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Molecular Breeding of Tomato Lines for Mass Production of Miraculin in a Plant Factory

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A transgenic tomato line (56B, "Moneymaker") that expresses the *miraculin* gene driven by the CaMV 35S promoter was crossed with a dwarf tomato ("Micro-Tom") for the molecular breeding of cultivars that are suitable for miraculin production in a closed cultivation system. Plant size, miraculin accumulation, and self-pruning growth were used as selection indicators for F_2 plants. Two lines were chosen for further analysis, bred to the F_6 or F_7 generation and cultivated in a closed cultivation system. In 56B and the two crossed lines, the concentrations of miraculin in the pericarp were 140, 367, and 343 μg/g FW, respectively. We also estimated that 26.2, 73.6, and 45.9 kg FW/m^2 of tomatoes and 2.2, 16.6, and 9.8 mg/m^2 of miraculin in the pericarp, respectively, could be harvested per year. These two crossed lines will be useful for the mass production of miraculin, especially in a closed cultivation system.

KEYWORDS: Crossbreeding; fruit yield; miraculin; taste-modifying protein; tomato

INTRODUCTION

Miraculin is a glycoprotein extracted from the miracle fruit (*Richadella dulcifica*), which is native to West Africa (*1*). Miraculin itself is not sweet, but it converts a sour taste into a sweet taste (*2*,*3*). This property means miraculin can be used as a diet and low-calorie sweetener. In Japan, miracle fruit production is limited because it is a tropical plant. Therefore, the production of miraculin has been attempted using transgenic *Escherichia coli* (*4*,*5*), yeast (*6*), *Aspergillus oryzae* (*7*), and various plants (*6*, *8*, *9*).

We previously developed a transgenic tomato line (56B, "Moneymaker") that expresses the *miraculin* gene driven by a constitutive promoter (*10*). The transgene and miraculin accumulation were genetically stable from generation T_1 to T_5 in 56B (*11*). Tomatoes are usually cultivated in a greenhouse only once or twice per year in Japan, and the fruit yield and quality are dependent on the environment (including temperature, solar radiation, soil nutrition condition, and so on). By contrast, the fruit yield and quality of tomato plants cultivated in a plant factory, which is a closed cultivation system, are relatively constant and less affected by environmental conditions (*12*). Another advantage of a closed cultivation system is that it prevents the spread of transgenic plants or pollen. Thus, we recently constructed a closed cultivation system in a plant factory for 56B (*13*). A single truss production system (*14*) was used due to the limited cultivation space available. In this system, the tomato plants were pinched, leaving a few leaves above the first truss, and only the first truss was harvested. The labor requirements were reduced using this production system because the training and management were easy (*14*). Tomato plants grew faster and exhibited more uniform growth in the plant factory than in a netted greenhouse (*13*). Miraculin accumulation levels in fruits from the plant factory were more stable than those from a netted greenhouse (*13*). However, both the initial and the operating costs of the plant factory were much higher than those of a netted greenhouse. Therefore, improving cost performance has been a critical issue for the plant factory.

"Micro-Tom", which was bred for home gardening (*15*), is a model cultivar of tomato (*16*, *17*). It is a miniature dwarf tomato cultivar with a short life cycle and a determinate-type growth habit. The recombinant miraculin protein accumulates strongly in the exocarp of 56B, and the weight ratio of the exocarp in "Micro-Tom" fruit is much higher than that of other six cultivars (*18*). These phenotypes of "Micro-Tom" make it suitable for the closed cultivation system in the plant factory and indicate that "Micro-Tom" is potentially well suited for miraculin production. The miraculin accumulation in an F_1 hybrid line between 56B and "Micro-Tom" was higher than that between 56B and wild type "Moneymaker" (unpublished data). However, the fruit size of "Micro-Tom" is too small, and a small fruit size increases harvesting costs.

In this study, we bred suitable tomatoes for the closed cultivation system by crossing the 56B and "Micro-Tom" varieties. We discuss the production of miraculin in these crossed lines.

