INTRODUCTION

Strawberry (Fragaria x ananassa Duch.) is among the most widely consumed fruits worldwide. The soft and juicy texture of ripe strawberries is highly favored by a wide range of consumers. Despite significant improvements in packaging and transport, deterioration of strawberry fruit quality during distribution remains a major problem (Tatara et al., 1999). The fruit is subject to bruising from its own weight as well as external forces, such as contact with other fruits and packages. Fruit bruising is a serious problem, as it cannot be efficiently detected using common color machine vision systems (Nagata et al., 2006), resulting in consumer complaints and economic losses. The segregation of bruised fruits from non-bruised fruits can be both labor- and time-intensive. Therefore, effective preventing fruit bruising is essential for improving economic benefits and reducing labor requirements for the distribution of strawberries. However, the precise mechanism of bruising in strawberries is poorly understood.

The location and the morphological structure of the bruise in strawberries are controversial. Previous studies showed that firmness enhancement of the fruit surface by pre-cooling and cold chain processing cannot adequately prevent bruise occurrence but considerably reduces the occurrence of scratches (Ootake and Tanaka, 1988). Accordingly, internal tissues are speculated to be the primary site of bruising, whereas the skin surface is thought to be that of scratching in strawberries. Cell rupture and juice release have been observed in bruised apple (Holt and Schoorl, 1977; Ingle and Hyde, 1968), which has also been predicted in bruised strawberry fruit (Holt and Schoorl, 1982). However, the results of previous studies and precise mechanism of bruising in strawberries remain inconclusive, as most did not directly examine the skin and flesh of the bruised fruit in detail. Because the skin of strawberry fruit is very thin and fragile, it may become bruised with or without external force to the internal tissues. In addition, tensile tests showed that strawberry cells can be isolated without fracturing (Harker et al., 2000), suggesting that flesh or pith cells are less likely to rupture. It is important to directly determine whether morphological changes in the fruit skin, flesh, and pith are associated with bruising.

We performed confocal laser scanning microscopy to determine the morphological structure of the skin and other tissues in bruised strawberry fruit of cultivars with different mechanical properties. It is difficult to observe the sur-
face of bruised strawberry fruit in detail using conventional scanning or transmission electron microscopy because sample pretreatment may destroy the three-dimensional structure. In confocal scanning laser microscopy, raw samples can be directly observed without preparation, thus avoiding these issues. The goal of this study was to determine the locations and mechanisms of bruising in strawberry fruit based on morphological changes at the cellular level in different parts of the fruit.

MATERIALS AND METHODS

Plant materials
‘Toyonoka,’ ‘Sachinoka,’ and ‘Koiminori’ strawberries were used in this study. Previous studies showed that the apparent modulus of elasticity of ‘Koiminori’ is higher than that of other cultivars (Hikawa-Endo et al., 2018). All strawberries were grown in a greenhouse at the Western Region Agricultural Research Center, NARO, Zentsuji City, Kagawa Prefecture, Japan. Fully ripe fruits (20–30 g) harvested on February 6, 2020 were used. Uniformly developed fruits selected by visual inspection were transferred to the laboratory immediately after harvest, packed in hammock-type packages (Cradle C type, Oishi Sangyo Co., Ltd., Fukuoka, Japan), and stored at 4°C.

Treatments and physical property measurement
Twenty-four fruits per cultivar were sampled at 0, 6, and 12 days after harvest and divided into two groups. The 12 fruits in each group were carefully bruised using a universal testing machine (MCT-2150, A&D Co., Ltd., Tokyo, Japan) equipped with a cylindrical plunger with a tip diameter of 15 mm and load cell of 500 N. The bruised position was set at the equator of each fruit, and the compression speed was set to 100 mm min⁻¹. Preliminary tests revealed that the tested strawberry fruits were completely crushed at a compression force of approximately 20 N or higher, but they were rarely crushed and were only bruised at a compression force of approximately 10 N or lower. In particular, no fruits were crushed or discolored at compression forces of 7 N or lower (data not shown). Therefore, the plunger was compressed at the equator area of each fruit at 7 N. The compression speed was set to 100 mm min⁻¹. The fruit diameter (d) and compression depth (dₗ) at 7 N were measured to calculate the apparent modulus of elasticity (Eₗ) (Eq. 1).

\[
Eₗ = \frac{7N \times dₗ}{d \times S},
\]

where \(S\) is the area of the plunger. No treatment and property measurements were applied to the 12 fruits in the other group.

