Sugar Accumulation Profile during the Ripening Process in the Pollination-Constant Non-Astringent Japanese Persimmon ‘Akiou’

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We monitored sugar accumulation profiles and developmental changes in fruit traits during the ripening process in pollination-constant non-astringent (PCNA)-type persimmon cultivars of ‘Akiou’, ‘Fuyu’ and ‘Taishuu’. As the fruit ripened, transverse and longitudinal diameter, fruit weight, soluble solids content (SSC), and flesh juiciness increased significantly, with a constant varietal ranking throughout the ripening process. Color development of ‘Akiou’ and ‘Taishuu’ seemed to be faster than that of ‘Fuyu’ during the initial stages of fruit ripening, but there was little difference in color chart values toward the latter stage of fruit ripening. Flesh firmness decreased steadily over time, whilst varietal ranking remained constant. The varietal ranking of SSC in the latter stage of ripening was ‘Akiou’ $\geq$ ‘Taishuu’ > ‘Fuyu’. Significant varietal differences were observed in sucrose, glucose, and fructose contents. Based on sugar accumulation profiles, the cultivars could be classified into two types: hexose accumulators (‘Taishuu’) and sucrose accumulators (‘Akiou’ and ‘Fuyu’). The transcriptional profiles of key sugar accumulation-related genes: sucrose synthase (*SuSy*), vacuolar acid invertase (*VAI*), and sucrose phosphate synthase (*SPS*), were also examined. Transcriptional levels of *SuSy* and *VAI* in sucrose accumulators were lower than those in the hexose accumulator. Conversely, transcriptional levels of *SPS* were higher in the sucrose accumulators than in the hexose accumulator. In particular, the concomitant and rapid increase of both *SPS* expression and sucrose accumulation in mid-October, suggests that this period is crucial for ‘Akiou’ in terms of the SSC elevation.

Key Words: *Diospyros kaki*, invertase, sucrose accumulator, sucrose phosphate synthase, sucrose synthase.

Introduction

In Japan, the Japanese persimmon (*Diospyros kaki* Thunb.) is a major fruit crop, ranking fourth in fruit production after citrus, apples, and Japanese pears (Ministry of Agriculture, Forestry and Fisheries, 2015). Persimmon cultivars can be generally classified into four types based on seed formation and the astringency of the mature fruit (reviewed by Yonemori et al., 2000): pollination-constant non-astringent (PCNA, e.g. ‘Fuyu’, ‘Jiro’); pollination-variant non-astringent (PVNA, e.g. ‘Nishimurawase’, ‘Zenjimarū’); pollination-constant astringent (PCA, e.g. ‘Atago’, ‘Ichidagaki’); and pollination-variant astringent (PVA, e.g. ‘Aizumishirazu’, ‘Hiratanenashi’). Among these types, PCNA-type cultivars are preferred for fresh market use in Japan because they do not require postharvest astringency reduction (Yonemori and Matsushima, 1985).

The PCNA-type cultivar is an attractive, highly palatable fruit with a yellowish to reddish skin color. Since 1990, PCNA-type cultivars exhibiting superior fruit quality traits have been bred in Japan by the National Institute of Fruit Tree Science (e.g. ‘Kanshu’, ‘Soshu’ and ‘Taishuu’: Sato and Yamada, 2016; Yakushiji and Nakatsuka, 2007). PCNA-type cultivars have also been released by prefectural agricultural research institutes in Japan such as ‘Tokyo Beni’ (Kikuchi et al., 2011) and ‘Neo sweet’ (Niikawa et al., 2017). A seedless nonaploid PCNA-type cultivar ‘Fukuoka K1 Gou’ (TM: Akiou, hereafter described as ‘Akiou’), was bred in our laboratory using an embryo culture derived from a cross between ‘Fuyu’ $\times$ ‘Taishuu’ (Chijiwa et al., 2008, 2013). This cultivar ripens in mid-season and has an excellent texture (very juicy and soft flesh), high soluble solids content (SSC), a brilliant skin color, and large fruit.