MATERIALS AND METHODS

Plant Materials. The transgenic tomato line 56B (upright type, "Moneymaker") possesses the *miraculin* gene driven by the CaMV 35S

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promoter, and the recombinant miraculin protein accumulates in all plant tissues (*10*). The homozygous line from the T_3 generation of 56B was crossed with a dwarf tomato cultivar ("Micro-Tom"), and the transgenic plants were grown in a netted greenhouse and allowed to self-pollinate. Plant size, miraculin accumulation, and self-pruning were used as selection indicators for F_2 plants. The two selected lines were named cross no. 1 and cross no. 2, and they were bred to the F_7 and F_6 generations, respectively, by self-pollination. Fruit yield was used as a selection indicator in subsequent generations. The plants of 56B and the crossed lines were cultivated in a closed cultivation system, which is described in the following section.

Construction of the Tomato Cultivation System. The tomato cultivation system in the plant factory was described previously (*13*). Transgenic tomato seeds of 56B (T_7), cross no. 1 (F_7), and cross no. 2 (F_6) were germinated on Petri dishes covered with moist filter paper at 25/20 °C (light/dark), with 16 h of light from a fluorescent lamp at 450 μmol/m^2/s (photosynthetic photon flux) and 8 h of dark in the plant factory. The CO_2 concentration was maintained at 600 ppm. One week after sowing, seedlings were transplanted to rockwool cubes (5 cm × 5 cm × 5 cm) and grown in a Naeterrace seedling raising system, which was developed commercially by Taiyo Kogyo Co., Ltd. (Tokyo, Japan). Each day, the plants were provided with a nutrient solution containing 565 mg/L NO_3^-, 15.7 mg/L NH_4^+, 202.2 mg/L PO_3^-, 218.4 mg/L K^+, 19.9 mg/L Mg^{2+}, 95.0 mg/L Ca^{2+}, and micronutrients. Each seedling was then transferred to a two-layer cultivation system (*13*). For 32 days after germination, the plants were grown under the same conditions as those used in the seedling raising system, except for the nutrient solution. Otsuka-A nutrient solution, with an adjusted electrical conductivity of 1.8 dS/m, was supplied every day in the two-layer cultivation system. The planting densities of 56B and the crossed lines were 13.3 and 26.7 plants/m^2, respectively. The 56B plants were pruned, leaving three leaves above the first truss, and axillary buds were removed during cultivation. Axillary buds of cross no. 1 plants were also removed approximately two weeks after transplanting. Extra leaves were removed from 56B and cross no. 1 plants. There was no need to defoliate cross no. 2 leaves.

Analysis of Tomato Fruit Quality and Yield. Fruits were harvested once per week from day 48 to day 104 after transplantation to the two-layer system. Fruits with blossom-end rot were removed, and dehiscent fruits were counted. The weight, length, and diameter of each fruit were measured. The fruit yield per area per year (kg FW/m^2/year) was calculated based on the fruit yield per plant (g FW/plant), planting density (plants/m^2), and growing period per year (days/year) in the two-layer system.

Separation of Fruit Tissue. To measure the fresh weight and detect the miraculin protein in different tissues of the tomato, the fruits were separated into two parts: the pericarp and all other tissues. The other tissues were first separated from the fruit, which was cut into halves. The pH was measured in part of the pericarp. Next, the pericarp was separated into the exocarp and mesocarp by removing the exocarp after the fruit was dipped into liquid nitrogen for a few seconds. The fresh weight of each part was measured, and the separated fruit tissues were immediately frozen with liquid nitrogen.

Immunoblot Analysis, Enzyme-Linked Immunosorbent Assay (ELISA), and Protein Assay. The accumulation and concentration of the miraculin protein in 56B and the crossed lines were determined using immunoblot analysis and ELISA, respectively, as reported previously (*10*, *18*). Collected fruit tissues described above were ground to powder in liquid nitrogen. The powder (0.1 g FW) was thawed in 200 μL extraction buffer consisting of 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 2% polyvinylpolypyrrolidone . The extracts were centrifuged at 15000 rpm for 20 min at 4 °C, and the supernatant was used for immunoblot and ELISA analyses. The extracts (the equivalent of 0.5 mg FW per lane) were 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.). Miraculin accumulation was determined using immunoblot analyses, which were conducted according to Sun et al. 2007 (*10*). To measure the concentration of miraculin, 100 μL of 500-, 1000-, or 2000-fold dilutions of supernatant were applied to a 96-well plate (Sumiron, Sumitomo Bakelite, Tokyo, Japan), and various concentrations of purified miraculin protein were used as standards. ELISA was conducted according to Kim et al., 2010 (*18*). The soluble protein concentrations in the fruit tissues were determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

