201–225 g in the control tree were 258.9 and 263.0 μg·cm^−3, respectively, and concentrations in apples of 176–200 g and ≥ 201 g in the tree bearing excess fruit were 82.7 and 124.2 μg·cm^−3, respectively. The anthocyanin concentrations of ‘Pink Pearl’ apples ≤ 200 g and 201–225 g in the control tree were 19.3 and 22.0 μg·cm^−3, respectively, and those in the 176–200-g and 201–225-g classes in the tree bearing excess fruit were 9.8 and 8.5 μg·cm^−3, respectively. In contrast, there was no significant difference in anthocyanin concentrations between the 226–250-g class in the control
tree and the ≥226-g class in the tree bearing excess fruit. In both cultivars, there were no significant differences in soluble solids concentrations between the thinning treatments at equal weight classes (data not shown). For example, the soluble solids concentrations in ‘Geneva’ apples weighing 201–225 g from the control tree and ≥201 g from the tree bearing excess fruit were 10.4 and 10.8°Brix, respectively. The soluble solids concentrations in ‘Pink Pearl’ apples weighing 226–250 g from the control tree and ≥226 g from the tree bearing excess fruit were 11.8 and 11.6°Brix, respectively.

The titration acidities in ‘Geneva’ apples from the control tree were higher in smaller classes, whereas the titration acidities did not differ significantly among the ‘Pink Pearl’ size classes (Fig. 3A, B). In contrast, titration acidities were higher in the larger size classes from

**Fig. 2.** Effect of excess fruit set on anthocyanin synthesis in the fruit flesh of ‘Geneva’ and ‘Pink Pearl’ cultivars. Anthocyanin concentration in the flesh is presented by fruit weight classes. A, ‘Geneva’ and B, ‘Pink Pearl’, where one fourth cluster had a single fruitlet (Control); C, ‘Geneva’ and D, ‘Pink Pearl’, where one cluster had a single fruitlet (Excess). The thinning experiment was conducted in 2014. Each bar represents the mean of three to six replicates; error bars indicate ±1 SE. Different letters indicate significant differences between weight classes at P < 0.05, as determined using Tukey–Kramer’s tests.

**Fig. 3.** Effect of excess fruit set on titration acidity in the fruit of ‘Geneva’ and ‘Pink Pearl’ cultivars. Titration acidity in the fruit is presented by fruit weight classes. A, ‘Geneva’ and B, ‘Pink Pearl’, where one fourth cluster had a single fruitlet (Control); C, ‘Geneva’ and D, ‘Pink Pearl’, where one cluster had a single fruitlet (Excess). The thinning experiment was conducted in 2014. Each bar represents the mean of three to six replicates; error bars indicate ±1 SE. Different letters indicate significant differences between weight classes at P < 0.05, as determined using Tukey–Kramer’s tests.
the trees bearing excess fruit in both cultivars (Fig. 3C, D). In both cultivars, excess fruit set lowered titration acidities of the fruit compared with the same weight classes in the control (t-test, \( P < 0.05 \)). The titration acidities of ‘Geneva’ apples of sizes \( \leq 200 \) and \( 201–225 \) g in the control were 1.06 and 0.98 g/100 mL, respectively, whereas apples of sizes 176–200 and \( \geq 201 \) g from the tree bearing excess fruit were 0.69 and 0.78 g/100 mL, respectively. The titration acidities of ‘Pink Pearl’ apples of sizes \( \leq 200 \), 201–225, and 226–250 g from the control were 1.36, 1.42, and 1.35 g/100 mL, respectively, whereas apples of sizes 176–200, 201–225, and \( \geq 226 \) g from the tree bearing excess fruit were 1.08, 1.17, and 1.12 g/100 mL, respectively.

**Effect of light conditions on anthocyanin accumulation in flesh**

The anthocyanin concentrations in the flesh of unbagged and bagged apples, which were randomly sampled from whole trees, were measured in 2013 (Table 1). When apples were bagged at 57 DAFB, the flesh of ‘Geneva’ apples was already red, whereas that of ‘Pink Pearl’ apples was intensely red at 57 DAFB. The concentration in bagged fruit was lower than that in unbagged fruit (Table 1). In contrast, the treatment did not significantly influence pigmentation in the flesh of ‘Pink Pearl’ apples, although the mean anthocyanin concentration in bagged fruit was lower than that in unbagged fruit (Table 1). The skin of bagged ‘Geneva’ apples was pale pink at harvest. It was not clear whether anthocyanin remained in the skin until harvest or accumulated after the bagging treatment as the skin of ‘Geneva’ apples was intensely red at 57 DAFB. The skin of bagged ‘Pink Pearl’ apples also looked pale pink at harvest because the red flesh showed through the translucent yellow skin.