Asakuma and Shiraishi (2017) proposed several descriptors which can be used to evaluate fruit skin color, flesh firmness, flesh juiciness, and sugar composition

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when breeding Japanese persimmons. They also suggested that improvement in the organoleptic properties of PCNA-type cultivars can be systematically achieved using a combination of these traits, which are particularly useful when attempting to improve sugar composition. As in other fruit crops (Kajiura et al., 1979; Suzuki et al., 1990), a higher proportion of sucrose is known to improve the organoleptic perception of sweetness in PCNA-type persimmon fruit. Previous studies have shown that the sugar composition of persimmon fruit differs between cultivars (Asakuma and Shiraishi, 2017; Hirai et al., 2004; Hirano et al., 1995; Suzuki et al., 2010). In addition, Zheng and Sugiura (1990) classified persimmon cultivars into two types; sucrose accumulators and hexose accumulators.

In the present study, we monitored changes in sugar composition, as well as other fruit quality traits, during the latter stage of the ripening process (stage III) in the PCNA-type Japanese persimmon ‘Akiou’ (mid-ripening and high SSC). Associations with the expression patterns of the genes linked to key enzymes controlling sugar accumulation were analyzed to improve our knowledge and increase the SSC of ‘Akiou’. To provide comparative data, we also examined the PCNA-type Japanese persimmon cultivars ‘Fuyu’ (late-ripening and moderate SSC) and ‘Taishuu’ (mid-ripening and high SSC), which were initially used to breed ‘Akiou’.

Materials and Methods

Plant materials

The study took place in 2016. Three trees per cultivar of ‘Akiou’, ‘Fuyu’, and ‘Taishuu’ were used in the experiment. Tree ages for the ‘Akiou’, ‘Fuyu’, and ‘Taishuu’ cultivars were 9, 36, and 19 years old, respectively. These cultivars are grown for commercially marketable fruit production using normal agricultural practices including pruning, flower and fruit thinning, irrigation, and soil and pest management (Yamada, 2006) in an open-field of the Fukuoka Agricultural and Forestry Research Center, Fukuoka, Japan (33°50' N and 130°57' E). From Sep 1 to Nov 15, the growth characteristics (transverse diameter, longitudinal diameter, and skin color of the apex, equatorial, and basal area) of 10 fruits per tree were examined at ~2 week intervals. Fruits were not removed from trees during measurement (Tables S1–S3). Four fruits were sampled per tree, chosen based on the results of the fruit growth examination mentioned above. Fruit quality traits—transverse diameter, longitudinal diameter, fruit weight, skin color (apex), flesh firmness, flesh juiciness, SSC, and sugar composition—were determined for each fruit sampled. Peeled and deseeded fruit flesh was ground in liquid nitrogen, and the resulting powder was stored at –30°C for later gene expression analysis.

Analysis of fruit quality traits

Fruit quality traits were assessed in accordance with

the methods described by Asakuma and Shiraishi (2017), with some modification. Skin color around the fruit apex was determined using a Chroma Meter (CR-300; Minoruta, Tokyo, Japan) and expressed as a color chart value using the following formula: color chart (CC) = $-7.274 \ln(\text{hue angle}) + 35.62$, $R^2 = 0.976$. Color chart values for Japanese persimmon were provided by the National Institute of Fruit Tree Science, Japan (Yamazaki and Suzuki, 1980), with a value of 0 indicating green skin and value of 10 indicating dark-red skin. Flesh firmness (kg) was determined using a hand-universal pressure tester with a 5.0-mm-diameter $\times$ 10.0-mm-height columnar plunger (KM-5; FUJIWARA SCIENTIFIC, Tokyo, Japan). Peeled flesh (5 to 10 g) was weighed and wrapped in one layer of medical gauze. After hand-pressing (a single press) for 15 s, the squeezed juice (flesh juiciness) was measured using a 25-mL mess cylinder and expressed as $\text{mL} \cdot \text{g}^{-1}$ FW. SSC (°Brix) of the resulting juice was determined using a portable calibrated electronic refractometer (PAL-1; Atago, Tokyo, Japan).

