

Fruit Photosynthesis and Phosphoenolpyruvate Carboxylase Activity as Affected by Lightproof Fruit Bagging in Satsuma Mandarin

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ABSTRACT. To clarify why fruit bagging reduces sugar content at harvest, we investigated its effect on carbon dioxide assimilation by Satsuma mandarin (*Citrus unshiu*) fruit through photosynthesis and phosphoenolpyruvate carboxylase (PEPC; enzyme code 4.1.1.31). Seasonal changes in gross photosynthesis ranged from 70 to 400 $\mu\text{mol}\cdot\text{d}^{-2}\cdot\text{h}^{-1}\text{O}_2$ with a peak at 99 days after full bloom (DAFB) when the assimilation rate of fruit was comparable to that of leaves. However, a peak showing net photosynthesis appeared at 112 DAFB because of high fruit respiration. When fruit were bagged at 85 DAFB, the net photosynthetic peak disappeared, perhaps as a result of the decline in chlorophyll content in the rind. Sugar and organic acid content in the bagged fruit were 0.3% and 0.16% less, respectively, than controls at the mature stage (204 DAFB). PEPC activity in the rind was much higher than in leaves on a protein basis; it increased between 92 and 112 DAFB and showed a peak of 72 units. The PEPC activity peak was also 90% of control after fruit bagging. Thus, just before their color development, mandarin fruit assimilate CO_2 actively through photosynthesis and PEPC. However, these activities are inhibited by bagging, likely resulting in lower sugar content at harvest. The concomitant activation of PEPC and photosynthesis between 99 and 126 DAFB indicates that CO_2 fixed by PEPC might be used for photosynthesis in mandarin fruit, because photosynthesis in several fruit such as apple (*Malus pumila*) and pea (*Pisum sativum*) is considered to have an intermediate status among C_3 , non-autotrophic tissue, and C_4/CAM photosynthesis.

In Japanese commercial fruit tree orchards, young fruit often are covered with paper bags to prevent fungal, insect, and physical damage and to promote color development on the fruit skin. Fruit bagging also seems to be useful for reducing the use of pesticides. However, bagging usually lowers sugar content at harvest (Arakawa et al., 1994; Huang et al., 2009; Proctor and Loughheed, 1976; Watanabe et al., 2011). This phenomenon generally is considered to be caused by inhibiting fruit photosynthesis by shading, weakening fruit sink strength resulting from lower light and increased temperature and moisture in the bag, or both. However, little supportive experimental evidence has been provided.

Photosynthesis occurs in fruit of many crop plants. Photosynthetic activity of coffee fruit (*Coffea arabica*) seems to contribute 20% to 30% of the total photosynthesis of the tree (Lopez et al., 2000). Pods of pea and soybean (*Glycine max*) photosynthesize in two distinct layers; the outer layer, comprising chlorenchyma of the mesocarp, captures CO_2 from the outside atmosphere and the inner layer, a chloroplast-containing epidermis lining the pod gas cavity, reassimilates the CO_2 released from respiring seeds in the pod cavity (Atkins et al., 1977; Quebedeaux and Chollet, 1975). However, in fleshy fruit, the subepidermal layers contain chlorophyll during the early developmental stages, and photosynthesis is confirmed in some plant species. CO_2 exchange through the epidermis has been best documented in young apple fruit (Blanke and Lenz, 1989;

Jones, 1981), and in citrus (*Citrus* sp.) fruit, $^{14}\text{CO}_2$ assimilation by the fruit and distribution among the fruit tissues have been well studied. In grapefruit (*Citrus paradisi*), the ^{14}C in the juice tissue was significantly higher when fruit were incubated in the dark than in the light between 2 and 6.5 months after anthesis, and little ^{14}C -photosynthate was detectable (Yen and Koch, 1990). Bean and Todd (1960) proved $^{14}\text{CO}_2$ uptake by young ‘Valencia’ orange (*Citrus sinensis*) fruit; incorporated ^{14}C was distributed into sugars, amino acids, and organic acids in rind tissues under light conditions, but not into sugars in the juice sacs. In experiments determining the role of fruit CO_2 assimilation in citric acid accumulation of *Citrus natsudaoidai* juice sacs, the level of juice ^{14}C was also greater in fruit placed into the dark than the light (Akao and Tsukahara, 1979). Thus, CO_2 fixed by photosynthesis in fruit does not seem to be accumulated in the juice, although carbon assimilated by PEPC would be stored as organic acids and/or amino acids in citrus.