Microscopy observation
The fruit skin surface of the equator part of both the bruised and intact groups was observed with a confocal laser scanning microscope (Optelics Hybrid, Lasertec Corp., Tokyo, Japan) equipped with a 50× objective and analysis software (LMeye7, Lasertec Corp.). Under the microscope, the surface smoothness and cell area of each tissues were assessed. The smoothness of the fruit surface was evaluated using the following two methods: first, three arbitrary cells per fruit were measured. The mean height difference (\(Rₖ\)) between the highest (\(Hₘₗₐₓ₁, Hₘₗₐₓ₂, Hₘₗₐₓ₃\)) and lowest areas of each cells (\(Hₘᵦₗ₁, Hₘᵦₗ₂, Hₘᵦₗ₃\)) was calculated (Eq. 2).

\[
Rₖ = \frac{(Hₘₗₐₓ₁ - Hₘᵦₗ₁) + (Hₘₗₐₓ₂ - Hₘᵦₗ₂) + (Hₘₗₐₓ₃ - Hₘᵦₗ₃)}{3}
\]

Second, an arbitrary straight line measuring 233.6 μm was drawn on the view screen, and the height (\(Hₗ\)) on the line was measured every 0.292 μm. The arithmetic mean roughness (\(Rₐ\)) was calculated (Eq. 3).

\[
Rₐ = \frac{1}{800} \int_{0}^{800} |Hₗ| \, dHₗ
\]

\(Rₖ\) and \(Rₐ\) indicate the smoothness of the fruit surface independent of each other. An RGB image of the surface was captured, and the mean area of 10 cells per image was calculated using Adobe Photoshop CS5 (Adobe Inc., San Jose, CA, USA). The cell area was defined as the area surrounded by the cell wall. No cells adjacent to stomata were observed during this study.

Thereafter, each fruit was cut into slices at its equator by using a sharp razor, and the flesh and pith of the cross-section of sliced fruit were observed at a depth of 4 mm from the surface and bundle zone using the same method. Four arbitrary view fields of the flesh and pith were observed. As described by Szczesniak and Smith (1969), the pith of strawberry fruit was defined as the inner part of the bundle zone in this study. The mean area of 10 cells per view field was measured in the same manner as for the fruit surface, and the mean area of each fruit was further calculated.

In all observations, the horizonality of the field of view was revised using analysis software. All observations were performed in a laboratory at 19°C and 40% humidity.

Statistics
All data were subjected to analysis of variance (ANOVA) and Tukey’s honest significant difference (HSD) test (for comparison between varieties and between days after harvest) or t-test (for comparison between treatments) at \(P < 0.05\) by using JMP® 8.0.2 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

The apparent modulus of elasticity of the ‘Koiminori’ test fruit was significantly greater than that of the ‘Toyonoka’ and ‘Sachinoka’ test fruits throughout the test period. At day 6 post-harvest, the apparent modulus of elasticity of the ‘Toyonoka’ test fruits was greater than that of the ‘Sachinoka’ test fruit. Changes in the apparent modulus of elasticity with storage duration were non-significant for all tested cultivars. No significant interaction between varieties and days after harvest was observed (Table 1).

Changes in fruit morphology associated with bruising were investigated for three cultivars with different elasticity, considering varietal differences and storage effects. Vis-
ual inspection of both the intact and bruised fruit surface revealed minimal discoloration or deformation. Under the microscope, no noticeable wrinkles or cracks were observed on the surface of both intact and bruised test fruits (Fig. 1). Additionally, no cell rupture was observed in the skin, flesh, or pith. Three-way ANOVA for skin cell size showed significant effects of variety, days after harvest, and their interactions. Varieties also significantly affected the flesh and pith cell size. Furthermore, interaction between varieties and days after harvest also significantly affected the flesh cell size. The skin and pith cell size were largest in the order of ’Toyonoka,’ ’Koimino-ri,’ and ’Sachinoka,’ whereas the flesh cell size was larger in ’Toyonoka’ and ’Koimino-ri’ than in ’Sachinoka.’ However, bruising did not affect on skin or pith cell size. Three-way ANOVA only for flesh cell size showed a significant effect of bruising. The flesh cell size in bruised fruit was significantly smaller than in intact fruit (Table 2).

Based on the morphological changes observed in this study, the flesh of strawberries was bruised, not the skin or pith. Thus, the external force on the fruit reaches the flesh and compresses the cells. Interestingly, bruised flesh cells were compressed without disintegration, rejecting the hypothesis that cells rupture in bruised tissue to release juice (Holt and Schoorl, 1982). In addition, the deformed bruised flesh cells did not return to their original size, at least until microscopic observation, i.e., plasticity was observed in this study. As bruising is thought to be caused by minor external forces (Hikawa-Endo and Sone, 2019; Kohyama et al., 2013), it is important to note that plastic deformation can be caused by these forces. An apparent linear region was observed in the load displacement curve obtained when pressure was applied to the strawberry fruit (data not shown). For homogeneous materials, such as metals, this region corresponds to the elastic region; however, because plant cells are a mixture of elastic and plastic bodies, some energy is used for plastic deformation in this region (Nonami, 2001).