To analyze sugar composition, peeled flesh (15 to 20 g) was weighed and transferred to a 50-mL heat-tolerant tube partially secured with a screw-cap. Flesh samples consisted of four replicates and following sampling were immediately microwave-irradiated at 730 W for 60 s before extracting the sugars. The irradiated sample was then ground in a laboratory blender along with ~40 mL of deionized water. The puree was centrifuged at $5000 \times g$ at 25°C for 10 min. The resulting supernatant was brought to 50 mL in volume using deionized water and then filtered through a 0.45- μm filter. The sugar composition was analyzed using a HPLC (LC-10A; Shimadzu, Kyoto, Japan) consisting of a SCL-10A system controller, LC-10AD pumps, a CTO-10A column oven, and a RID-10A refractive index detector. The column (SCR-101N, 7.9×300 mm; Shimadzu) was operated at 60°C with $0.8 \text{ mL} \cdot \text{min}^{-1}$ of water. The injection volume was 10 μL .

Gene expression analysis

One milliliter of Fruit-mate for RNA Purification (Takara, Kyoto, Japan) containing 1% [v/v] of 2-mercaptoethanol was added to approximately 400 mg of frozen powder and vortexed for 1 min. The mixture was centrifuged at $10,000 \times g$ for 2 min at room temperature. A part (350 μL) of the supernatant was then transferred to a new 1.5 mL RNase-free tube. Then, 350 μL of RLT buffer from a RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was added, and the mixture was vortexed for 1 min at room temperature. Subsequent procedures for RNA extraction were performed according to the RNeasy Plant Mini Kit manufacturer’s instructions. cDNA was synthesized using the PrimeScript II cDNA synthesis kit (Takara) as described in the manufacturer’s manuals. qRT-PCR was performed using a 7500 Real-Time PCR system (Applied Biosystems, Forster

City, CA, USA) and a SYBR Premix Ex Taq II (Takara) as described in the manufacturer's manuals. The PCR protocol was as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s, 60°C for 34 s; 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The primer sequences used were as follows (Suzuki et al., 2010): sucrose synthase, *SuSy* (forward 5'-CCATTGCTCATGCTTTGGAG-3', reverse 5'-ATGTACCGGTAAAGCTCACC-3'); vacuolar acid invertase, *VAI* (forward 5'-TGGAATGTGG GAGTGTCTG-3', reverse 5'-CCAACCCCAACAAG ATTCTTC-3'); sucrose phosphate synthase, *SPS* (forward 5'-TGTTATTCCTCCTGGGATGG-3', reverse 5'-GCTTGCTGATCATGAGGATC-3'). Relative gene expression was calculated using the $\Delta\Delta C_t$ method with three biological replicates per cultivar. The amounts of transcripts were normalized against β -actin and converted using 'Taishuu' on Sep 1 (relative gene expression 1.0).

Results and Discussion

Fruit development

Development of persimmon fruit is a complex process exhibiting a double sigmoid growth curve with three distinct phases; namely, two periods of growth (stages I and III) separated by a lag phase (stage II) during which cell expansion slows and seeds mature (George et al., 1997; Nii, 1980; Yakushiji and Nakatsuka, 2007). Our current study was based on the agricultural records of 'Akiou', 'Fuyu', and 'Taishuu' from 2013 to 2015 (Fig. S1; Table S4), in which stage II of each cultivar was estimated according to the definition (the increase rate of the transverse diameter at $< 0.3 \text{ mm} \cdot \text{d}^{-1}$) by Zheng et al. (1990). In this study, we monitored developmental and biochemical profiles of persimmon fruit from stage III ('Akiou' and 'Taishuu') and from the latter part of stage II ('Fuyu'). As previously reported (Hirano et al., 1995; Hirata and Hayashi, 1978; Sugiura and Tomana, 1983; Yonemori and Matsushima, 1987), it is likely that both seeded and seedless persimmon cultivars exhibit similar patterns of fruit development. However, the growth curve of seedless 'Akiou' and the less seeded 'Taishuu' seemed to be continuous, with an unclear transition into stage II, as compared to that of seeded 'Fuyu'. As suggested by Ishida et al. (1990), the cessation of embryo growth associated with seed degeneration may occur in the fruit of 'Akiou' and 'Taishuu'.

