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ARTICLE

Spatial and Developmental Profiling of Miraculin Accumulation in Transgenic Tomato Fruits Expressing the Miraculin Gene Constitutively

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We previously developed a transgenic tomato that expresses the miraculin gene using a constitutive promoter. In this study, we profiled the developmental and spatial accumulation of the miraculin protein and mRNA in transgenic tomato fruits. Miraculin mRNA expression was almost constant up to orange stage, and then the expression increased at red stage. The miraculin protein accumulated gradually during fruit development and reached its highest level at the overripe stage. At the red stage of fruit, miraculin protein was accumulated at the highest level in the exocarp, and similar in other fruit tissues: mesocarp, dissepiment, upper placenta, lower placenta and jelly. Moreover, the pattern of miraculin accumulation in fruit tissues was the same regardless of genetic background and position at which the miraculin gene was inserted in the genome. We also discuss suitable tomato types expressing miraculin for their commercial use.

KEYWORDS: Fruit tissue; taste-modifying protein; miraculin; 35S promoter

INTRODUCTION

Miraculin, a glycoprotein extracted from the miracle fruit (*Richadella dulcifica*), is native to West Africa (*1*). Miraculin itself is not sweet, but once the human tongue is exposed to it, its taste-modifying function causes a sour taste to be perceived as sweet, and the modification effect can last as much as 1−2 h (*2*, *3*). Miraculin has great value as a diet and low calorie sweetener, but the miraculin protein is still dependent on its natural source; thus, it is extracted from the miracle fruit and sold in tablets, or its seedlings and fruits are sold at an expense by importing the plant from its origin.

Miraculin production in *Escherichia coli* (*4*), yeast, and tobacco (*5*) has been attempted but failed to express miraculin with taste-modifying activity. Recently, the production of miraculin in *E. coli* was reattempted. This study showed that the taste-modifying activity, without glycosylation, was no more than 72% that of native miraculin even with 1.6 times higher density than the refined native glycoprotein (*6*).

We have expressed miraculin in transgenic lettuce (*7*), tomato (*8*), and strawberry (*9*). Among these studies, the miraculin gene was successfully expressed in tomato using the constitutive CaMV 35S promoter, and the transgenic tomato accumulated miraculin in the entire plant at high levels. The CaMV 35S promoter is used to drive transgenes in plants with its high level of expression activity, but shows no specific occurrence (*10*−*12*). The 35S promoter functions in most cells and tissue types but at different levels depending on the developmental stage and tissue type (*13*). The 35S promoter also includes modular sequences, which demonstrate various developmental and tissue-specific expression patterns (*14*). When the 35S promoter was used in cucumber with the thaumatin gene, which produces a sweet-tasting protein, transgene expression was detected in the vascular tissue but not in the pollen due to gene silencing (*15*). This implies that the expression and accumulation pattern of transgenes by the 35S promoter may vary depending on the transgene or plant species to be transformed. Studies are currently being conducted to refine and use miraculin from tomatoes, but no previous studies have examined the profile of miraculin accumulation in such tomatoes.

Tomatoes are an important crop throughout the world with 126 million tons produced on 4,626 ha of land in about 170 countries [Food and Agriculture Organization (FAO) 2007]. Tomato consumption is distinguished between fresh and processed tomatoes such as paste, juice, and ketchup. Especially in Asia, the consumption of fresh tomatoes includes about 94% of the total tomato consumption, and Asia has a much higher consumption of fresh tomatoes than other regions of the world [World Processing Tomato Council (WPTC)]. Tomato cultivars are divided into various types depending on processing and fresh use. These tomatoes have different tissue compositions in the fruits, which is likely to affect miraculin accumulation pattern in the fruits. Therefore, it is important to select tomato cultivars for expressing miraculin depending on their commercial use.

In this study, we profiled the spatial and developmental characteristics of miraculin accumulation in transgenic tomato fruits stably expressing the miraculin gene to effective use of miraculin from transgenic tomatoes. We also discuss what the

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appropriate tomato cultivars are for accumulating miraculin for processing and fresh use.