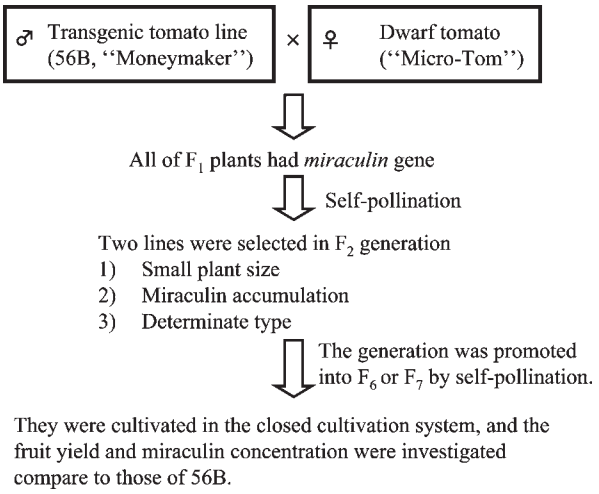


Figure 1. Flowchart of selection during the breeding of the crossed lines. The "Micro-Tom" phenotypes are the recessive mutations *dwarf* and *self-pruning*. F_1 plants show a dominant phenotype that combines the characteristics of the two parent plants. F_2 plants were first screened for dwarf size and then screened for the *self-pruning* phenotype. The selected plants were analyzed for recombinant miraculin protein accumulation. Finally, two crossed lines were selected and named cross no. 1 and cross no. 2. The F_3 generation was allowed to self-pollinate until the F_6 or F_7 generations, respectively, were produced. They were used to examine cultivation in a closed cultivation system.

Histochemical Analysis. Fruits at the green stage were sliced at the appropriate size to observe the tissue in 56B and the crossed lines. Transverse slices (100 μm) were prepared using a Leica vibratome (VT1200S; Leica, Nussloch, Germany). The separated sections were stained with safranin and observed under an optical microscope.

RESULTS

Selection of Crossed Lines. A flowchart of the selection process during the breeding of the crossed lines is shown in **Figure 1**. The 56B variety was crossed with a dwarf tomato ("Micro-Tom") that exhibited a self-pruning growth habit and short height, hypocotyl, and petiole phenotypes. The hypocotyl length of "Micro-Tom" is much shorter than that of "Moneymaker". Therefore, we used this marker to select F_2 individuals as candidates possessing the dwarf phenotype. The hypocotyl lengths of the F_1 plants were similar to those of the parental line 56B (data not shown). Ten F_1 plants were allowed to self-pollinate and produce F_2 seeds, which were sown and grown in a netted greenhouse. Out of 560 F_2 seedlings, 86 were selected 2 weeks after sowing on the basis of hypocotyl length.

As a second screen, we tested miraculin accumulation in the 86 F_2 seedlings and found 23 candidate lines that showed miraculin protein accumulation. These 23 plants were then grown for 8 more weeks, at which time we selected 7 plants with the self-pruning growth habit. Finally, two F_2 plants, named cross no. 1 and cross no. 2, were selected for further analysis on the basis of fruit yield and plant size. The plants of cross no. 1 and cross no. 2 produced higher fruit yields and were much smaller than 56B and the other six F_2 candidates, respectively.

Fruit yield was used as a selection indicator in subsequent generations, and homozygous lines expressing *miraculin* were fixed at generation F_4. The crossed lines were bred to generations F_6 (cross no. 2) or F_7 (cross no. 1) by self-pollination.