Skin cells were prevented from being deformed by external forces, possibly because these cells are much

| Days after harvest | ‘Toyonoka’ | ‘Sachinoka’ | ‘Koimino-ri’ |
|-------------------|------------|-------------|--------------|
| 0                 | 0.39±0.01 a| 0.39±0.01 c | 0.58±0.02 a  |
| 6                 | 0.47±0.01 b| 0.34±0.01 c | 0.62±0.03 a  |
| 12                | 0.42±0.01 bc| 0.38±0.01 c | 0.59±0.03 a  |

Fig. 1 Laser microscopy images of strawberry fruit skin surface.
A: Intact fruit at day 0 post-harvest, B: Bruised fruit at day 0 post-harvest, C: Intact fruit at day 6 post-harvest, D: Bruised fruit at day 6 post-harvest, E: Intact fruit at day 12 post-harvest, F: Bruised fruit at day 12 post-harvest. Black bars indicate 200 μm.
cells that are easily disintegrated. Further research is needed to determine which components give rise to this characteristic skin or flesh.

Smaller cells are thought to be stronger than larger cells because of their higher percentage of cell wall per area. In this study, different varieties had different skin cell sizes. Deformations possibly occur when varieties with extreme-ly large skin cells are subjected to bruising treatment. In addition, no deformation was observed in the pith with smaller than those of the flesh in strawberries (Table 2). Smaller cells are thought to be stronger than larger cells because of their higher percentage of cell wall per area. In this study, different varieties had different skin cell sizes. Deformations possibly occur when varieties with extreme-ly large skin cells are subjected to bruising treatment. In addition, no deformation was observed in the pith with large cells (Table 2). This is likely because the external force in this experiment was small (7 N) and the bundle zone on the outside of the pith was strong, preventing the external force from reaching the pith.

In conclusion, this study provides compelling evidence that bruising in strawberry fruit is caused by external forces that induce flesh cell compression without deforming the skin surface. Based on the results obtained for different cultivars and storage days, compression of flesh cells is relatively common in bruised fruit. Although bruising is not effectively detected by common machine vision systems (Nagata et al., 2006), it can be detected by target-ing the flesh cells. Moreover, fruits may not require large amounts of fungicides or other treatments for surface disinfection after bruising, as their surface morphology remains intact. Further empirical studies are warranted to confirm whether bruising affects the generation of volatile com-pounds in wounds, mycelial growth, and spore formation. To prevent bruising, a breeding objective is to obtain skin that does not transmit external forces to internal tissues rather than skin that is not prone to cell deformation or cell disintegration. Similarly, the breeding objective should be flesh cells that are not easily deformed, rather than flesh cells that are easily disintegrated. Further research is needed to determine which components give rise to this characteristic skin or flesh.

Table 2 Comparison of surface smoothness and cell area of test fruits in varieties, days after harvest and treatments

| Source of variation | Treatment | Fruit surface smoothness (µm²) | Cell area (mm²) |
|---------------------|-----------|-------------------------------|---------------|
|                     |           | R²                           | R²             |
| Varieties (A)       | ‘Toyomaka’| 0.67 ± 0.02                   | 3,625 ± 99 a   |
|                     | ‘Sachinoka’| 0.63 ± 0.03                   | 4,108 ± 127 b  |
|                     | ‘Koiminori’| 0.60 ± 0.02                   | 4,086 ± 107 b  |
| Days after harvest (B) | 0          | 0.60 ± 0.02                   | 4,527 ± 125 a  |
|                     | 6         | 0.64 ± 0.02                   | 3,917 ± 120 b  |
| Treatments (C)      | Bruised   | 0.62 ± 0.02                   | 4,283 ± 104    |
|                     | Intact    | 0.61 ± 0.02                   | 4,059 ± 93     |

Three-way ANOVA*

| A | NS  | NS  | **  | **  | **  |
| B | NS  | NS  | NS  | NS  | NS  |
| C | NS  | NS  | NS  | NS  | NS  |
| A×B | NS  | NS  | **  | *  | NS  |
| A×C | NS  | NS  | NS  | NS  | NS  |
| B×C | NS  | NS  | NS  | NS  | NS  |
| A×B×C | NS  | NS  | *  | NS  | NS  |

Three-way ANOVA*:  Different letters indicate significant differences (Tukey’s HSD test or t-test, P < 0.05). Values are the mean ± SE (n = 12).
R² indicates the mean height difference between the highest and lowest areas of each cell.
R indicates the arithmetic mean roughness.
NS: non-significant, *: significant at P < 0.05; **: significant at P < 0.01.

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