MATERIALS AND METHODS

Plant Materials. The transgenic tomato lines expressing the miraculin protein were produced in our previous work (*8*). The two lines, 56B and 7C (upright type, cv. 'Moneymaker') were selected depending on miraculin accumulation and grown to the T_6 and T_4 generations, respectively. These two tomato lines possess the miraculin gene driven by the CaMV 35S promoter, and recombinant miraculin protein accumulates in the entire plant. Line 56B was crossed with a dwarf tomato (cv. 'Micro-Tom') and grown to the F_6 generation to investigate the effect of a difference in genetic background on miraculin accumulation. Miraculin accumulation, self-pruning habit, fruit size, and plant height were used as selection indicators. The selected line (56B × 'Micro-Tom'; F_6) producing the miraculin protein has a self-pruning habit, which is a characteristic of 'Micro-Tom', and bigger fruits than 'Micro-Tom', and the plant size is more compact than 'Moneymaker'. These plant lines (56B, 7C, and 56B × 'Micro-Tom'; F_6) were cultivated in a closed hydroponics system under a netted greenhouse.

The fruits were harvested at various growth stages. The developmental stages were classified by days after flowering and fruit ripening stage as follows: flower (0), 7 days (7), 14 days (14), 28 days (28), 35 days (35), mature green (MG), yellow (YL), orange (OG), red (RE), and overripened (OR). The harvested fruits were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Eight tomato cultivars ('Micro-Tom', 'Aiko', 56B × 'Micro-Tom' (F_6), 'M82', 'Moneymaker', 'Reiyo', 'Momotaro-fight', and 'Aichi-first') were used for the fresh-weight determination of each fruit tissue. 'Micro-Tom (accession number TOMJPF00001)', 'M82 (accession number TOMJPF00005)', and 'Moneymaker (accession number TOMJPF00002)' were obtained from the National Bio Resource Project (NBRP) (http://tomato.nbrp.jp/). 'Aiko', 'Reiyo', 'Momotaro-fight', and 'Aichi-first' were bought at a local market.

Separation of Fruit Tissue. To measure fresh weight and/or detect miraculin mRNA and protein from different tissues in the tomato, fruit from the eight cultivars was separated into six parts: the exocarp, jelly, mesocarp, dissepiment, upper placenta, and lower placenta. The exocarp was separated from the fruit after dipping in liquid nitrogen for a few seconds, and then the fruit without the exocarp was cut into halves. Jellies were separated out, and the remaining fruit tissue parts were separated into the mesocarp, dissepiment, upper placenta, and lower placenta. The fresh weight of each separated part was measured, and the parts were frozen immediately in liquid nitrogen. Separated fruit tissues from the line 56B were frozen with liquid nitrogen immediately after measuring the fresh weight for the miraculin accumulation profiling analysis. Each data was shown as average of seven fruits. Moreover, another set of fruit tissues from the line 56B were freeze-dried to analyze the miraculin protein content per dry weight.

Immunoblot Analysis. The accumulation of miraculin protein in lines 56B, 7C, and 56B × 'Micro-Tom' F_6 transgenic tomatoes was determined using immunoblot analysis. One fruit sample from three independent plants was randomly collected at every stage of fruit development. Collected fruit tissues were ground to powder in liquid nitrogen. The powder (0.1 g) was thawed in 100 μL of extraction buffer consisting of 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 2% polyvinylpolypyrrolidone. The extracts were centrifuged at 15,000 rpm for 20 min at 4 °C, and the supernatant was used for immunoblot analyses. Ten microliters of the extract (2.5 mg of dry weight and 5 mg of fresh weight equivalents per lane) was separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred onto Hybond-P polyvinylidene fluoride membranes (GE Healthcare Ltd., Amersham, Buckinghamshire, U.K.). Immunoblot analyses were conducted according to Sun et al. (*8*).