Unbagged and bagged fruit were sampled based on fruit position (sunlight or shade) in 2015. Anthocyanin was extracted from the flesh on opposite sides (sun-exposed or shaded side) for both fruit positions. ANOVA was conducted for each cultivar to compare the effects of bagging, fruit position, and fruit side on anthocyanin development in the flesh (Tables 2 and 3). The results of the bagging treatment were similar to those in 2013. The anthocyanin concentration in the flesh of bagged ‘Geneva’ apples was significantly reduced (Table 2), whereas there was no significant difference in anthocyanin concentrations between unbagged and bagged ‘Pink Pearl’ apples (Table 3). In

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**Table 1.** Effect of bagging on anthocyanin synthesis in the fruit flesh of the ‘Geneva’ and ‘Pink Pearl’ cultivars.

| Cultivar      | Anthocyanin conc. (μg·cm\(^{-3}\)) | Unbagged | Bagged |
|---------------|-----------------------------------|----------|--------|
| Geneva        | 270.5a                            | 195.4b   |        |
| Pink Pearl    | 55.4c                             | 42.9c    |        |
| ANOVA         |                                   |          |        |
| Cultivar (C)  | **                                |          |        |
| Bagging (B)   | **                                |          |        |
| C×B           | **                                |          |        |

* The bagging treatment was conducted in 2013, and 30 apples were sampled for each treatment.

**Table 2.** Effect of light conditions on anthocyanin synthesis in the fruit flesh of the ‘Geneva’ cultivar.

| Main effect | Anthocyanin conc. (μg·cm\(^{-3}\)) | Unbagged | Bagged |
|-------------|-----------------------------------|----------|--------|
| Fruit Position |                                   |          |        |
| Sunlight    | 154.5a                            | 83.9b    |        |
| Shade       | 94.7b                             | 88.9b    |        |
| ANOVA       |                                   |          |        |
| Bagging (B) | *                                 |          |        |
| Fruit position (FP) | **            |          |        |
| Fruit side (FS) | NS                        |          |        |
| B×FP        | *                                 |          |        |
| B×FS        | NS                                |          |        |
| FP×FS       | NS                                |          |        |
| B×FP×FS     | NS                                |          |        |

* The bagging treatment was conducted in 2015, and 12–20 apples with or without bagging treatment were sampled for each fruit position.

NS, *, and ** indicate not significant or significant differences of \( P < 0.05 \), and \( P < 0.01 \), respectively, by ANOVA.

**Table 3.** Effect of light conditions on anthocyanin synthesis in the fruit flesh of the ‘Pink Pearl’ cultivar.

| Main effect | Anthocyanin conc. (μg·cm\(^{-3}\)) | Unbagged | Bagged |
|-------------|-----------------------------------|----------|--------|
| Fruit Position |                                   |          |        |
| Sunlight    | 59.0a                             | 45.0b    |        |
| Shade       | 37.1c                             | 49.3b    |        |
| ANOVA       |                                   |          |        |
| Bagging (B) | NS                                |          |        |
| Fruit position (FP) | *                             |          |        |
| Fruit side (FS) | NS                        |          |        |
| B×FP        | **                                |          |        |
| B×FS        | NS                                |          |        |
| FP×FS       | NS                                |          |        |
| B×FP×FS     | NS                                |          |        |

* The bagging treatment was conducted in 2015; 12–20 apples that were bagged or not bagged were sampled for each fruit position. NS, *, and ** indicate not significant or significant differences of \( P < 0.05 \), and \( P < 0.01 \), respectively, by ANOVA.
both cultivars, fruit position had a main effect on pigmentation (Tables 2 and 3). Anthocyanin concentrations in the flesh of unbagged apples that were in sunlight were significantly higher than those in apples that were in shade, but there was no difference between positions for bagged fruit. There was no interaction effect for fruit side in either cultivar (Tables 2 and 3), indicating that anthocyanin concentrations in the flesh from the sun-exposed and shaded sides were the same regardless of fruit position.

