

Translocation and accumulation of fruit-fixed photosynthate in Satsuma mandarin

Shin Hiratsuka¹ · Shogo Nakayama¹ · Shohei Tamura¹ · Kazuyoshi Nada¹

Received: 31 March 2016 / Accepted: 1 August 2016 / Published online: 4 August 2016
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Abstract Satsuma mandarin fruit (*Citrus unshiu* Mark.) photosynthesizes as comparable to leaf at about 100 days after full bloom (DAFB). In this study, translocation and accumulation of fruit-fixed photosynthate were investigated by using ¹⁴CO₂. When fruit at 108 DAFB was exposed to ¹⁴CO₂ for 48 h under 135 photosynthetic photon flux density (PPFD), ¹⁴C-sucrose, ¹⁴C-glucose and ¹⁴C-fructose were detected not only in flavedo but juice sac; more than 50% of fruit assimilated ¹⁴C-sugars were present in juice sac. Thus, majority of rind-fixed photosynthate are infiltrated into juice sac and accumulated there within 48 h after assimilation. Although ¹⁴C-sucrose was predominant at flavedo where high SS (sucrose synthase) activity toward synthesis was present, the amount decreased gradually from the outside (flavedo) to the inside (juice sac) of fruit. In vascular bundle, strong SS toward cleavage and soluble acid invertase activities were involved, and ¹⁴C-fructose was predominant in juice sac. Accordingly, rind-fixed photosynthate is once converted to sucrose, the translocated sugar in *Citrus*, at flavedo by SS toward synthesis, and loaded on vascular bundle through symplastic and/or apoplastic movement in the albedo tissue. In the vascular bundle, sucrose may be degraded by SS toward cleavage and invertase, and resulting hexoses transported symplastically to the juice sac through juice stalk.

Keywords *Citrus unshiu* · Fruit photosynthesis · Photosynthate pathway · Sugar conversion · Translocated sugar

Introduction

Sugar content is one of the most important characters for determining fruit quality, and the sugar usually depends on photosynthate assimilated at leaf. Shaded fruits or fruits bearing inside the canopy, however, are often impeded sugar accumulation compared with sunny fruits. Fruit bagging also causes negative effects on sugar increase in the fruit (Arakawa et al. 1994; Huang et al. 2009; Watanabe et al. 2011; Hiratsuka et al. 2012; Ryuhan et al. 2013), which is often conducted in Japanese commercial orchards to protect fruits from fungal, insect, and physical damages, and to develop coloration on fruits as well. These facts suggest the significance of light on fruit surface for accumulating sugars in the fruit, but no experimental proof has been made.

Fruit photosynthesis is known in several plant species including *Citrus* (Blanke and Lenz 1989; Blanke and Bower 1991; Blanke 1995; Hiratsuka et al. 2012), and the photosynthate largely contributes to crop production in *Coffea* (Lopez et al. 2000). However, almost no data is available on the role of fruit-fixed photosynthate in sugar increase in the fruit. Meanwhile, the transportation of leaf-fixed photosynthate is well known in *Citrus*; the photosynthate is once converted to translocated sugar, the sucrose, at leaf, then it is loaded on vascular bundle and transported to the fruit (Koch 1984; Lowell et al. 1989). Inside the fruit, thereafter, the sucrose is mainly loaded on dorsal vascular bundle, and moved to juice sac symplastically and/or apoplastically through non-vascular juice stalk (Koch 1984; Lowell et al.

Electronic supplementary material The online version of this article (doi:10.1007/s10725-016-0204-9) contains supplementary material, which is available to authorized users.

✉ Shin Hiratsuka
hiratuka@bio.mie-u.ac.jp

¹ Graduate School of Bioresources, Mie University, Tsu, Mie 514-8507, Japan

1989; Koch and Avigne 1990). However, it is completely unclear how the fruit-fixed photosynthate is translocated to the inside of fruit.