Phenotypes of 56B and the Crossed Lines. After we obtained stable and nonsegregating F_6 (cross no. 2) or F_7 (cross no. 1)

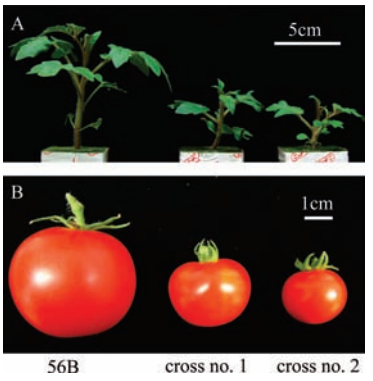


Figure 2. Seedlings 22 days after germination (**A**) and average red fruit (**B**) of 56B and the crossed lines. Seedlings were grown in the Naeterrace for 22 days.

Table 1. Fruit Characteristic of 56B and the Crossed Lines in the Closed Cultivation System[a]

line	no. fruits per plant	fruit weight (g FW)	fruit length (mm)	fruit diameter (mm)
56B	4.0 ± 0.3	51.2 ± 2.4	39.9 ± 0.8	48.4 ± 0.9
cross no. 1	21.9 ± 2.4	13.3 ± 0.5	25.5 ± 0.3	29.4 ± 0.4
cross no. 2	36.6 ± 5.7	6.7 ± 0.3	20.6 ± 0.3	22.2 ± 0.4

[a] The data presented are the means ± standard errors of eight plants.

plants, they were cultivated in the closed cultivation system in the plant factory. Seedlings of the crossed lines were more compact than those of 56B (**Figure 2**), so they were cultivated at higher planting densities than 56B. Cross no. 1 plants showed succulent and bushy growth approximately two weeks after transplanting, so extra axillary buds and leaves were removed. By contrast, cross no. 2 plants did not require such treatments for growth.

The plants of cross no. 2 were grown without pinching or defoliating and therefore produced more fruits than 56B and cross no. 1 (**Table 1**). The average number of fruits produced by 56B was approximately four because the fruits were harvested only from the first truss, and approximately two fruits with blossom-end rot were removed per plant. On the other hand, there were no fruits with blossom-end rot found in the crossed lines. There were some dehiscent fruits in 56B and cross no. 1, but they were counted because the fruits were harvested only once per week. By contrast, there were no dehiscent fruits in cross no. 2. The fruit weights and sizes of 56B were the largest of all lines, and the fruits of cross no. 1 were two times heavier than those of cross no. 2 (**Table 1**, **Figure 2**).

Fruit Yields in 56B and the Crossed Lines. The fruit yield per plant of cross no. 1 was higher than that of 56B (**Figure 3A**), and the fruit yield of cross no. 2 was similar to that of 56B. The fruit yields per plant of 56B and cross no. 1 were greatly reduced 76 days after transplanting into the two-layer cultivation system because their axillary buds were removed. On the other hand, the fruit yield per plant of cross no. 2 increased linearly until the end of the cultivation period. The maximum fruit yields per area per year for 56B, cross no. 1 and cross no. 2 were 26.2, 73.6, and 45.9 kg FW/m^2/year, respectively (**Figure 3B**). In this study, the maximum fruit yield of cross no. 1 per area per year was much higher than that of 56B, and that of cross no. 2 was also higher than that of 56B. Thus, cross no. 1 was the best line with respect to fruit yield.

Miraculin Accumulation and Concentration and pH level in the Pericarp of Fruits of 56B and the Crossed Lines. To examine miraculin production in the pericarp, the accumulation and concentration of miraculin in the pericarp were investigated. The levels of miraculin accumulation in the pericarp of the crossed lines were much higher than those of 56B (**Figure 4**). The miraculin concentrations in the pericarp of the crossed lines were also approximately 2.5 times higher than that of 56B (**Figure 4**). These results show that the crossed lines performed better with respect to miraculin production in the same amount of pericarp than 56B. The native miraculin protein was stable under acidic conditions (*1*), so the pH levels in the transgenic tomato fruits were measured. The pH levels of 56B and the crossed lines were 4.15 and approximately 4.05, respectively.