Enzyme-Linked Immunosorbent Assay (ELISA). 100 μL of 1000 times diluted supernatant was applied to a 96 well plate (Sumiron, Sumitomo Bakelite, Tokyo, Japan), and various concentrations of purified miraculin were applied as standard and incubated for 1 h at 37 °C. After incubation, samples were combined with 100 μL of Starting Block solution (Thermo Fisher Scientific Inc., Rockford, IL) for blocking, incubated for 10 min at room temperature and washed with PBS (pH 8.0) four times, and then HRP-labeled miraculin antibody was reacted to samples on the well for 1 h at room temperature. After reaction, samples were washed with PBS four times, combined with 100 μL of ABTS (Thermo Fisher Scientific Inc., Rockford, IL), incubated for 15 min at room temperature for color development and combined with 100 μL of 1% SDS to stop color development, and then color development was detected by 405 nm absorbance and miraculin concentration was calculated by 405 nm absorbance.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The miraculin mRNA expression levels at various stages and in tissues from the transgenic tomato plants were determined using quantitative RT-PCR. Total RNA was isolated from frozen tomato tissues and treated with DNase using an RNeasy Plant Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Total RNA (1.5 μg) was used to synthesize the first-strand cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). The first-strand cDNA diluted in a total volume of 500 μL of cDNA (1 μL) was used for quantitative RT-PCR with SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan), and the PCR thermal cycling conditions followed the manufacturer's instructions. Relative quantification of miraculin gene expression was calculated using the tomato ubi3 gene (accession number X58253) as an internal control (*16*, *17*). Primer sequences were as follows: miraculin forward, 5′-CACCCAATCCGGTTCTTGAC-3′, and reverse, 5′-GTGGTGGCGGATACTGTAAGG-3′; ubi3 forward, 5′-CACCAAGCCAAAGAAGATCA-3′, and reverse, 5′-TCAGCATTAGGGCACTCCTT-3′.

Histochemical Analysis. Red stage fruit was sliced at the appropriate size to observe the fruit tissue in 'Micro-Tom'. Fruits were fixed in 45% ethanol, 5% formaldehyde, and 5% acetic acid for 60 h at 4 °C. After dehydration in a graded 2-methyl-2-propanol series, the sliced fruit was embedded in Paraplast Plus (Fisher Scientific, Loughborough, Leicestershire, U.K.) and sectioned to a 12 μm in thickness. Sections were applied to slide glasses, which were treated with 3-aminopropyltrichlorosilane (Shinetsu Chemicals, Tokyo, Japan), and stained with the periodic acid−Schiff reaction as described by Jensen (*18*). The section was observed under an optical microscope.

RESULTS

Miraculin Accumulation during Fruit Development in Transgenic Tomato. The transgenic tomato fruits were harvested at the various growth stages to characterize the time course accumulation of miraculin during the fruit development. It appeared that the vegetative and fruit phenotypes of the transgenic tomato plants accumulating miraculin were not significantly influenced, suggesting that the miraculin may not be associated with these developments. Miraculin mRNA expression and protein accumulation appeared to be present at the indicated developmental stage (**Figure 1**). The mRNA accumulation of the miraculin in the transgenic plants was significantly observed until the orange stage, and then increased during the red and overripe stages. The highest mRNA accumulation was observed at the red stage, which was about five times higher than that of the orange stage (**Figure 1A**). The immunoblot analysis showed that the miraculin protein accumulation was observed at the expected size (47 kDa), due to that the miraculin protein is known to undergo dimerization (*19*).

Accumulation of the miraculin protein appeared to be gradually increased during fruit maturation, and the highest accumulation was observed at the overripe stage (**Figure 1B**).

Miraculin Accumulation in Various Tissues of the Transgenic Tomato Fruit. In order to examine the spatial profiling of miraculin protein accumulation, we used fruit at red stage that showed a high level of miraculin mRNA and protein accumulation (**Figures 2** and **3**). In order to examine detailed expression pattern in fruit in the transgenic tomato plants, the fruit tissue was categorized into seven portions: exocarp, mesocarp, dissepiments, upper placenta, lower placenta, jelly, and seeds (**Figure 2A**).