CO_2 assimilation by fruit PEPC has been well studied (Diakou et al., 2000, Moing et al., 2000, Munoz et al., 2001), and a possible role for PEPC in organic acid accumulation in the juice was reported in peach (*Prunus* sp.) (Moing et al., 2000), although other metabolic factors such as vacuolar storage ability also were linked to organic acid accumulation (Moing et al., 1999). The pods of pea and soybean possess higher PEPC activity than the leaves, and pod PEPC seems to participate in CO_2 assimilation (Atkins et al., 1977; Quebedeaux and Chollet, 1975). However, there is little information on the physiological role of fruit PEPC in sugar accumulation of mature fruit. According to Blanke and Lenz (1989), fruit have an intermediate status between C_3 and C_4/CAM photosynthesis, and thus fixation of CO_2 by PEPC might contribute to sugar accumulation in fruit. Recently, Walker et al. (2011) suggested that organic acids

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dissimilated through phosphoenolpyruvate carboxykinase (PEPCK) are used for gluconeogenesis in cherry (*Prunus avium*) fruit.

The present study was conducted to verify the lightproof fruit bagging effect on sugar concentration at harvest in Satsuma mandarin. Then, the effect of fruit bagging on fruit photosynthesis and PEPC activity was characterized during fruit development.

Materials and Methods

PLANT MATERIALS. Three adult trees of Satsuma mandarin (cv. Okitsuwase) were used at an experimental farm of Mie University, Tsu, Mie, Japan. The 90 fruit (30 fruit per tree) were covered with paper bags (18 cm long × 14 cm wide; Kobayashi Bag Mfg. Co., Iida, Japan) coated black inside, which cut the transmitted light more than 99%, at 85 d after full bloom. This bag has been used for improving fruit coloration of peach in practical culture of Japan. Nine fruit from three trees (three fruit per tree) were sampled at 92, 99, 112, 126, 140, and 161 DAFB and used for determination of chlorophyll content, photosynthesis and respiration rate, and PEPC activity. Nine unbagged fruit from three trees (three fruit per tree) were used as controls for each date.

To determine the effect of bagging on sugar and acid content in mature fruit, 15 bagged fruit and 15 unbagged fruit were sampled randomly from the canopy surface of the same trees (five fruit per tree) at 204 DAFB.

DETERMINATION OF CHLOROPHYLL CONTENT IN THE RIND. For chlorophyll determination, 15 rind discs (4 mm diameter) were prepared from three orientations of five fruit using a cork borer, and albedo tissue was removed by a razor blade. The five randomly selected discs were homogenized in a glass homogenizer containing 80% acetone at 0 °C. After centrifugation at 15,000 g_n for 5 min, the supernatant volume was adjusted to 3 mL with 80% acetone. Based on the method of Arnon (1949), chlorophyll a and b concentrations were determined by measuring optical density (OD) of the extract at 645 and 663 nm. The experiment was repeated three times.

DETERMINATION OF PHOTOSYNTHESIS AND RESPIRATION RATE. Immediately after sampling, 10 rind discs (3 mm diameter) were prepared from three fruit with the albedo tissue removed, and O₂ evolution was measured using an oxygen electrode apparatus (Rank Brothers, Cambridge, U.K.) under 1000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light supplemented with halogen lamps (Sumita Optical Glass, Saitama, Japan). Briefly, the discs prepared were soaked in 0.05 M phosphate buffer (pH 7.0) for 30 s and then placed in an electrode tank with 3 mL of fresh phosphate buffer. The tank was maintained at 25 °C and the buffer was stirred by a small stirrer. After injection of 100 μL of 0.31 M NaHCO₃ into the tank as a CO₂ source, the O₂ increase in the buffer was recorded for 15 min. Then the tank was covered with three layers of aluminum foil and the O₂ decrease was recorded for 10 min. Before use, the buffer had been bubbled with N₂ gas for 1 h and stored in a desiccator containing 2 M NaOH to remove CO₂. The experiment was repeated three times. The CO₂ exchange of the leaves was also measured by the same method at each sampling date using five leaves.