The present study, therefore, aimed to determine whether the fruit-fixed photosynthate accumulates in juice sac, and the pathway of the photosynthate, from flavedo to juice sac, by using fruits exposed to ^{14}C . Fruits were used at ~100 days after full bloom (DAFB), because Satsuma mandarin fruit photosynthesizes actively at this stage as reported earlier (Hiratsuka et al. 2012), perhaps due to normal stoma function on the fruit (Hiratsuka et al. 2015). Finally, sucrose-metabolizing enzyme activities were determined to understand the “translocated sugar” at each fruit tissue, because fruit-fixed photosynthate was proved to be once converted to sucrose at flavedo in above experiments.

Materials and methods

Plant materials

Three fully-grown trees of Satsuma mandarin (*Citrus unshiu* Marc., cv. Uenowase) were used at the Experimental Farm of Mie University, Tsu, Mie, Japan. In 2013, to know ^{14}C -sugar accumulation into fruits at different developmental stages, i.e. small green fruit (70 DAFB, ~23 mm diameter), photosynthetically active fruit (98 DAFB, ~42 mm diameter) (Hiratsuka et al. 2012, 2015) and fully developed fruit (209 DAFB, ~60 mm diameter), three fruits were subjected to ^{14}C feeding study, respectively. In 2014, to understand detailed translocation manners of fruit-fixed photosynthate, fruits at 108 DAFB were subjected to ^{14}C feeding and enzyme activity determination, respectively. At 108 DAFB, fruits were ~1 month before the onset of coloration and their average diameter was 45 mm.

^{14}C feeding

Developmentally uniform three fruits were placed on wet filter paper in a transparent acryl box to make the stem cut surface contact with the wet paper, and 3.49 g NaHCO_3 and 5.6 M Bq of $\text{NaH}^{14}\text{CO}_3$ (American Radiolabeled Chemicals Inc., USA) were put in a bottle equipped inside the box. Into the bottle, 80% lactic acid was introduced to generate ^{14}C , where the CO_2 concentration was 500 ppm. Then, the box was irradiated with 135 photosynthetic photon flux density (PPFD) light supplied by halogen lamps (Sumita Optical Glass, Saitama, Japan), and incubated for 48 h at 25 °C. After incubation, fruits were brought out, washed with distilled water and stored at -80 °C until use.

Extraction of sugars and HPLC analysis

To compare differences in ^{14}C -sugar accumulation into fruits between different developmental stages, juice was squeezed from juice sacs of 70, 98 and 209 DAFB fruits respectively, centrifuged at 8000×g for 5 min, and resulting supernatant was subjected to HPLC analysis. Before subjecting to HPLC analysis, sample solution was filtered through Millipore membrane (0.45 μm, Japan Millipore Co. Ltd., Japan). Meanwhile, to examine detailed translocation and accumulation manners of rind-fixed photosynthate, ^{14}C -fed fruit at 108 DAFB was separated into flavedo, albedo, vascular bundle, segment epidermis and juice sac, respectively (Fig. 1). Then, the tissue was ground by mortar and pestle in 70% EtOH made by 0.1 M phosphate buffer (pH 7.0), and boiled for 15 min. After the sample was centrifuged at 17,000×g for 5 min, the supernatant evaporated in vacuo and adjusted the volume with H_2O . In juice sac sample, juice centrifuged was used directly as described above.

The HPLC equipment and analytical conditions were as follows; pump=LC-10AD (Shimadzu Co. Ltd., Japan), column=ZIC®-pHILIC PEEK (Shimadzu Co. Ltd., Japan), column temperature=40 °C, eluent=200 mM ammonium formate:acetonitrile=25:75, flow rate=2 ml min⁻¹, detector=RID-10AD (Shimadzu Co. Ltd., Japan). The eluent was fractionated at 30 s intervals and ^{14}C was counted in each fraction. Each sugar was identified by comparing its retention time with standard one. Experiments were repeated, at least, three times using three fruits and data were expressed as dpm g⁻¹ fw or dpm fruit⁻¹.