Transverse diameter significantly increased as fruit ripened, and varietal ranking was constant over the course of development with 'Taishuu' > 'Akiou' > 'Fuyu' after Sep 1, 2016 (Fig. 1A). Similarly, longitudinal diameter increased over the season, again following the pattern of 'Taishuu' > 'Fuyu' > 'Akiou' (Fig. 1B). The respective ratio of length to diameter (longitudinal/transverse) in 'Akiou', 'Fuyu', and 'Taishuu' was 0.64, 0.75, and 0.70, respectively, indicating that the fruit of 'Akiou' is flat-shaped, being more similar to

Fig. 1. Changes in transverse diameter (A), longitudinal diameter (B), and fruit weight (C), during the ripening stages of three persimmon cultivars. Each point represents the mean of 3 trees $\pm$ SE. SE bars smaller than the symbol width are not visible. Means with the same letter on each sampling date are not significantly different (Tukey's test, $P < 0.05$).

'Hiratanenashi' (0.66: Zhou et al., 2010) than 'Fuyu' or 'Taishuu'. A significant increase was observed in fruit weight over time, and varietal ranking was constant throughout ripening, with 'Taishuu' > 'Akiou' > 'Fuyu' (Fig. 1C). Changes in color chart value (fruit apex) of the cultivars during ripening are shown in Figure 2. Color development in 'Akiou' and 'Taishuu' seemed to occur faster than in 'Fuyu' during the period from Sep 1 to Oct 1, but there was little difference in the color chart value during the latter stage of fruit ripening (after Oct 15). The ranking of color chart values on Nov 15 was 'Fuyu' (5.7) > 'Akiou' (5.3) > 'Taishuu' (5.1), at which point all cultivars reached the appropriate level for commercial use (5.0) (Niikawa et al., 2014).

Flesh firmness and juiciness are crucial factors when determining the palatability of persimmon fruit, as a soft and juicy texture are traits highly regarded by consumers (Ban et al., 2010; Mitani et al., 2015; Yamada et al., 1998). Flesh firmness of the cultivars decreased steadily, with a significant decrease in firmness occurring over the period from Oct 1 to Nov 15. The ranking on Nov 15 was 'Fuyu' (1.9 kg) > 'Akiou' (1.3 kg) >

Fig. 2. Changes in the color chart of 'Akiou', 'Fuyu', and 'Taishuu' during the ripening stages of persimmon cultivars. The color chart value was obtained using the following formula: color chart = $35.62 - 7.274 \cdot \ln(\text{hue angle})$, $R^2 = 0.976$. Means with the same letter on each sampling date are not significantly different (Tukey's test, $P < 0.05$).

'Taishuu' (0.9 kg) (Fig. 3A). Flesh juiciness increased rapidly at the start of ripening and subsequently remained constant until the end of the experiment (Fig. 3B). Flesh juiciness of 'Akiou' and 'Taishuu' ($> 0.35 \text{ mL} \cdot \text{g}^{-1} \text{ FW}$ in both) was significantly higher than that of 'Fuyu' ($< 0.26 \text{ mL} \cdot \text{g}^{-1} \text{ FW}$). Both 'Akiou' and 'Taishuu' are thus considered to have a soft and very juicy texture. SSC increased gradually throughout ripening, and the SSC of 'Akiou' and 'Taishuu' was significantly higher than that of 'Fuyu' from Oct 15 to Nov 15 (Fig. 3C). The varietal ranking of SSC on Nov 15 was 'Akiou' (18.1) $\geq$ 'Taishuu' (18.0) $>$ 'Fuyu' (16.2). Based on the agricultural records (Table S4), the SSC values at the optimum fruit ripening time of 'Akiou' (Nov 7) and 'Taishuu' (Oct 26) in this study (Fig. 3C) were estimated at 17.9 and 16.2, respectively. As for 'Fuyu', it is likely that the SSC exceeded 16.2 at an optimum fruit ripening time of Nov 24, based on the SSC curve of Fig. 3C. Consequently, the varietal ranking of each fruit trait among the cultivars (Figs. 1 to 3) agrees with results reported by other studies (Asakuma and Shiraishi, 2017; Chijiwa et al., 2013). This suggests that varietal differences in fruit quality traits may be (genetically) determined before stage III of fruit development. Furthermore, it is of significant note that higher SSC accumulation was observed in 'Akiou' and 'Taishuu' in terms of the breeding stock, even though these cultivars had already passed their optimum fruit ripening time.