DETERMINATION OF PHOSPHOENOLPYRUVATE CARBOXYLASE ACTIVITY IN THE RIND. For the experiment, three fruit and five leaves were used. The rind of the fruit or leaf was homogenized in 0.05 M Tris-HCl buffer (pH 7.8) containing 30% Polyclar-AT (Sigma-Aldrich, St. Louis, MO), 150 mM NaCl, 1 mM CaCl₂, 10 mM L-cysteine, 1 mM ascorbic acid, and 1 mM Na₂-EDTA

using a glass homogenizer. The homogenate was centrifuged at 20,000 g_n for 10 min and the supernatant was passed through a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column to remove polyphenols. The protein fraction gathered was saturated with 80% ammonium sulfate and the protein was collected by centrifuging at 20,000 g_n for 10 min. The sediment was redissolved in a small amount of the buffer and dialyzed against the same buffer overnight. All procedures were carried out below 4 °C. The protein concentration of the sample was determined according to the method of Bradford (1976) and the samples were stored at -80 °C until use.

PEPC activity was determined as NADH oxidation using a coupling reaction with malate dehydrogenase (MDH) (Kumar et al., 1989). The reaction mixture consisted of 100 μg protein, 20 units MDH (Wako Chemical, Osaka, Japan), 1.5 mM NADH, 1 mM dithiothreitol, 10 mM NaHCO₃, and 5 mM MgCl₂ in 0.05 M Tris-HCl buffer (pH 7.8). The total volume of the mixture was 1.4 mL. The reaction was started by adding phosphoenolpyruvate (PEP) at a final concentration of 2.3 mM; the reaction was allowed to stand for 2 h at 25 °C and the decrease in OD 340 nm was recorded. As a control, buffer without any protein was used. Before use, the buffer had been bubbled with N₂ gas for at least 1 h and stored in a desiccator containing 2 M NaOH. The experiment was repeated three times. Enzyme activity was expressed in units (1 unit = 1 μmol NADH oxidation per milligram protein per hour).

DETERMINATION OF SUGAR AND ACID CONTENT. At fruit maturation (204 DAFB), 15 fruit each were sampled from bagged and unbagged treatments as described previously, and some juice was squeezed from each fruit. Then, the sugar content was determined by a refractometer (Atago, Tokyo, Japan) and the organic acid content by titration with 0.1 M NaOH. Based on the titration value, the citric acid content was calculated (1 mL of 0.1 M NaOH = 6.4 mg citric acid).

STATISTICAL ANALYSIS. The experiment was repeated 2 years, from 2006 to 2007. Because similar results were obtained in both years, data in 2006 were presented here. Three trees were used as a randomized complete block, and three fruit samples with and without treatment were taken from each tree at each sampling date. Then, randomly selected fruit were subjected to respective experiments as described previously. Statistical analyses were performed for the data obtained by 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 Satsuma mandarin fruit were bagged at 85 DAFB, the sugar content at the mature stage (204 DAFB) was 10.07% \pm 0.1% in contrast to 10.33% \pm 0.12% in control (data not shown). The organic acid contents were 1.61% in bagged fruit and 1.76% in control, respectively. Thus, both sugar and organic acid contents were reduced 0.3% and 0.16% than controls, respectively, by fruit bagging. Although fruit growth was promoted by bagging during 110 and 130 DAFB, almost no effect was observed thereafter (Fig. 1). During later stages of fruit development, bagging inhibited chlorophyll degradation and carotenoid formation in the rind as described subsequently, resulting in pale yellow fruit at harvest.

The chlorophyll content in the rind showed unique changes during fruit development (Fig. 2). The rind contained \approx 3.4 mg·d⁻²

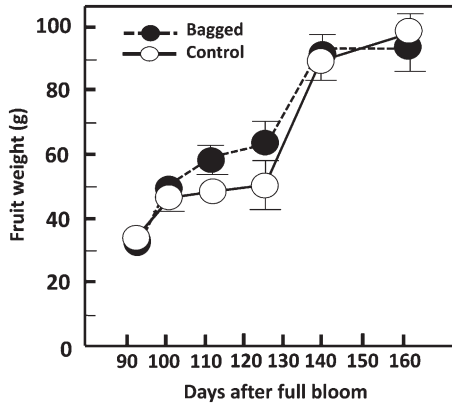


Fig. 1. Effect of lightproof fruit bagging on fruit growth in Satsuma mandarin. Vertical bars indicate SE.