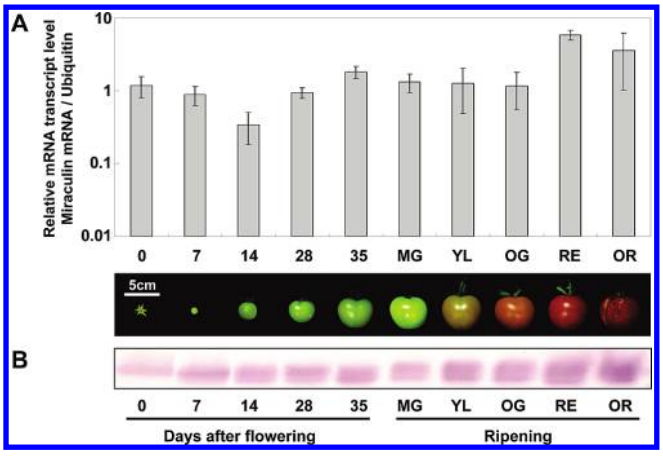

Figure 1. Changes in miraculin mRNA expression and protein accumulation in a transgenic tomato during fruit development and ripening. (**A**) Miraculin mRNA expression during fruit development in a transgenic tomato using real-time quantitative RT-PCR analysis. Line 56B was used as the transgenic tomato. The developing fruits were harvested at days 0, 7, 14, 28, and 35 after flowering and at the fruit ripening stage as follows: mature green (MG), yellow (YL), orange (OG), red (RE), and overripe (OR). Vertical bars show the standard deviation from three independent experiments. (**B**) Immunoblot analysis of the miraculin protein during development in transgenic tomato fruits. Five-milligram fresh weight equivalents of tomato fruit were separated by SDS−PAGE and blotted onto a PVDF membrane. The membrane was hybridized with antibodies to miraculin.

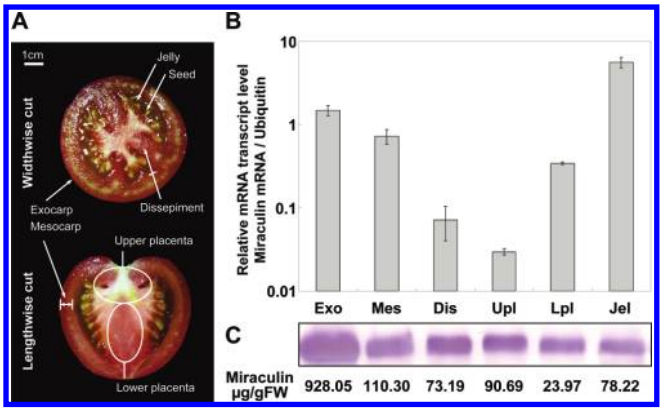

Figure 2. Miraculin mRNA expression and protein accumulation in red fruit tissues of a transgenic tomato. (**A**) Cross section (top) and longitudinal section (bottom) of transgenic tomato fruit. Line 56B was used as the transgenic tomato. (**B**) Miraculin mRNA expression in the fruit tissues of a transgenic tomato using real-time quantitative RT-PCR analysis. Vertical bars show the standard deviation from three independent experiments. Exo, exocarp; Mes, mesocarp; Dis, dissepiment; Upl, upper placenta; Lpl, lower placenta; Jel, jelly; Sed, seed. (**C**) Immunoblot analysis of the miraculin protein in the fruit tissues of a transgenic tomato. Five-milligram fresh weight equivalents of fruit tissue were separated by SDS−PAGE and blotted onto a PVDF membrane. The membrane was hybridized with antibodies to miraculin. The miraculin concentration of fruits was analyzed by ELISA.

This analysis demonstrated that the miraculin mRNA accumulation in the jelly tissue was 3.7 times higher than that in the exocarp (**Figure 2B**). In contrast, the highest miraculin protein accumulation was observed in the exocarp (**Figure 2C**). Meanwhile, similar protein accumulation was observed in the other tissues examined. The miraculin protein accumulation appeared to be observed at