Sucrose, glucose, and fructose are the primary sugars in persimmon fruit (Asakuma and Shiraishi, 2017; Hirai

et al., 2004; Hirano et al., 1995; Tsuji and Komiyama, 1987). Significant varietal differences in sucrose content were found during ripening, and the varietal ranking on Nov 15 was 'Akiou' ($10.8 \text{ g} \cdot 100 \text{ g}^{-1} \text{ FW}$) $\geq$ 'Fuyu' ($9.9 \text{ g} \cdot 100 \text{ g}^{-1} \text{ FW}$) $>$ 'Taishuu' ($8.0 \text{ g} \cdot 100 \text{ g}^{-1} \text{ FW}$) (Fig. 4A). In addition, a significant and rapid rise in sucrose accumulation was observed in 'Akiou' over the period from Oct 15 to Nov 1. Although there was a significant varietal ranking change in the seasonal change in glucose and fructose content over the course of fruit development (Fig. 4B, C), this variation was small when compared to the variation in sucrose content. Hexose sugars accumulated over the period from Sep 1 to Sep 15 in 'Taishuu', and remained constant until Nov 15. In 'Akiou' and 'Fuyu', levels of hexose sugars remained unchanged over the course of fruit development.

From the sugar accumulation profiles, the three cultivars fell into two broad types: hexose accumulators ('Taishuu'); and sucrose accumulators ('Akiou' and 'Fuyu'). In other fruits such as the tomato, melon, strawberry, Asian pear, and apple, both types as well as intermediates have been reported (Yamaki, 2010). Zheng and Sugiura (1990) reported that six persimmon cultivars could be classified as hexose accumulators ('Hiratanenashi', 'Kikuhira', 'Nigorokonashiba') and sucrose accumulators ('Atago', 'Hanagosho', 'Mikatanigosho'). Suzuki et al. (2010) also showed that hexose accumulators ('Kishu', 'Soshu', 'Taishuu') and sucrose accumulators ('Fuyu') are present among persimmon cultivars. Varietal differences in the sugar com-

Fig. 3. Changes in flesh firmness (A), flesh juiciness (B), and soluble solid content (C) during the ripening stages of three persimmon cultivars. Each point represents the mean of 3 trees $\pm$ SE. SE bars smaller than the symbol width are not visible. Means with the same letter on each sampling date are not significantly different (Tukey's test, $P < 0.05$).

positions of persimmon fruit analyzed in the current study suggest the involvement of genetic controls on sugar-metabolizing enzymes, especially in sucrose cleavage.

Gene expression analysis

Sucrose is the translocated sugar in persimmon fruit (Zimmermann and Ziegler, 1975) and is generally broken down into glucose, fructose, or UDPglucose by SuSy or invertase. After translocation, SPS functions actively to resynthesize sucrose within the fruit (Yamaki, 2010). In this study, the transcriptional profiles of *SuSy*, *VAI*, and *SPS* were examined to determine the expression level of key sugar accumulation-related genes during the ripening of persimmon cultivars. The primers used in this study were not derived from the base sequences specific to persimmon (Suzuki et al., 2010), but were instead derived from a highly conserved region among plant species obtained from the NCBI database (Maglott et al., 2006).

Transcriptional levels of *SuSy* and *VAI* exhibited similar expression patterns throughout the ripening process

Fig. 4. Changes in sucrose (A), glucose (B), and fructose (C) content during the ripening stages of three persimmon cultivars. Each point represents the mean of 3 trees $\pm$ SE. SE bars smaller than the symbol width are not visible. Means with the same letter on each sampling date are not significantly different (Tukey's test, $P < 0.05$).