**Comparison of fruit quality between two growing regions**

Fruit quality of ‘Pink Pearl’ apples harvested at Suzaka, a warmer region, and Morioka, a cooler region, was compared over two years (Table 4). Although there was no significant difference in fruit weight between the two locations, the apples harvested at Suzaka had lower firmness, starch index, and titration acidity, and higher soluble solids concentration compared with apples at Morioka. Annual variations were insignificant in fruit traits, except firmness. The anthocyanin concentration in the flesh of apples from Morioka was significantly higher than that in fruit from Suzaka. Preharvest drop was severe in ‘Pink Pearl’. It is difficult to judge the optimum harvest time by tasting fruit because apples have a low sugar content and are highly acidic. Thus, fruit was harvested at the beginning of the preharvest drop at both Suzaka and Morioka. At this time, senescent apple breakdown was not apparent in either region, as revealed by the fruit firmness data (Table 4). We found that the soluble solids concentration in Suzaka fruit was higher than that in Morioka fruit (Table 4). Therefore, we suggest that the lower anthocyanin concentration in fruit harvested at Suzaka was not attributable to early harvesting.

The anthocyanin concentrations in the flesh of ‘Pink Pearl’ apples harvested at Morioka on 4 and 9 September 2014 were 21.5 and 47.3 μg·cm⁻³, respectively. In 2015, the anthocyanin concentrations in the flesh of fruit harvested at Morioka on 21 August and 2 September were 2.4 and 48.9 μg·cm⁻³, respectively. These results suggest that anthocyanin in the flesh rapidly accumulated during the last 10 days at Morioka. Changes in air temperature at Suzaka and Morioka in 2014 and 2015 are shown in Figure 4. In both years, the maximum air temperature from full bloom to harvest was higher at Suzaka than at Morioka, whereas the minimum air temperature from mid-July to mid-August was similar at both locations. After mid-August, the minimum air temperature at Morioka decreased compared with that at Suzaka. The initiation of anthocyanin synthesis coincided with this decrease in minimum air temperature at Morioka. These results suggest that the continuous higher air temperature during fruit development and maturation at Suzaka inhibited anthocyanin accumulation in the fruit flesh, while the lower air temperature after mid-August at Morioka promoted its accumulation.

**Discussion**

Thinning treatments are important for producing high-quality fruit and preventing biennial bearing in apple trees. An early study showed that a low leaf/fruit ratio reduced the soluble solids concentration and red coloration of ‘Delicious’ apples (Magness, 1928). Recent reports also demonstrated that the soluble solids content was lower in high-cropping trees of ‘Braeburn’ (Wünsche et al., 2000) and that ‘Gala’ apples were lower in soluble solids content and acidity with excess fruit (Kon et al., 2013). In this study, we showed that the anthocyanin concentration of apple flesh, fruit weight, and titration acidity of apples from trees bearing excess fruit were lower compared with the control trees in red-fleshed ‘Geneva’ and ‘Pink Pearl’ cultivars (Figs. 1, 2, and 3). As the titration acidities of both cultivars are quite high compared with those of common eating apples, excess fruit set may improve fruit quality; however, our study demonstrated that thinning was essential to produce larger fruit with redder flesh. In

| Location | Year  | Anthocyanin conc. (μg·cm⁻³) | Fruit weight (g) | Firmness (N) | Starch index (0–5) | Soluble solids conc. (°Brix) | Titration acidity (g/100 mL) |
|----------|-------|-----------------------------|------------------|--------------|-------------------|-----------------------------|-----------------------------|
| Suzaka   | 2014  | 2.7b                        | 255.9            | 74.3c        | 1.6b              | 12.8a                       | 1.07c                       |
|          | 2015  | 4.1b                        | 269.1            | 79.6c        | 2.1b              | 12.8a                       | 1.15bc                      |
| Morioka  | 2014  | 47.3a                       | 280.4            | 81.0bc       | 2.7a              | 12.2b                       | 1.27ab                      |
|          | 2015  | 48.9a                       | 258.1            | 88.1ab       | 2.7a              | 11.7c                       | 1.38a                       |

**Significance**

- **P** < 0.05, and **P** < 0.01, respectively, by ANOVA.

* Seven apples were sampled for each region.

7 The harvest days at Suzaka were 29 August 2014 (120 DAFB) and 19 August 2015 (115 DAFB). The harvest days at Morioka were 9 September 2014 (121 DAFB) and 2 September 2015 (123 DAFB).