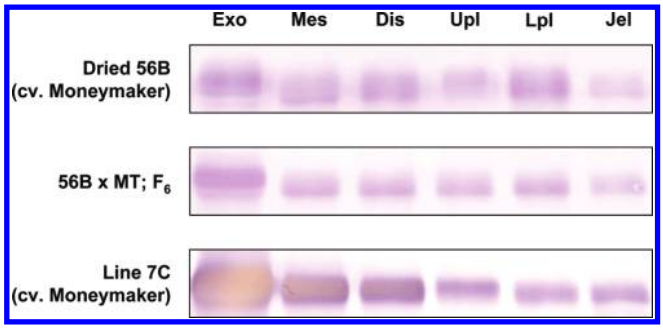

Figure 3. Effect of various factors on miraculin accumulation in fruit tissues. Using immunoblot analysis, miraculin accumulation was detected in the dried red fruit of a transgenic tomato (line 56B; T_6) (top) and fresh red fruit of the hybrid between line 56B and 'Micro-Tom' (56B × MT; F_6) (middle) and another transgenic line, 7C (T_4) (bottom). The fruit tissue materials are described in **Figure 2**. Each 2.5 mg dry weight (top) and 5 mg fresh weight (middle and bottom) equivalent of fruit tissues was separated by SDS−PAGE and blotted onto a PVDF membrane. The membrane was hybridized with antibodies to miraculin.

an extremely high level in exocarp, which was nine times higher than that in the other tissues.

In order to confirm the effect of the water amount on the miraculin protein accumulation, an immunoblot analysis was performed using the freeze-dried tissues of each fruit (**Figure 3**, **top**). Similar miraculin protein accumulation was observed among the exocarp, mesocarp, dissepiments, upper placenta, and lower placenta, although the lower protein accumulation was observed in the jelly.

The F_6 generation of the hybrid between line 56B and 'Micro-Tom' was analyzed to assess the possible effect of genetic background on the miraculin accumulation. Prior to this analysis, we have confirmed that the transgene encoding the miraculin gene was introgressed from the same line 56B by Southern blot analysis (data not shown). Consistent with **Figure 3** (top), the highest miraculin protein accumulation was observed in the exocarp (**Figure 3**, middle). In addition, line 7C showed the highest miraculin protein accumulation in the exocarp, which was consistent with the fact that the 56B and the hybrid lines showed similar protein accumulation (**Figure 3**, bottom).

In order to explore a possible effect of miraculin accumulation on the tomato fruit tissues, fruit tissue was sectioned and observed under light microscopy (**Figure 4**). This histological analysis demonstrated that the exocarp cell size appeared to be smaller than that of the mesocarp, dissepiment, placenta, and jelly.

Ratios of Fresh Weight of Each Fruit Tissue in Various Cultivars. In order to examine whether higher miraculin protein accumulation in fruit tissue was due to increased cell numbers or weight, the percentages of ratio in the fruit tissues was determined in the different seven cultivars (**Figure 5**). The percentages of ratio were determined from the average weight of seven independent fruits. We divided the tomato cultivar into three groups based on total weight of fruits, small-sized fruit (less than 20 g), medium-sized fruit (20 g < weight < 100 g), and large-sized fruit (more that 100 g). The small-sized group included 'Micro-Tom', 'Aiko', and the F_6 generation of the hybrid between line 56B and 'Micro-Tom'. Interestingly, the exocarp weight percent ratio in 'Micro-Tom' was 8.16%, which was at least 5% higher than in the other varieties, but the mesocarp ratio was somehow lower than the others. The mesocarp weight percent ratio in 'Aiko' was relatively high, 53.67%, and the exocarp ratio was 3.27%, which was the second highest ratio after that of 'Micro-Tom'. The F_6 generation of the hybrid between line 56B and 'Micro-Tom' showed similar

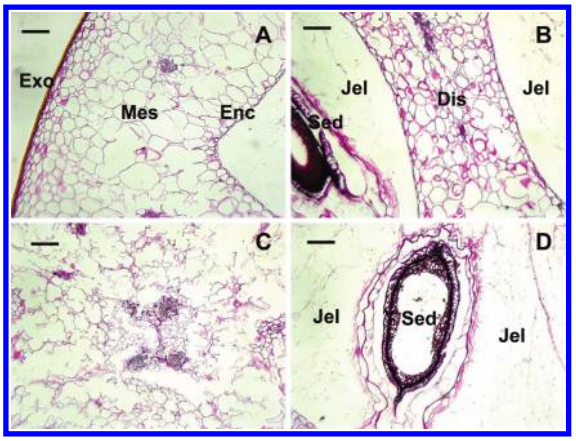

Figure 4. Histochemical analysis of 'Micro-Tom' fruit at red stage. Cross sections of the pericarp (**A**), dissepiment (**B**), placenta (**C**) and seed and jelly (**D**) were observed. Scale bars indicate 0.3 mm. Exo, exocarp; Mes, mesocarp; Enc, endocarp.