among the cultivars (Fig. 5), and their transcriptional ranking was 'Taishuu' > 'Fuyu' > 'Akiou' in accordance with the observed decline in sucrose accumulation. Based on the sugar and transcriptional profiles observed during ripening, the activity of SuSy in persimmon fruit is considered to promote sucrose breakdown in a way similar to that seen in other plants (Yamaki, 2010). Invertase enzymes are generally divided into three types according to their cellular localization; cell wall-bound (apoplastic) acid invertase (BAIV), cytoplasmic neutral invertase (CNIV), and vacuolar acid invertase (VAI). As reported previously (Hirano et al., 1995; Tsuji and Komiyama, 1987; Zheng and Sugiura, 1990), VAI activity during ripening could be the principal cause of varietal differences in sucrose accumulation in persimmon fruit. Zheng and Sugiura (1990) also showed that BAIV activity in hexose- and sucrose-accumulating cultivars is similar throughout fruit development. Suzuki et al. (2010) demonstrated by RT-PCR analysis that the expression levels of *SuSy* and *VAI* are higher in hexose-accumulating persimmon cultivars than in sucrose-accumulating cultivars, which agrees with our results. In addition, they postulated that varietal differ-

Fig. 5. Relative gene expression of *SuSy* (sucrose synthase), *VAI* (vacuolar acid invertase) and *SPS* (sucrose phosphate synthase) in fruit flesh during the ripening stages of three persimmon cultivars: 'Akiou', 'Fuyu', and 'Taishuu', represented by black, gray, and white squares, respectively. Data are presented as means of 3 replicates $\pm$ SE using quantitative real-time PCR analysis ($\Delta\Delta C_t$ method; β -actin and 'Taishuu' on Sep 1 were used as a reference gene and calibrator, respectively). Means with the same letter on each sampling date are not significantly different (Tukey's test, $P < 0.05$).

ences in the proportion of sucrose may result from the balance between *SuSy* and *VAI* expression. The ratio of sucrose to hexose in the tomato is primarily controlled by the level of *VAI* activity (Klann et al., 1993; Stommel, 1992), whereas sucrose accumulation in the melon depends on the level of *SPS* activity (Hayata et al., 2001; Hubbard et al., 1989). Similar to the grape (Deluc et al., 2007; Martínez-Esteso et al., 2011; Zhang et al., 2006), it is possible that *SuSy* and *VAI* act in a co-operative manner to achieve sucrose cleavage in persimmon fruit.

Sucrose resynthesis by *SPS* activity in mature sink organs has been reported in sucrose-accumulating crops

such as sugar beet (Fieuw and Willenbrink, 1990), sweet melon (Lingle and Dunlap, 1987), and wild tomato (Miron and Schaffer, 1991). Hubbard et al. (1989) proposed a scheme whereby sucrose accumulation in sweet melon is determined by the relationship between *SPS* activity and sucrose breakdown (facilitated by *VAI* and *SuSy*) processes. Komatsu et al. (2002) reported that *SPS* and *SuSy* during ripening play important roles in building sink strength when sucrose is accumulated in citrus fruit. From these findings, higher *SPS* activity and lower *VAI* and/or *SuSy* activity may actively contribute to sucrose accumulation in persimmon fruit during maturation. In this study, transcriptional levels of *SPS* in persimmon fruit remained broadly constant during ripening (Fig. 5), although a significant increase was observed in accumulated sucrose in 'Akiou' and 'Fuyu' by Oct 15, with a subsequent rise in sucrose accumulation from Nov 1 to Nov 15. In particular, the concomitant and rapid increase in both *SPS* activity and sucrose accumulation in 'Akiou' by Oct 15 suggests that this stage is crucial for the increase in SSC.

On the other hand, the sweetness of fruits is generally determined by the total sugar content and by the ratios among constitutive sugars: the higher the sucrose content, the stronger the organoleptic perception of sweetness (Asakuma and Shiraishi, 2017; Kajiura et al., 1979; Suzuki et al., 1990). Therefore, in terms of future persimmon breeding, heightened *SPS* activity and reduced *SuSy* and *VAI* activity (i.e. sucrose accumulation) is a promising mechanism to improve the sweetness of persimmon fruit. To achieve this, marker-assisted selection (MAS) of persimmon offspring would be useful for selecting desirable genotypes as mentioned above. Primers specific to *VAI* activity associated with sucrose accumulation were developed for the tomato (Harada et al., 1995) and Japanese pear (Itai et al., 2010) to enable reliable MAS in breeding studies. Further research needs to be undertaken to determine the functional significance of key sugar accumulation-related genes using specific primers based on the genome sequences of persimmon cultivars.

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