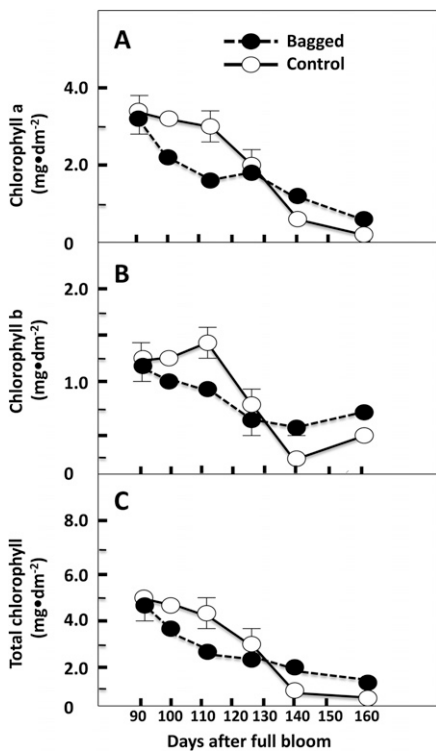


Fig. 2. Seasonal changes in chlorophyll a (A), chlorophyll b (B), and total chlorophyll (C) content of Satsuma mandarin rind as affected by lightproof fruit bagging. Vertical bars indicate SE.

chlorophyll a at 92 DAFB, which decreased gradually until 112 DAFB and then rapidly thereafter (Fig. 2A). At 161 DAFB, the amount reached almost zero. The chlorophyll b content remained unchanged during the first 20 d at 1.3 mg·d⁻² and was degraded promptly thereafter (Fig. 2B), but the amount somewhat increased again between 140 and 161 DAFB. The changes in total chlorophyll content were similar to those of chlorophyll a (Fig. 2C). After fruit bagging, both chlorophyll a and b content decreased considerably during the first 20 d, especially at 112 DAFB, when they were 57% and 36% of controls, respectively (Fig. 2). Therefore, a clear decrease in total chlorophyll content was found in this interval. However, at ≈126 DAFB, there was no difference in the pigment content between bagged and

unbagged fruit because of prominent chlorophyll degradation in control but not in bagged fruit. Therefore, beginning at 140 DAFB, bagged fruit contained more chlorophyll than controls.

The chlorophyll a:b ratio was 2.9 at 92 DAFB and decreased through 112 DAFB and then increased to ≈3.0 at 140 DAFB (Fig. 3). The decrease between 92 and 112 DAFB was the result of a decrease in chlorophyll a content, and the increase between 112 and 140 DAFB was the result of a rapid decrease in chlorophyll b. After 140 DAFB, the ratio was lowered to 1.3 as a result of chlorophyll a degradation. After fruit bagging, the ratio sharply declined during the first 20 d but recovered at 126 DAFB and was maintained at a lower ratio than controls thereafter.

Mandarin fruit photosynthesized in quite a different manner than leaves (Fig. 4). In leaves, the gross photosynthetic rate decreased gradually from 450 to 270 μmol·d⁻²·h⁻¹ O₂ during the experiment (Fig. 4A), whereas in the fruit, it increased during the first week and peaked at 99 DAFB at 390 μmol·d⁻²·h⁻¹ O₂ and then decreased. At 99 DAFB, the fruit photosynthetic rate was the same as that of leaves. The peak of net photosynthesis of the control fruit, however, was at 112 DAFB (Fig. 4C) because of high fruit respiration at 99 DAFB (Fig. 4B). For the control fruit, there were two respiration peaks: the first peak was at 99 DAFB and the second was at 126 DAFB. After fruit bagging, gross photosynthesis was reduced to 70% of controls at 99 DAFB and to almost zero at 126 DAFB, when the rate of control fruit photosynthesis was ≈70 μmol·d⁻²·h⁻¹ O₂. As a result, zero or negative net photosynthesis was observed in bagged fruit during the experiment, because the respiration rate was not always reduced by bagging. Thus, fruit bagging negated the net photosynthetic peak that occurred in control fruit at 112 DAFB.