Miraculin Accumulation and Concentrations in Various Fruit Tissues of 56B and the Crossed Lines. We examined the spatial profiles of miraculin accumulation in 56B and the crossed lines. Fruits were divided into three parts: the exocarp, the mesocarp, and all other tissues. In the fruits of all lines, the miraculin accumulation and concentration in the exocarp were much higher than in the other tissues (**Figure 5**). In the crossed lines, miraculin accumulation levels and concentrations in the mesocarp and other tissues were higher than those in the 56B line.

Ratios of Fresh Weight of Each Tissue, Soluble Protein Concentration, and Histochemical Analysis in 56B and the Crossed Lines. To understand the molecular mechanism of the higher miraculin accumulation in the crossed lines compared with the parental line 56B, the percentages of weight and soluble protein contents in the fruit tissues were determined in these three lines (**Figure 5**). The weight percentages were determined from the average weight of eight different fruits. The exocarp weight percentages in 56B, cross no. 1, and cross no. 2 were 3.2%, 4.3%, and 6.1%, respectively. The weight percentages of the mesocarp and other tissues were similar among all lines. The soluble protein concentration in the exocarp of all lines was higher than in the other tissues. The soluble protein concentration in the mesocarp of the crossed lines was similar to that in 56B, and the soluble protein concentration in the other tissues of the crossed lines was higher than that in 56B. The histological data showed that the exocarp cells were smaller than the mesocarp cells in 56B and the crossed lines (**Figure 6**). The mesocarp cells of 56B were larger than those of the crossed lines.

DISCUSSION

Calculated Miraculin Production from the Pericarp of 56B and the Crossed Lines. Both the fruit yields and the miraculin concentrations of the crossed lines were much higher than those of 56B (**Figures 3** and **4**). Miraculin production from the pericarp was calculated from the miraculin concentration in the pericarp, the maximum fruit yield per area per year (kg FW/m^2/year), and the weight ratio of pericarp to all fruit tissues (percentage, Materials and Methods). The calculated values indicated that miraculin production in the pericarp of cross no. 1 and cross no. 2 was 7.5 and 4.5 times higher, respectively, than that in 56B (**Table 2**).

Planting Density. Light is one of the most important factors that affect tomato productivity (*19*). Light interception depends on the planting density, plant size, and defoliation. The planting density was very high using the single truss cropping system in the plant factory (*13*), and it was necessary to remove extra axillary buds and leaves. The crossed lines could be cultivated in a 2-fold higher planting density than the 56B line. However, cross no. 1 plants showed succulent and bushy growth. The extra axillary buds and leaves had to be removed because of reduced light interception. Cross no. 2 plants grew normally, and it was not necessary to remove axillary buds and leaves. "Micro-Tom" grows normally even under fluorescent light conditions (*17*). Cross no. 2 might have inherited this characteristic of