Estimation of ^{14}C -sugar amount per fruit

Because sucrose, glucose and fructose were detected as major sugars in the sample (Supplementary Fig. 1), the sum of ^{14}C -sugar per fruit was calculated by using data on the amount of ^{14}C -sucrose, ^{14}C -glucose, ^{14}C -fructose and fresh weight of respective tissues. At 70, 98 and 209 DAFB in

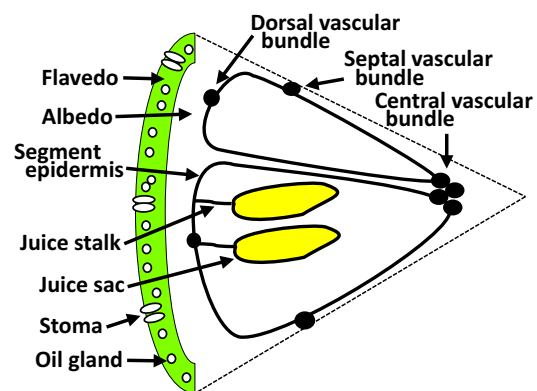


Fig. 1 Schematic diagram of horizontal section in mandarin fruit

2013, average fresh weight of juice sacs was 9.4, 47, and 91.3 g, respectively. At 108 DAFB in 2014, each tissue per fruit was as follows; flavedo=11.4 g, albedo=9.7 g, vascular bundle=0.6 g, segment epidermis=7.4 g, and juice sac=65.0 g.

Determination of sucrose-metabolizing enzyme activity

Since ^{14}C -sucrose was predominant at flavedo but decreased at the inside of fruit in above experiments, enzyme activities for both the synthesis and cleavage of sucrose were measured in respective tissues. Enzymes tested were sucrose synthase toward synthesis (SS-syn, EC: 2.4.1.13), sucrose phosphate synthase (SPS, EC: 2.4.1.14), sucrose synthase toward cleavage (SS-cleav, EC: 2.4.1.13), soluble acid invertase (INV-SA, EC: 3.2.1.26), insoluble acid invertase (INV-IA, EC: 3.2.1.26), soluble neutral invertase (INV-SN, EC: 3.2.1.26), and insoluble neutral invertase (INV-IN, EC: 3.2.1.26).

Developmentally uniform three fruits without $^{14}\text{CO}_2$ treatment were used. Each fruit was separated into respective tissues as described above, and proteins were extracted according to the method of Kubo et al. (2001) with slight modifications. Briefly, the tissue was powdered in liquid nitrogen by using motor and pestle, and Polyclar-AT[®] ($0.3 \text{ g g}^{-1} \text{ fw}$, Sigma, USA) and extraction buffer (0.05 M Tris-HCl (pH 7.8) containing 150 mM NaCl, 1 mM CaCl_2 , 10 mM KCl, 10 mM L-cysteine, 1 mM ascorbic acid, 1 mM $\text{Na}_2\text{-EDTA}$) were added. In juice sac preparations, 2 M Tris-HCl was used instead of 0.05 M buffer because of its high acidity. After stirring the homogenate for 15 min on ice, it was centrifuged at $16,000\times g$ for 15 min and supernatant was obtained. To the resulting sediment, extraction buffer was added again, shaken vigorously, centrifuged, and the supernatant was combined with the first obtained one. Then, proteins in the supernatant were partially purified by sedimenting them between 20 and 100% $(\text{NH}_4)_2\text{SO}_4$ saturation, and subjected to measuring SS, SPS and soluble invertase activity, respectively. For determining insoluble invertase activity, sedimented debris was used.

Protein concentration was determined by the method of Bradford (1976) using BSA as a standard, and enzyme assay was carried out according to the methods of Kubo et al. (2001). Experiments were repeated, at least, three times using three fruits and data were expressed as unit ($\mu\text{mol mg}^{-1} \text{ protein h}^{-1}$).

Statistical analysis

The experimental design was carefully set up based on several preliminary experiments and repeated 2 years, from 2013 to 2014. Because similar results were obtained in both years, data in 2014 were presented here, except for data on

Supplementary Fig. 2 which were from results in 2013. Statistical analyses were performed for the data obtained by, at least, three replications in each experiment by using Excel (Version 12.3.2; Microsoft, Redmond, WA). Data were expressed means \pm SES unless otherwise indicated.