Mandarin fruit also assimilated CO₂ through PEPC. At 92 DAFB, both rind and leaves had ≈38 units of PEPC activity (Fig. 5). Thereafter, leaf PEPC decreased gradually until 161 DAFB, whereas rind PEPC increased and peaked at 112 DAFB at 66 units. At this stage, rind PEPC was 2.3 times higher than leaf PEPC on a protein basis. Throughout the experiment, the PEPC activity of the fruit was more than twice as high as that of the leaves except at 92 DAFB. When the activity was compared on both a fresh weight and an area basis, the rind showed 217 units/g fresh weight (FW) and 15,472 units/d² at 99 DAFB, whereas the leaf had 300 units/g FW and 990 units/d², respectively. These data are because the rind contained only one-third the amount of proteins compared with the leaf on

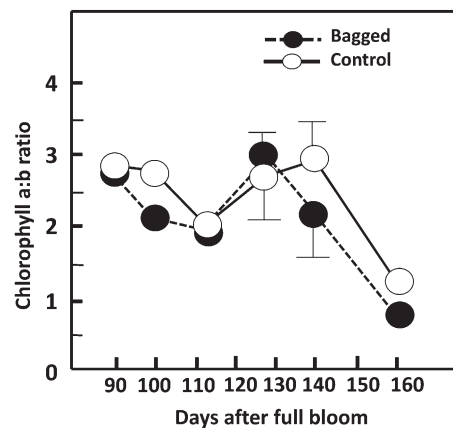


Fig. 3. Seasonal changes in chlorophyll a:b ratio in Satsuma mandarin rind as affected by lightproof fruit bagging. Vertical bars indicate SE.

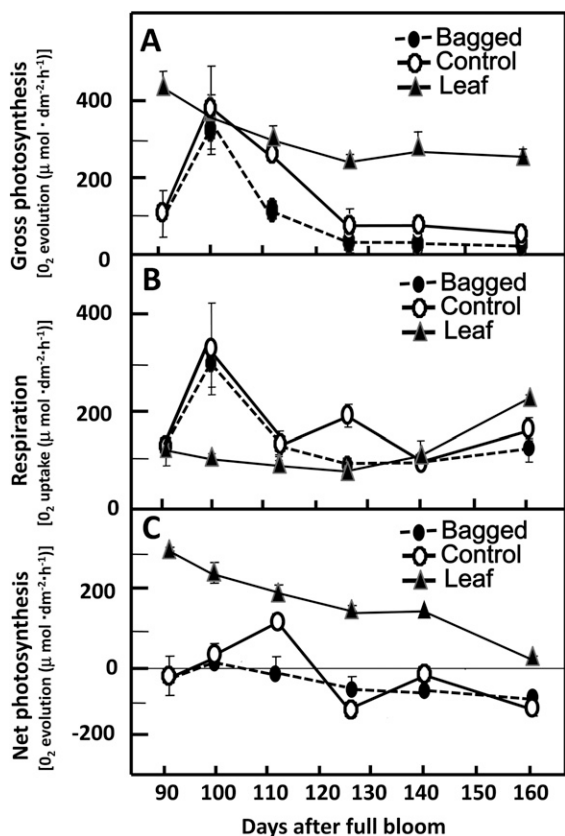


Fig. 4. Seasonal changes in gross photosynthesis (A), respiration (B), and net photosynthesis (C) in Satsuma mandarin rind as affected by lightproof fruit bagging. Vertical bars indicate SE.

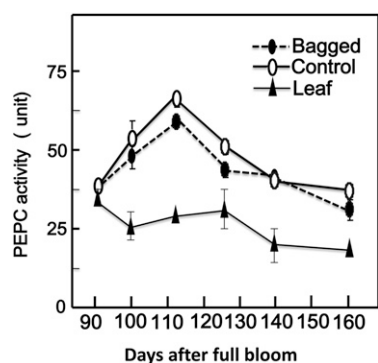


Fig. 5. Seasonal changes in phosphoenolpyruvate carboxylase (PEPC) activity in Satsuma mandarin rind as affected by lightproof fruit bagging. Vertical bars indicate SE; 1 unit = 1 μmol NADH oxidation per milligram protein per hour.

a FW basis, but the rind protein was much greater on an area basis. The activity peak found at 112 DAFB in the rind coincided with the net photosynthetic peak of the fruit. When fruit were bagged, rind PEPC activity was partially suppressed and $\approx 10\%$ inhibition was observed throughout the experimental period.