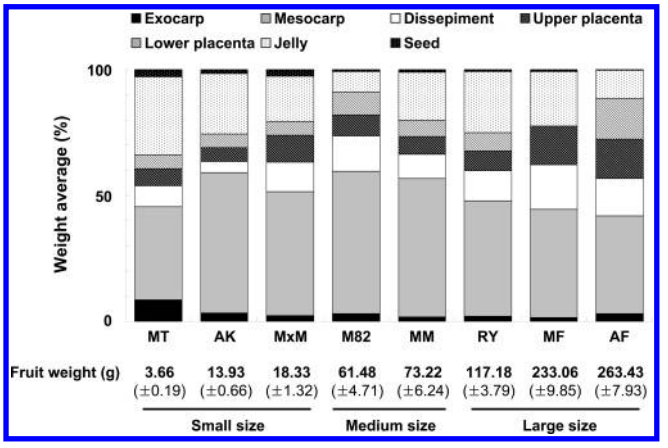

Figure 5. Ratio of fruit tissues to fruit weight in various tomato cultivars. The weight percent ratios of fruit tissue were averaged from seven fruits. The eight cultivars used were 'Micro-Tom' (MT), 'Aiko' (AK), 56B × 'Micro-Tom' (F_6) (M × M), 'Moneymaker' (MM), 'Reiyo' (RY), 'Momotaro-fight' (MF), and 'Aichi-first' (AF). The fruit tissues examined are described in **Figure 2**. Fruit weights are the means ± SE ($n = 7$).

trends on weight ratio with 'Moneymaker' than 'Micro-Tom'. 'M82' and 'Moneymaker' were placed into the medium-sized group in which the average weight was between 50 and 70 g. The mesocarp weight percent ratio in the medium-sized fruit was over 50%. The ratio of jelly was especially low in 'M82' at 6.63%. 'Reiyo', 'Momotaro-fight', and 'Aichi-first' were classified into the large-sized group (> 100 g). The mesocarp weight percent ratio was below 50% in the large-sized group, and the ratios of the dissepiment, placenta, and jelly were comparatively higher than in the other groups, although the rates among these tissues varied depending on the cultivar. Overall, a significant character was observed in the weight percent ratio of each fruit tissue in different cultivars but not between size groups.

DISCUSSION

Spatial and Developmental Profiling of Miraculin Accumulation. The CaMV 35S promoter is the most commonly used promoter for driving expression of transgenes in plants (*12*). However, some reports suggest that the expression levels are different in cells, tissue types, and developmental stages (*13*). Previously, we developed transgenic tomatoes expressing the miraculin gene using the CaMV 35S promoter (*8*). Although identifying the accumulation profile of materials produced for commercial purposes is important, the miraculin accumulation pattern has not been characterized. In this study, we profiled the spatial and developmental accumulation pattern of miraculin in transgenic tomato fruit. The miraculin mRNA expression level was almost uniform from the flower to orange stage and then increased from the red to overripe stage. The miraculin protein accumulation was gradually increased during fruit development and ripening and reached at the peak in overripe fruit. Higher miraculin accumulation was observed in the exocarp of red fruit, and miraculin also accumulated in other tissues at lower levels. The various fruit tissues were also investigated for miraculin accumulation per protein amount. The miraculin accumulation was the highest in exocarp and almost the same with that per fresh weight (data not shown). These results demonstrate that miraculin mRNA expression and protein accumulation can be detected at every stage and tissue of transgenic tomato fruit, although the levels are different. Similar results have been reported for GUS activity driven by the CaMV 35S promoter in transgenic rice and soybean. Different levels of GUS activity were detected in the leaves, roots, and flower organs of transgenic rice (*20*). Additionally, GUS activity has been higher at pericycle cells in root, parenchyma cells in xylem, phloem tissues of stem and leaf and observed in different level at various tissue types such as procambium cells in root, vascular cambium cells in stem and cortex cells in leaf midrib in transgenic soybean (*21*).