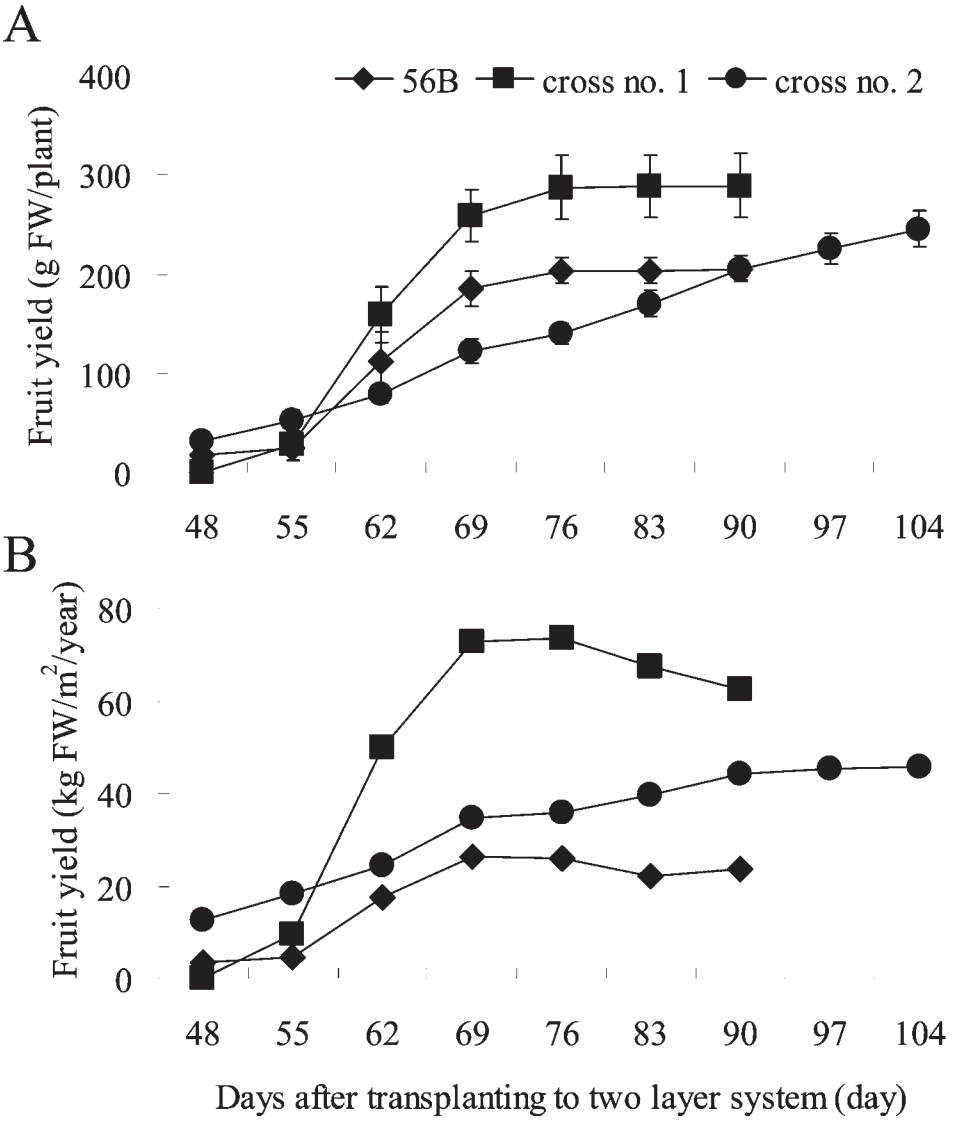
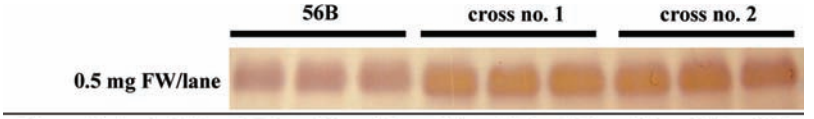


Figure 3. Fruit yield per plant (**A**) and per area per year (**B**) for 56B and the crossed lines in the closed cultivation system. The data presented in (**A**) are the means ± standard errors of eight plants. The data presented in (**B**) were calculated from the data presented in (**A**), the planting density (plants/m^2), and the growing period in the two-layer system per year (days/year). The planting density of 56B and the crossed lines were 13.3 and 26.7 plants/m^2, respectively.



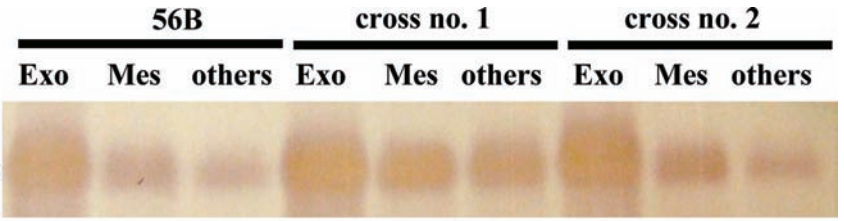
Miraculin (μg/g FW)	156	146	119	394	337	370	349	357	323
pH	4.15±0.05			4.05±0.01			4.06±0.01		

Figure 4. Accumulation levels and concentrations of miraculin and pH levels in the pericarp of 56B and the crossed lines. Miraculin accumulation was detected by immunoblot analysis. Protein samples were extracted from 0.5 mg samples (fresh weight). The miraculin concentration was measured by ELISA. Miraculin accumulation and concentration data were obtained from three different bulk pericarp tissues from three fruits. The pH data are the means ± standard errors of three different bulk pericarp tissues.