Discussion

We confirmed a negative effect of bagging young fruit on sugar concentration at harvest. Although the degree of sugar reduction was less than that in apple (Arakawa et al., 1994;

Proctor and Lougheed, 1976; Watanabe et al., 2011), a small reduction in mandarin sugar has sometimes great importance to the market; at the packing house for Satsuma mandarin, fruit containing less than 10% sugar are usually unacceptable in Japan. On the other hand, fruit bagging caused serious decrease in soluble solid content in ‘Seminar’ (*C. paradisi* \times *C. reticulata*); early bagging (1 Oct.) induced 1.6% reduction and later treatment (15 Nov.) showed 0.5% reduction, respectively, than controls at 12 weeks storage after harvest (Sato, 1997).

The cause of sugar decrease has been considered to be a result of either photosynthesis inhibition or weakening of the sink strength of the fruit (Arakawa et al., 1994; Proctor and Lougheed, 1976), but detailed data have not been available. Fruit bagging may raise humidity and temperature surrounding the fruit, which perhaps affects water content in the fruit. If the bagging promotes fruit enlargement, sugar reduction can be explained by juice dilution. However, the growth was unaffected during later stages of development (Fig. 1). Another possibility is that sink strength of the fruit may be weakened as a result of inhibiting fruit transpiration by bagging. This is likely, but the photosynthates entered into the fruit will be used not only for sugar accumulation, but fruit growth itself, and thus fruit growth would be reduced by bagging. Therefore, we consider that humidity and temperature in the bag may not directly affect the sugar content at harvest in Satsuma mandarin, because bagging showed almost no effect on fruit weight at harvest.

The pathway of rind-fixed carbon into citrus fruit is unclear. Citrus fruit have three types of vascular bundles (i.e., central, septal, and dorsal). In grapefruit, the majority of photosynthates from the leaf enter into the non-vascular segment epidermis through dorsal vascular bundles (Koch, 1984). The photosynthates then move to the non-vascular juice stalk and finally accumulate in the juice sac. Thus, the carbon in the fruit can be transferred not only through vascular bundles, but also symplastic and/or apoplastic systems. Therefore, the carbon fixed at the rind may move symplastically and apoplastically to the dorsal vascular bundle first, because we detected high levels of ^{14}C in the dorsal vascular bundle when mandarin fruit were exposed to $^{14}\text{CO}_2$ at 100 DAFB (data not shown). Then, the carbon may be unloaded into the segment epidermis and transported symplastically to the juice sac. During the carbon translocation, some carbon may be used for respiration in the fruit tissues and the released CO_2 will be reassimilated by PEPC in the fruit. We detected PEPC activity in the albedo, segment epidermis, and juice sac tissues, respectively, although their activities were less than that of the flavedo tissue (data not shown).

In this study, fruit growth was not inhibited by bagging, but carbon fixing was, especially between 99 and 126 DAFB, when fruit assimilated CO_2 most actively by photosynthesis and PEPC activity during fruit development. Therefore, we consider that photosynthesis and PEPC activity in young fruit contribute to sugar concentration at harvest. However, because little $^{14}\text{CO}_2$ fixed by photosynthesis in fruit is reportedly transported into juice sacs in citrus (Bean and Todd, 1960; Yen and Koch, 1990), sugar reduction by fruit bagging cannot be explained only by photosynthetic inhibition. In this respect, possible mechanisms of sugar formation from organic acids are discussed subsequently.

Chlorophyll content in unbagged rind maintained a similar level until 112 DAFB when a net photosynthetic peak occurred. However, that in bagged fruit decreased immediately after

bagging and the decrease continued to 112 DAFB when the net photosynthetic peak disappeared completely. Thus, photosynthetic inhibition by fruit bagging may be the result of incomplete chlorophyll development in the rind.