NS, *, and ** indicate not significant or significant differences of *P*<0.05, and *P*<0.01, respectively, by ANOVA.
both cultivars, the soluble solids concentration was not influenced by the thinning treatment when apples of comparable weight were compared. The soluble solids concentrations of ‘Pink Pearl’ fruit harvested at Morioka in 2014 and 2015 were 12.2 and 11.7°Brix, respectively (Table 3), which were similar to levels in the 2014 thinning experiment. This suggests that the fruit/leaf ratio was adequate to accumulate sugar in apples from the trees bearing excess fruit, as soluble solids concentrations in ‘Geneva’ and ‘Pink Pearl’ apple fruits were originally lower compared with those of other cultivars grown for table use. Sugar accumulation in apples is required for anthocyanin synthesis because UDP-glycosides are direct substrates for cyanidin 3-glycosides, which are pigments in the apple skin and flesh (Rupasinghe et al., 2010; Saure, 1990). In ‘Geneva’ and ‘Pink Pearl’ apples, even lower soluble solids concentrations in the fruit may be sufficient to synthesize the substrates for anthocyanin synthesis in the flesh.

Anthocyanin synthesis is highly light dependent in common apple cultivars with white flesh and red skin (Saure, 1990; Ubi, 2004). In such apples, no anthocyanin accumulates in the skin under dark conditions (Arakawa, 1991). In our study, however, the bagging treatment demonstrated that anthocyanin accumulated in the flesh of ‘Geneva’ and ‘Pink Pearl’ apples under dark conditions (Tables 1, 2, and 3). Although the flesh of the ‘Geneva’ apples was already red during bagging, anthocyanin was considered to be synthesized after bagging because the fruit weight and anthocyanin concentration in the flesh increased seven- and two-fold, respectively, during the maturation stages. Sunlight irradiation was associated with an increase in anthocyanin synthesis in the flesh of unbagged apples in both cultivars (Tables 2 and 3). No interaction effect for fruit side was found in either cultivar (Tables 2 and 3), suggesting that in the unbagged fruit grown in sunlight, the flesh from the shaded side was just as red as that on the sun-exposed side. Therefore, it is important for ‘Geneva’ and ‘Pink Pearl’ fruit to be fully exposed to sunlight to increase anthocyanin synthesis in the flesh, although fruit accumulated a certain level of anthocyanin without sunlight irradiation. Light-dependent signal transduction pathways responsible for expression of the MYB TF genes and anthocyanin synthetic genes in apples may differ between the flesh and the skin.

Our preliminary experiment showed that the air temperature inside bags of fruit exposed to sunlight was maximally 6°C higher than the outside air temperature, as was that inside bags of fruit kept in the shade (when the outside air temperature was 26°C on a sunny day).
(data not shown). Meanwhile, air temperatures inside bags of fruit and the outside air temperatures were the same at night (data not shown). The surface temperature of unbagged fruit exposed to sunlight was maximally 8°C higher than the surface temperature of shaded fruit under the same conditions (data not shown). As warmer climatic conditions during ripening suppress anthocyanin accumulation in apple skins (Honda et al., 2014; Lin-Wang et al., 2011), it may be that the high temperature reached inside bags of fruit exposed to sunlight reduced anthocyanin accumulation in the flesh compared with that of unbagged fruit (Tables 2 and 3). However, in the bagged fruit of both cultivars, the anthocyanin concentrations in the flesh were the same when the bags were exposed to sunlight or shade (Tables 2 and 3), although the air temperature inside bags exposed to sunlight was higher than that inside shaded bags. In the unbagged fruit of both cultivars, the anthocyanin concentrations in the flesh of fruit exposed to sunlight were significantly higher than those of shaded fruit (Tables 2 and 3), although the surface temperature of fruit exposed to sunlight was higher. Therefore, in the bagging experiment, sunlight irradiation may have had a more critical influence on fruit flesh pigmentation than daytime temperature conditions.

Sugiura et al. (2013) reported that the annual mean temperature rose by 3.1°C per decade at Suzaka over the last 40 years. Over this period of time in ‘Tsugaru’ apples, a major early-ripening cultivar in Japan, firmness and acid concentration significantly decreased, whereas soluble solids concentration increased, although not significantly. In our study, ‘Pink Pearl’ apples from the trees grown at Suzaka were lower in terms of firmness and titration acidity, and higher in soluble solids concentration than those at Morioka (Table 4). Decreases in firmness and acid concentration, and increases in soluble solids concentration in apples from trees grown under warmer conditions are considered common in both early-ripening cultivars.