The miraculin protein was highly accumulated in the exocarp of line 7C, which is independent of the line 56B, and the crossed line of 56B ('Moneymaker') and 'Micro-Tom' (**Figure 3**). This result indicates that the miraculin accumulation pattern was similar regardless of the genetic background or the position at which the foreign DNA was inserted into the genome. Sunikumar et al. (*13*) suggested that the expression level varies among independent transgenic lines when using the CaMV 35S promoter connected to the GFP reporter gene, although the expression pattern was very similar in all lines.

Generally, epidermis cells are densely arranged in higher plants. Our histological data also showed that the exocarp cells were extremely small and contained more mass compared to other tomato fruit tissues (**Figure 4**). Recent research has reported that miraculin accumulates in the intercellular layer space of the miracle fruit and transgenic tomato (*22*). We speculated that the abundant miraculin protein accumulation in the exocarp was caused primarily by cell size and the amount of intercellular layer space per fresh weight. This was supported by our result showing contraction of the difference in the accumulation level between the exocarp and other tissues when examined per dry weight.

Utilization of Transgenic Tomato Fruit Containing Miraculin. Throughout the world, tomatoes are eaten as fresh fruit, cooked, or as a seasoning. Choosing a tomato variety based on the intended use is necessary to utilize transgenic tomatoes accumulating miraculin. According to an estimation of miraculin content, homogenized tomato fruit mixtures of line 56B contained about 90 μg of miraculin per g fresh weight. This estimation indicated that 100 g of fresh tomato contains 9 mg of miraculin. On the other hand the taste-modifying function was induced by 50 μg of miraculin. Therefore, fresh fruits or juice of line 56B contain enough of miraculin for commercial use.

When a miraculin-producing tomato is eaten fresh, it is usually consumed as a whole fruit. Although miraculin accumulates in the exocarp, thick skin is unsuitable for eating raw varieties such as 'Micro-Tom'. The fruit should be small with a thin skin so that it is easy to eat if one wishes to acquire the effect of miraculin. Among the examined cultivars (**Figure 5**), 'Aiko' might be a suitable tomato cultivar expressing miraculin for eating fresh fruits.

In general, the exocarp and seeds of tomatoes are removed by commercial processing to produce juice, ketchup, puree, and paste, but the exocarp is important to recover miraculin more efficiently. However, according to our results (**Figure 5**), the ratio of exocarp to whole fruit did not exceed 5% except in 'Micro-Tom'. In particular, the proportions were less than 2.3% in 'Momotaro-fight'. Less miraculin loss may be achieved by selecting a tomato cultivar in which the ratio of exocarp to whole fruit is low such as 'Momotaro-fight'.

In the case of purifying miraculin from tomato fruit, it is able to utilize the exocarp. Therefore selection of tomato cultivars should be considered about the exocarp ratio because transgenic tomato exocarp contained a significant amount of miraculin per flesh weight when the miraculin gene was driven by the 35S promoter. We have produced transgenic tomatoes with miraculin using 'Moneymaker' as the background, but the percent weight ratio of the exocarp was 1.94%. In contrast, the ratio in 'Micro-Tom' was 8.16%, which was higher than the other varieties (**Figure 5**). Therefore 'Micro-Tom' might be a better cultivar for expressing recombinant miraculin for miraculin purification than other varieties.

In conclusion, we showed the spatial and temporal characteristics of miraculin accumulation in transgenic tomatoes, in which the miraculin gene was driven by the constitutive 35S promoter. In addition, we provided information on the tissue rates in several tomato cultivars. These data will be helpful for selecting tomato cultivars suitable for expressing recombinant miraculin based on the types of commercial use.

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