Although the chlorophyll a:b ratio is known to change from 1:1 to 4:1 as fruit develops in a species-specific manner (Knee, 1972; Phan, 1975), mandarin fruit showed a 1.6:1 between 92 and 161 DAFB. However, when this developmental change was examined in detail, the ratio showed a drastic alteration with two peaks of the ratio. Furthermore, fruit bagging varied the pattern considerably, indicating that great modifications in photosynthetic machinery occur not only in unbagged, but also in bagged rind. Generally, plant leaves adapt to surrounding light conditions and alter the chlorophyll a:b ratio to use the light efficiently; with decreasing light intensity, the ratio decreases. It has been reported that the chlorophyll a:b ratio decreases with decreasing light intensity in storied *Chamaecyparis obtusa* forests (Tanaka et al., 1994), and a similar adaptation was observed in the bagged mandarin fruit in this study. However, even under unbagged conditions, the fruit showed a similar decrease as it developed. Thus, the fruit are photosynthetically unique organs completely different from a leaf.

The photosynthetic rate of unbagged fruit did not always parallel the amount of chlorophyll content; the pigment levels were almost the same between 92 and 112 DAFB, but gross photosynthesis changed drastically from 110 to 390 $\mu\text{mol}\cdot\text{d}^{-2}\cdot\text{h}^{-1}\text{O}_2$ during this very short duration (Fig. 4). At the same time, the chlorophyll a:b ratio declined from 2.9 to 2.0 (Fig. 3), and prominent morphological and physiological changes have been reported during this stage: 1) fruit growth is retarded and reaches the final stage of its sigmoid growth curve (Kubo and Hiratsuka, 1998); 2) the rind surface becomes smooth; 3) sucrose accumulation begins and citric acid content decreases sharply in the juice sacs (Kubo et al., 2001); and 4) chlorophyll degradation and carotenoid formation occur just after this stage. Considering these observations, factors other than chlorophyll concentration control photosynthetic activity in the rind such as activation of the photosynthetic electron transport system or the concentration or activation of RuBisCO protein.

Mandarin fruit showed much higher PEPC activity than leaves on both a protein and an area basis, and a concomitant decrease in its activity and the organic acid content was found after fruit bagging, indicating that fruit PEPC plays a role in citric acid accumulation in Satsuma mandarin. Actually, CO_2 fixed by fruit PEPC is considered to contribute to organic acid accumulation in peach (Moing et al., 1999, 2000). On the other hand, to activate PEPC, phosphorylation of the enzyme through PEPCK is necessary in the C_4 plant guinea grass (*Panicum maximum*) (Bailey et al., 2007) and phosphorylation is light-dependent in C_4 and other plants (Chollet et al., 1996). The interruption of light by bagging may have lowered PEPC activity in this study (Fig. 5). Another possibility is that bagging modifies the activity of allosterically regulated PEPC such as by increasing the malate (inhibitor) or decreasing the sugar phosphate (activator) content in the rind, as observed in C_4 plants (Chollet et al., 1996), resulting in a reduction in citric acid accumulation.

PEPCK activity is positively correlated with organic acid reduction in several fruit (Famiani et al., 2009; Famiani and Walker, 2009; Walker et al., 2011). This cytosolic enzyme catalyzes the ATP-dependent decarboxylation of oxaloacetate (OAA) to PEP, i.e., catalysis of a reaction in the opposite direction of PEPC (Leegood and Walker, 2003), and the

dissimilated organic acids may be used in gluconeogenesis (Walker et al., 2011). Our findings suggest that mandarin fruit uses organic acids for sugar production and that bagging inhibits both organic acid synthesis and PEPCK activity, resulting in reduction of sugar accumulation.

In this study, a concomitant increase in photosynthesis and PEPC activity in the rind was found between 92 and 126 DAFB, when photosynthesis and PEPC activity were most prominent during fruit development. After fruit bagging, these activities were considerably inhibited and sugar content was lowered. These observations may indicate the presence of a C_4/CAM photosynthetic mechanism in the fruit of C_3 mandarin plants. Investigations are now in progress using $^{14}\text{CO}_2$.

In conclusion, mandarin rind actively assimilates CO_2 both through photosynthesis and PEPC, especially between 92 and 126 DAFB, but these activities are considerably hampered by fruit bagging. Because fruit bagging reduces sugar content at harvest, the fixed CO_2 would ordinarily contribute to sugar accumulation in mature fruit.

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