"Micro-Tom". Thus, the limited space in the closed cultivation system could be effectively used for the cultivation of cross no. 2. The plant height of cross no. 2 was especially low, so the two-layer cultivation system could be changed into a three-layer cultivation system in the same space. These plants could also grow entirely in the Naeterrace until harvesting time (data not shown).

Fruit Characteristics and Yield. Blossom-end rot is induced by an imbalance in the nutrient solution, such as a Ca^{2+} deficiency (*20*, *21*), a K^+ deficiency (*22*), differences in the ammonium-to-nitrate ratio (*23*), and high electrical conductivity (*24*). In this study, the rate of blossom-end rot detected in 56B was similar to a previously determined rate (*13*). The crossed lines exhibited no blossom-end rot. This fruit characteristic was inherited from "Micro-Tom" because "Micro-Tom" rarely shows blossom-end rot. The 56B and cross no. 1 plants had some dehiscent fruit, but cross no. 2 had none.

In our previous work, the maximum fruit yield of 56B was approximately 45 kg FW/m^2/year under strong light conditions (*13*).



	56B			cross no. 1			cross no. 2		
	Exo	Mes	others	Exo	Mes	others	Exo	Mes	others
Miraculin (μg/g FW)	1352	141	38	1098	291	250	1200	237	107
Fruit weight (g FW)	50.7 ± 3.8			15.0 ± 5.3			9.9 ± 1.7		
The percentage of weight ratio (%)	3.2	57.2	39.6	4.3	57.1	38.6	6.1	55.9	40.0
Soluble protein (mg protein/g FW)	12.2	8.2	7.3	12.1	7.8	9.0	12.3	9.3	10.6

Figure 5. Accumulation levels and concentrations of miraculin in fruit tissues, ratios of fresh weight of each tissue and soluble protein concentrations in 56B, and the crossed lines. Miraculin accumulation was detected by immunoblot analysis. Protein samples were extracted from 0.5 mg samples (fresh weight); the data presented are representative of three independent experiments. The miraculin concentration was measured by ELISA; the data presented are the averages of three independent experiments. The data of fruit weight are the means ± standard errors of eight fruits. The ratios of fresh weight of each tissue are the averages of measurements from eight fruits. The soluble protein concentration in fruit tissue was determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The data presented are the means ± standard errors of three independent bulk tissues. Exo, exocarp; Mes, mesocarp; Others, other tissues.

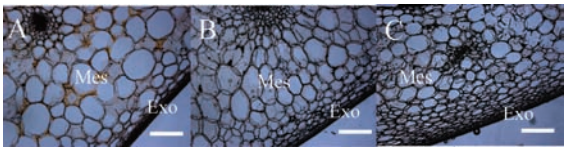


Figure 6. Histochemical analysis of 56B (**A**), cross no. 1 (**B**), and cross no. 2 (**C**) fruits at the green stage. Cross sections of the pericarp were observed. Scale bars indicate 0.5 mm. Exo, exocarp; Mes, mesocarp.

Table 2. Calculated Miraculin Production from the Pericarp of 56B and the Crossed Lines in the Closed Cultivation System

line	56B	cross no. 1	cross no. 2
miraculin concentration (μg/g FW)[a]	140 ± 11	367 ± 16	343 ± 10
maximum fruit yield (kg FW/m^2/year)[b]	26.2	73.6	45.9
weight ratio of pericarp (%)[c]	60.4	61.5	62.0
miraculin production (mg/m^2/year)[d]	2.2	16.6	9.8

[a] According to **Figure 4**. The data presented are the means ± standard errors of three independent bulk pericarps from three fruits. [b] According to **Figure 3B**. [c] According to **Figure 5**. [d] Miraculin production was calculated from the data (a), (b) and (c).

We also investigated the fruit yield per area per year of "Micro-Tom" grown at a high planting density entirely in the Naeterrace until harvesting time. The maximum fruit yield of "Micro-Tom" was approximately 40 kg FW/m^2/year. The fruit yield of cross no. 1 was much higher than that of 56B and "Micro-Tom". Moreover, the fruit yield of cross no. 1 per area per year could be improved by delaying the time of seedling raising because this time was adapted to 56B in this study.