As previous studies have reported, anthocyanin synthesis in apple skin was inhibited under warmer climatic conditions throughout the day during ripening (Honda et al., 2014; Lin-Wang et al., 2011). We found that anthocyanin accumulation in the flesh of apples grown at Suzaka was markedly suppressed compared with those grown at Morioka (Table 3). During fruit development and maturation stages in 2014 and 2015, the maximum air temperature at Suzaka was higher than that at Morioka (Fig. 4). The decrease in minimum air temperature coincided with rapid anthocyanin accumulation in the apple flesh at Morioka in two different years (Fig. 4). These results indicate that high maximum air temperature during the growing period and low minimum air temperatures before harvest had inhibitory and inductive effects on pigmentation in the flesh of ‘Pink Pearl’ apples, respectively. This is similar to the findings of Blankenship (1987), who showed that the fruit of ‘Red Chief’ apples from potted trees grown under warm night conditions had paler red skin than did those grown under cool night conditions. One month before harvest in 2014, the maximum air temperature at Morioka remained almost unchanged (approximately 25°C) (Fig. 4A), but it decreased from 27 to 20°C in 2015 (Fig. 4B). In both years, anthocyanin concentrations in the flesh of ‘Pink Pearl’ apple were the same (Table 4). These results indicate that a decrease in maximum air temperature at Morioka after mid-August in 2015 did not affect anthocyanin synthesis in the flesh of ‘Pink Pearl’ apples.

To summarize, an appropriate fruit load, growth in sunlight, and growth under cooler climatic conditions, all redden the flesh of red-fleshed apples, as is also true of common apples with white flesh and red skin. ‘Ruby Sweet’ and ‘Rose Pearl’ are mid-ripening cultivars whose fruits are harvested in mid-October at Morioka. The genotype of their MYB TF gene is the same as that of ‘Pink Pearl’ (type 2) (S. Moriya, personal communication). Future studies should investigate how other red-fleshed type 2 apples, including these two cultivars, respond to changes in air temperature and accumulate anthocyanin in fruit flesh during the development and ripening periods.

Acknowledgements

We thank Dr. K. Yoshimura of Yamaguchi Prefectural Industrial Technology Institute, for measuring the light permeability of the double-layer paper bag, and Dr. S. Moriya of NARO Institute of Fruit Tree and Tea Science, for critically reading this paper and providing information on the genotype of the MYB TF gene of ‘Ruby Sweet’ and ‘Rose Pearl’.

Literature Cited

Abe, K., J. Soejima, H. Iwanami, N. Kotoda, S. Moriya, H. Bessho, S. Komori, Y. Ito, S. Takahashi, K. Okada, H. Kato, T. Haji, M. Ishiguro, T. Masuda and S. Tsuchiya. 2013a. New red-fleshed apple cultivar ‘Rose Pearl.’ <http://www.naro.affrc.go.jp/project/results/laboratory/fruit/2013/fruit13_s05.html> (In Japanese).

Abe, K., J. Soejima, H. Iwanami, N. Kotoda, S. Moriya, S. Takahashi, Y. Ito, H. Bessho, K. Okada, H. Kato, S. Komori, T. Haji and M. Ishiguro. 2013b. New red-fleshed apple cultivar ‘Ruby Sweet.’ <http://www.naro.affrc.go.jp/project/results/laboratory/fruit/2013/fruit13_s04.html> (In Japanese).

Arakawa, O. 1991. Effect of temperature on anthocyanin accumulation in apple fruit as affected by cultivars, stages of fruit ripening and bagging. J. Hort. Sci. 66: 763–768.

Ban, Y., C. Honda, Y. Hatusuyama, M. Igarashi, H. Bessho and T. Moriguchi. 2007. Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. Plant Cell Physiol. 48: 958–970.

Blankenship, S. M. 1987. Night-temperature effects on rate of apple fruit maturation and fruit quality. Sci. Hortic. 3: 205–
Chagné, D., C. M. Carlisle, C. Blond, R. K. Volz, C. J. Whiteworth, N. C. Oragaucie, R. N. Crowhurst, A. C. Allan, R. V. Espley, R. P. Hellens and S. E. Gardiner. 2007. Mapping a candidate gene (MdMYB10) for red flesh and foliage colour in apple. BMC Genomics 8: 212.