Results

When tissue extract was separated by HPLC, sucrose, glucose and fructose were detected as major sugars (Supplementary Fig. 1), and fractions corresponding to respective sugars contained radioactivity. At gfw base, ^{14}C -sugar accumulation into juice sacs was much larger in the young fruit (70 DAFB), especially in ^{14}C -fructose, compared to developed ones (Supplementary Fig. 2A). At fruit base, however, respective ^{14}C -sugar amounts tended to be larger in 98 DAFB fruit than those in 70 DAFB one (Supplementary Fig. 2B). In 209 DAFB fruit, the accumulation was strictly low at both gfw and fruit base. Although the degree of photosynthate accumulation altered considerably depending on developmental stages of the fruit, active accumulation was confirmed in the fruit at ~ 100 DAFB.

Comparing the amount of ^{14}C -sugar among tissues from 108 DAFB fruit (Fig. 2A), ^{14}C -sucrose showed large fluctuations; it was predominant at flavedo with $\sim 3600 \text{ dpm g}^{-1} \text{ fw}$ but decreased gradually advancing toward the inside of fruit, $\sim 200 \text{ dpm g}^{-1} \text{ fw}$ at juice sac. The ^{14}C -glucose level was relatively constant in all tissues (~ 400 to $\sim 600 \text{ dpm g}^{-1} \text{ fw}$), though vascular bundle contained somewhat large

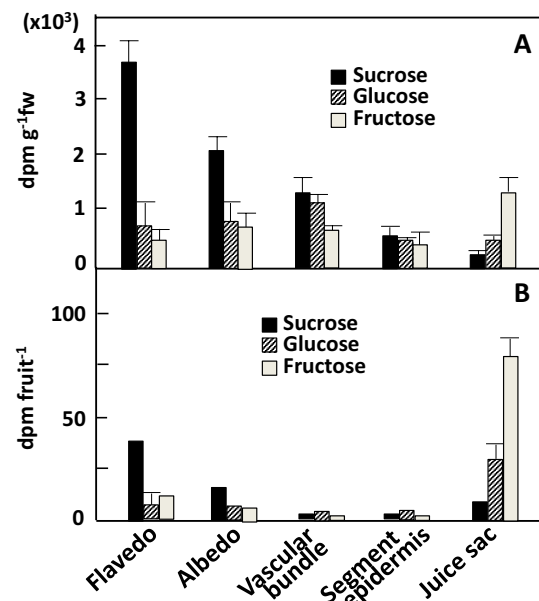


Fig. 2 Comparison of ^{14}C -sucrose, ^{14}C -glucose and ^{14}C -fructose amount among fruit tissues. **A** Radioactivity on fresh weight base. **B** Radioactivity on fruit base. Vertical bars indicate SE

amount. Although the ^{14}C -fructose was predominant in the juice sac, almost constant radioactivity was detected in other tissues, ~ 400 to 600 dpm g^{-1} fw. When ^{14}C -sugar amount per fruit was calculated based on fresh weight of each tissue, ^{14}C -sucrose was also major in flavedo and ^{14}C -fructose was in juice sac, respectively; ^{14}C -sucrose occupied $\sim 64\%$ of total radioactive sugars in flavedo and ^{14}C -fructose $\sim 67\%$ in juice sac (Fig. 2B).

The sum of ^{14}C -sugars in each tissue is shown in Fig. 3A; ~ 4600 dpm g^{-1} fw sugars presented in flavedo, and the amount decreased gradually to ~ 1800 dpm g^{-1} fw with advancing toward the inside of fruit. When ^{14}C -sugar amount was compared on fruit base, more than 50% of fruit-assimilated sugars were infiltrated into the juice sac (Fig. 3B). Thus, rind-fixed photosynthate was transported to the inside of fruit and majority of them accumulated in juice sacs within 48 h after CO_2 assimilation.