The fruit yield of cross no. 2 per area per year was similar to the fruit yields of 56B and "Micro-Tom". However, the two-layer cultivation system could be changed into a three-layer cultivation system, and the fruit yield of cross no. 2 could be also improved by delaying the time of seedling raising similar to modifications that could be made for cross no. 1. Cross no. 2 plants could also be cultivated entirely in the Naeterrace for seedling raising. Thus, the fruit yield of cross no. 2 per unit area could be increased if the cultivation system was optimized for this cross line.

Miraculin Concentration. The native miraculin concentration in the pericarp of miracle fruit is approximately 400 μg/g FW (data not shown). In this study, the concentration of recombinant miraculin in the pericarp of the crossed lines was approximately 350 μg/g FW, which is almost equal to the level found in miracle fruit itself. The fresh weight of miracle fruit is approximately 1 g FW/fruit, which is sufficient to modify a sour taste into a sweet taste. Purified recombinant miraculin from the transgenic tomato has a similar taste-modifying activity as that of purified native miraculin from the miracle fruit (*10*). That is, one fruit of the crossed lines should have sufficient levels of miraculin for taste modification. Miraculin concentration in the pericarp was significantly correlated with exocarp weight percentages (data not shown). The miraculin concentration in the mesocarp of the crossed lines was also higher than that of 56B (**Figure 5**). These results were due to higher miraculin concentration in the pericarp of the crossed lines than that of 56B.

Miraculin accumulates in the intercellular layer of the miracle fruit and transgenic tomato (*25*). We speculated that the amount of miraculin in the intercellular layer per fresh weight of exocarp was higher than the amount found in other tissues of 56B because the exocarp cell size is much smaller than the cell sizes in other tissues (*18*). In this study, the miraculin concentration in the mesocarp of the crossed lines was higher than in 56B (**Figure 4**), although the soluble protein concentration in the mesocarp of the crossed lines similar to that in 56B (**Figure 5**). Fruit weight appears to be significantly correlated with cell size in the pericarp (*26*). The fruit size of the crossed lines was much smaller than that of 56B, and the cell sizes in the mesocarp of the crossed lines were smaller than those of 56B (**Figure 6**). The small cell size in the mesocarp of the crossed lines might be related to the high concentrations of miraculin. The pH of the crossed lines was slightly lower than that of 56B (**Figure 4**). This result might also be related to the high concentration of miraculin in the mesocarp of the crossed lines, as native miraculin is stable under acidic conditions (*1*).

Cost Performance. Cost performance is very important when recombinant miraculin is being produced commercially. It is necessary to increase miraculin productivity while reducing the

initial and operating costs to improve cost performance. We bred two suitable lines for the production of recombinant miraculin in a closed cultivation system. The miraculin productivity of cross no. 1 was especially high (**Table 2**). The labor requirement was reduced when a single truss production system was used (*14*), but it was necessary to remove extra axillary buds and leaves during the cultivation of the 56B line. The amount of work required for the cultivation of cross no. 2 was much less than the other lines because there was no need to remove axillary buds and leaves during cultivation. The plants of cross no. 2 did not have dehiscent fruits. This is an advantage for harvest because the fruits can be harvested together. Thus, the labor costs for the cultivation of cross no. 2 would be reduced, although the cost of harvesting the fruits might increase because of the smaller fruit size of cross no. 2.

The plants of cross no. 2 might also grow under lower light conditions than those used in this study, so the electricity costs for lighting might be reduced. Furthermore, plants of cross no. 2 could also grow entirely in a Naeterrace (a commercial system) for seedling raising until harvest, so the initial costs would also be reduced.

In conclusion, the levels of miraculin production of the crossed lines were much higher than that of 56B because both the fruit yields and the concentrations of miraculin were higher in the crossed lines. Thus, two suitable lines were bred for this closed cultivation system. In the future, the fruit yields of the crossed lines could be increased with further improvements in the cultivation system.

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