Chagné, D., K. Lin-Wang, R. V. Espley, R. K. Volz, N. M. How, S. Rouse, C. Brendolise, C. M. Carlisle, S. Kumar, N. De Silva, D. Micheletti, T. McGhie, R. N. Crowhurst, R. D. Storey, R. Velasco, R. P. Hellens, S. E. Gardiner and A. C. Allan. 2013. An ancient duplication of apple MYB transcription factors is responsible for novel red fruit-flesh phenotypes. Plant Physiol. 161: 225–239.

Faramarzi, S., A. Yoddollahi and B. M. Soltani. 2014. Preliminary evaluation of genetic diversity among Iranian red fleshed apples using microsatellite markers. J. Agric. Sci. Technol. 16: 373–384.

Honda, C., H. Bessho, M. Murai, H. Iwanami, S. Moriya, K. Abe, M. Wada, Y. Moriya-Tanaka, H. Hayama and M. Tatsuki. 2014. Effect of temperature on anthocyanin synthesis and ethylene production in the fruit of early- and medium-maturing apple cultivars during ripening stages. HortScience 49: 1510–1517.

Iwanami, H., Y. Moriya-Tanaka, T. Hanada, C. Honda and M. Wada. 2016. Labor-saving production of apple with red coloration and marked eating quality by effective use of a chemical defoliant. Hort. Res. (Japan) 15: 29–37 (In Japanese with English abstract).

Kon, T. M., J. R. Schupp, H. E. Winzeler and R. P. Marini. 2013. Influence of mechanical string thinning treatments on vegetative and reproductive tissues, fruit set, yield, and fruit quality of ‘Gala’ apple. HortScience 48: 40–46.

Lin-Wang, K., D. Micheletti, J. Palmer, R. Volz, L. Lozano, R. Espley, R. P. Hellens, D. Chagné, D. D. Rowan, M. Troggio, I. Iglesias and A. C. Allan. 2011. High temperature reduces apple fruit colour via modulation of the anthocyanin regulatory complex. Plant Cell Environ. 34: 1176–1190.

Magness, J. R. 1928. Observations on colour development in apples. Proc. Amer. Soc. Hort. Sci. 25: 289–292.

Rupasinghe, H. P. V., G. M. Huber, C. Embree and P. L. Forsline. 2010. Red-fleshed apple as a source for functional beverages. Can. J. Plant Sci. 90: 95–100.

Saure, M. C. 1990. External control of anthocyanin formation in apple. Sci. Hortic. 42: 181–218.

Siegelman, H. W. and S. B. Hendricks. 1958. Photocontrol of anthocyanin synthesis in apple skin. Plant Physiol. 33: 185–190.

Soejima, J., Y. Yoshida, T. Haniuwa, H. Bessho, S. Tsuchiya, T. Masuda, S. Komori, T. Sanada, Y. Ito, S. Sadamori and Y. Kashimura. 2010. New dwarfing apple rootstocks ‘JM1, JM7’ and ‘JM8’. Bull. Natl. Inst. Fruit Tree Sci. 11: 1–16 (In Japanese with English abstract).

Sugiura, T., H. Ogawa, N. Fukuda and T. Moriguchi. 2013. Changes in the taste and textural attributes of apples in response to climate change. Sci. Rep. 3: 2418.

Takos, A. M., F. W. Jaffé, S. R. Jacob, J. Bogs, S. P. Robinson and A. R. Walker. 2006. Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. Plant Physiol. 142: 1216–1232.

Ubi, B. E. 2004. External stimulation of anthocyanin biosynthesis in apple fruit. J. Food Agric. Environ. 2: 65–70.

Umemura, H., S. Otagaki, M. Wada, S. Kondo and S. Matsumoto. 2013. Expression and functional analysis of a novel MYB gene, MdMYB110a_JP, responsible for red flesh, not skin color in apple fruit. Planta 238: 65–76.

Volz, R. K., S. Kumar, D. Chagné, R. Espley, T. K. McGhie and A. C. Allan. 2013. Genetic relationships between red flesh and fruit quality traits in apple. Acta Hort. 976: 363–368.

Wünsche, J. N., J. W. Palmer and D. H. Greer. 2000. Effects of crop load on fruiting and gas-exchange characteristics of ‘Braeburn’/M.26 apple trees at full canopy. J. Amer. Soc. Hort. Sci. 125: 93–99.