Activities of sucrose-metabolizing enzymes were conspicuous in flavedo and vascular bundle (Fig. 4). At the flavedo, higher activities of SS toward synthesis ($\sim 2.5\text{U}$) and SPS ($\sim 1\text{U}$) were found compared to other tissues ($<0.4\text{U}$) except for vascular bundle ($\sim 3\text{U}$ SS-syn and $\sim 1.5\text{U}$ SPS) (Fig. 4A). Meanwhile, though extremely higher activities of SS toward cleavage ($\sim 6\text{U}$) and soluble acid invertase ($\sim 3\text{U}$) were detected in vascular bundle, other tissues showed

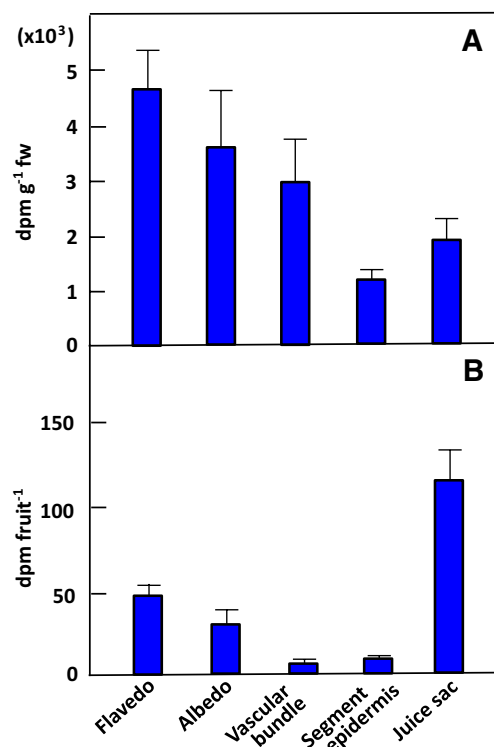


Fig. 3 Comparison of total ^{14}C -sugar amount (^{14}C -sucrose + ^{14}C -glucose + ^{14}C -fructose) among fruit tissues. **A** Radioactivity on fresh weight base. **B** Radioactivity on fruit base. Vertical bars indicate SE

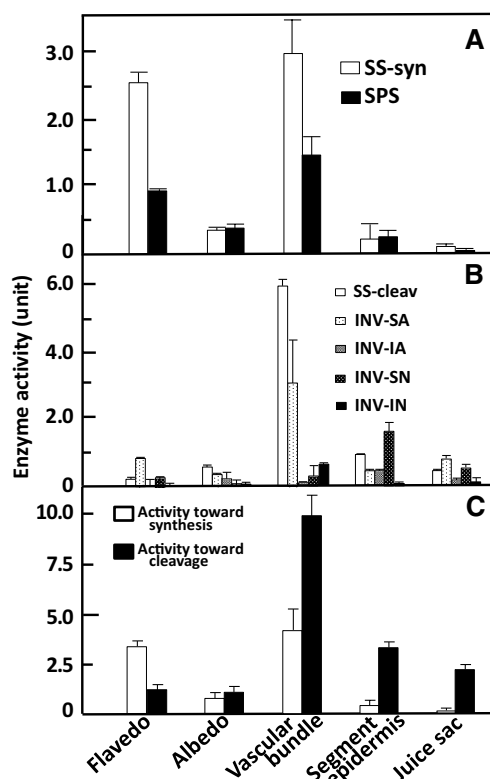


Fig. 4 Sucrose-metabolizing enzyme activities in each tissue of the fruit. **A** Activity toward synthesis. **B** Activity toward cleavage. *SS-syn* sucrose synthase toward synthesis, *SPS* sucrose phosphate synthase, *SS-cleav* sucrose synthase toward cleavage, *INV-SA* soluble acid invertase, *INV-IA* insoluble acid invertase, *INV-SN* soluble neutral invertase, *INV-IN* insoluble neutral invertase. **C** Total activity for sucrose synthesis and cleavage. Activity toward synthesis = *SS-syn* + *SPS*, Activity toward cleavage = *SS-cleav* + *INV-SA* + *INV-IA* + *INV-SN* + *INV-IN*

considerably lower activities of sucrose cleavage ($<1\text{U}$) (Fig. 4B). When total units of activities for sucrose synthesis and cleavage were calculated at each tissue, activity for the synthesis ($\sim 3.5\text{U}$) was largely superior to degradative one ($\sim 1.5\text{U}$) in flavedo, whereas cleavage action ($\sim 10.5\text{U}$) far surpassed the synthesis one ($\sim 4.5\text{U}$) in vascular bundle (Fig. 4C). Thus, sucrose synthesis and cleavage occurred simultaneously at each fruit tissue, and sucrose seemed to be synthesized at flavedo by SS toward synthesis and SPS using rind-assimilated hexoses, and it degraded gradually on the way to juice sac, especially at vascular bundle by SS toward cleavage and soluble acid invertase.

Discussion

When accumulation of rind-fixed photosynthate was compared among juice sacs from young, middle and fully matured mandarin fruits, ^{14}C -sugar amount was conspicuous in young fruit on gfw base, but larger in middle fruit on fruit base. Although young fruit tissue may function very

actively on photosynthate accumulation, middle fruit rind seemed to contribute much larger to CO₂ assimilation and sugar increase in the fruit, which may be due to both the increase in number of intact stomata on the fruit (Hiratsuka et al. 2015) and the fruit surface area. Detailed analyses of ¹⁴C-sugar behavior in the fruit, therefore, were conducted using fruits at ~100 DAFB in this study.

More than 50% of rind-fixed photosynthate were accumulated as sugars in juice sac of 108 DAFB fruit within 48 h after the CO₂ assimilation (Fig. 3B), implies that fruit-assimilated carbon functions on sugar increase in the fruit. Therefore, sugar decrease by fruit bagging is, at least in part, due to inhibition of fruit photosynthesis. Actually, fruit shading caused ~0.3% sugar reduction in Satsuma mandarin at harvest (Hiratsuka et al. 2012), and ~1.0% reduction in Japanese pear (Ryuhan et al. 2013). Thus, fruit photosynthate considerably contributes to sugar increase in the fruit, though contribution degree seems to be different largely among plant species. However, since we examined fruits at ~45 days before maturation in this study, it should be confirmed whether photosynthate fixed by young fruit contributes to the sugar content at harvest.

Majority of fruit-fixed photosynthate were present as hexoses in the juice sac, especially as fructose (Fig. 2A), though major accumulated sugar is sucrose in Satsuma mandarin (Kubo et al. 1996, 2001). Since young mandarin fruit usually contains sucrose, glucose, and fructose almost equally, and sucrose concentration increases with fruit maturation (Kubo et al. 1996, 2001), the fructose may be converted to sucrose at later ripening stages in the juice sac. Actually, activity for sucrose synthesis was very low in the juice sac (Fig. 4), but it is activated thereafter with fruit maturation (Kubo et al. 2001). Alternatively, rind-fixed photosynthate may mainly contribute to hexose accumulation. However, it is unexplainable that young fruit accumulated more ¹⁴C-sucrose than middle or mature fruit (Supplementary Fig. 2); vascular bundle of young fruit might have much less ability for sucrose cleavage in Satsuma mandarin.

The pathway of leaf-fixed photosynthate into *Citrus* fruit is relatively well understood (Koch 1984; Lowell et al. 1989). Glucose, the first product of leaf photosynthesis, is once converted to sucrose as a translocated sugar at leaf, and the sucrose is transported to fruit through phloem. Then, it enters mainly into dorsal vascular bundle in the fruit, where the sucrose is partially degraded by SS toward cleavage and invertase. The resulting hexoses are moved to juice sac through non-vascular juice stalk (Koch and Avigne 1990), where the hexoses are converted to sucrose by SS toward synthesis (Kubo et al. 2001). Meanwhile, the route of fruit-fixed photosynthate is completely unclear. Judging from mandarin fruit structure (Fig. 1) and ¹⁴C-sugar distribution among fruit tissues (Figs. 2, 3), photosynthate in flavedo may apoplastically move to the inside of fruit through

albedo and load on dorsal vascular bundle. Then, it is transported to juice sac apoplastically through juice stalk and accumulated there as the same way with leaf photosynthate.

Although rind-fixed ¹⁴C-sugars were detected in the segment epidermis (Figs. 2, 3), it is obscure whether they come via vascular bundle or directly from albedo. Since the leaf ¹⁴C-photosynthate is also detectable not only in segment epidermis but albedo of the grapefruit (*C. paradise* Macf.) (Lowell et al. 1989), the rind photosynthates seem to load on the dorsal vascular bundle first, then a part of them may be provided to segment epidermis to deposit and/or utilize them by its tissue cell.

The amount of ¹⁴C-sucrose was predominant at flavedo (Fig. 2), suggests that the hexose produced by fruit photosynthesis would be turned to sucrose there. The high enzyme activity for sucrose synthesis at flavedo (Fig. 4) supports this hypothesis, and this sugar processing is very similar with that of leaf photosynthate (Koch 1984; Lowell et al. 1989); the flavedo functions just like a leaf. During the sucrose transportation to the inside of fruit, more than 60% of them were degraded at both albedo and vascular bundle (Fig. 2) by SS toward cleavage and soluble acid invertase (Fig. 4B, C). This degradation process of sucrose is also similar to that in leaf photosynthate (Lowell et al. 1989). Meanwhile, each fruit tissue showed both activities for the sucrose synthesis and cleavage simultaneously (Fig. 4). Especially in vascular bundle, though remarkably higher activity for sucrose synthesis (SS-syn+SPS=~4.5U) was detected, activity toward cleavage (SS-cleav+invertases=~10.5U) far surpassed the synthesis one (Fig. 4C), may result in severe sucrose degradation there. Thus, reactions for sucrose synthesis and cleavage occur concomitantly at each fruit tissue; the cleavage action may account for constructing and nourishing respective tissue cells. On the other hand, since activities of SS and SPS are known to be controlled by their upstream enzymes such as protein phosphatase and UDP-glucose pyrophosphorylase (Huber and Huber 1996; Coleman et al. 2006), these enzymes may be activated first in the flavedo of Satsuma mandarin. Gene expression of SS (Zhao et al. 2016) and possible occurrence of SS isozymes (Suzuki et al. 1996) may also be involved in SS activation at flavedo. Taken together, the translocated sugar in fruit-fixed photosynthate seems to be sucrose at the outside of Satsuma mandarin fruit but hexoses at the inside, which is controlled precisely by SS toward synthesis, SPS, SS toward cleavage and invertases at each fruit tissue.

The mechanism of fruit photosynthesis is considerably complex; it may be intermediate status between C3, C4, CAM and shade leaf photosynthesis (Blanke and Lenz 1989; Hiratsuka et al. 2015). In addition, since the photosynthetic mechanism may alter during fruit development (Blanke and Lenz 1989; Hiratsuka et al. 2012), detailed research is necessary to understand the translocated sugar and role

of fruit-fixed photosynthate in sugar increase in the fruit. Meanwhile, ^{14}C feeding experiments were conducted using detached fruits in this study because of strictly legal limitation of radioisotope use in Japanese orchards. It should be examined whether the same reaction occurs in fruits on the tree.

In conclusion, majority of fruit-assimilated photosynthate are accumulated as sugars in juice sac of Satsuma mandarin. The photosynthate may be once converted to sucrose at flavedo by both SS toward synthesis and SPS, then, the sucrose moved to the inside of fruit with gradual degradation by SS toward cleavage and invertase, and finally accumulated mainly as hexoses in the juice sac.

Acknowledgments The authors thank to Mr. T. Kurosawa of Mie University for his help in radioisotope operation. This work was supported in part by the Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 19658013 and 23658029).

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