DOI:10.1021/jf100414v

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Production of Recombinant Miraculin Using Transgenic Tomatoes in a Closed Cultivation System

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We constructed a cultivation system with a controlled light period, light intensity, temperature, and CO_2 concentration for mass production of the taste-modifying protein miraculin from transgenic tomatoes. The tomato plants exhibited normal growth and produced over 270 g of fresh weight (FW) fruit per plant, with the recombinant miraculin concentration reaching up to 90 μg per g FW of tomatoes. The recombinant miraculin content of transgenic tomatoes was compared to that of plants grown in a netted greenhouse. The recombinant miraculin content of transgenic tomatoes grown in a closed cultivation system was more stable than that of tomatoes grown in a netted greenhouse, suggesting that the closed cultivation system is suitable for the production of recombinant miraculin. We estimate that 45 tFW of tomatoes and 4 kg of recombinant miraculin per 1,000 m^2 of cultivation area can be harvested per year.

KEYWORDS: Miraculin; transgenic tomato; artificial light; closed cultivation system; mass production

INTRODUCTION

Sweet-tasting proteins such as thaumatin (*1*), monellin (*2*), mabinlin (*3*), brazzein (*4*), pentadin (*5*), egg white lysozyme (*6*), neoculin (*7*), and miraculin (*8*, *9*) have been described for many years. Because calorie intake can be reduced by using these proteins instead of sucrose, they have attracted a great deal of attention. Miraculin is unique, as it is not a sweet-tasting protein but rather a taste modifier; a sour taste can be changed into a sweet taste by miraculin. Whereas thaumatin, monellin, mabinlin, pentadin, brazzein, and curculin have been investigated for their suitability for mass production (*10*), miraculin has not been thoroughly studied.

Miraculin was discovered in the red berries of the miracle fruit (*Richadella dulcifica*), which is a shrub native to tropical western Africa. Mass production of miraculin is challenging because the cultivation of miracle fruit outside of its native climate is difficult. To overcome the barriers standing in the way of miraculin mass production, the miraculin gene has been introduced into other organisms to produce recombinant miraculin. As such, we have produced recombinant miraculin in transgenic lettuce (*11*), transgenic tomatoes (*12*), and transgenic strawberries (*13*). Among these transgenic plants, tomatoes are eaten in many countries and have great potential for productivity.

The production of plant-derived recombinant proteins has been studied over the past decade (*14*). These methods provide a potentially more efficient and economical system than existing technologies, such as fermentation or bioreactor systems, and they can be easily scaled up (*15*, *16*). Tomatoes have been used as a source of many kinds of recombinant proteins, including components of a cholera vaccine (*17*) and a plague vaccine (*18*), and hepatitis B virus antigen (*19*). For the commercial production of recombinant protein, it is important to increase the amount of protein per fruit and to reduce the variation in the protein content within each fruit. Stable production of recombinant protein is achieved by growing transgenic plants in a uniform environment, which can be obtained by using a closed plant cultivation system.

Plants provided with optimal environmental conditions regarding light intensity, light period, CO_2 concentration, and nutrient levels in environmentally controlled rooms show stable and fast growth. At present, closed plant growth systems have only been reported for leafy vegetable plants. Leafy vegetable plants have simple shapes and adapt to low light conditions, making them easy to grow in closed environments. Although tomatoes have been cultivated in the field and in greenhouses by many methods, their yield is affected by weather conditions. Therefore, if tomatoes can be cultured and harvested in a closed cultivation system, recombinant miraculin production would be possible. Since the closed cultivation system is isolated from the outer environment, it can prevent gene flow from genetically modified plants (GMPs) to the outer environment and also prevent pest ingression from the outer environment, resulting in the reduction of pesticide use.

To achieve the stable production of recombinant miraculin, we constructed a tomato cultivation system in a closed environment. Using this new cultivation system, we grew transgenic tomatoes that accumulated recombinant miraculin. Here, we discuss the efficacy of the mass production of stable, recombinant miraculin using tomatoes.

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 Published on Web 04/28/2010

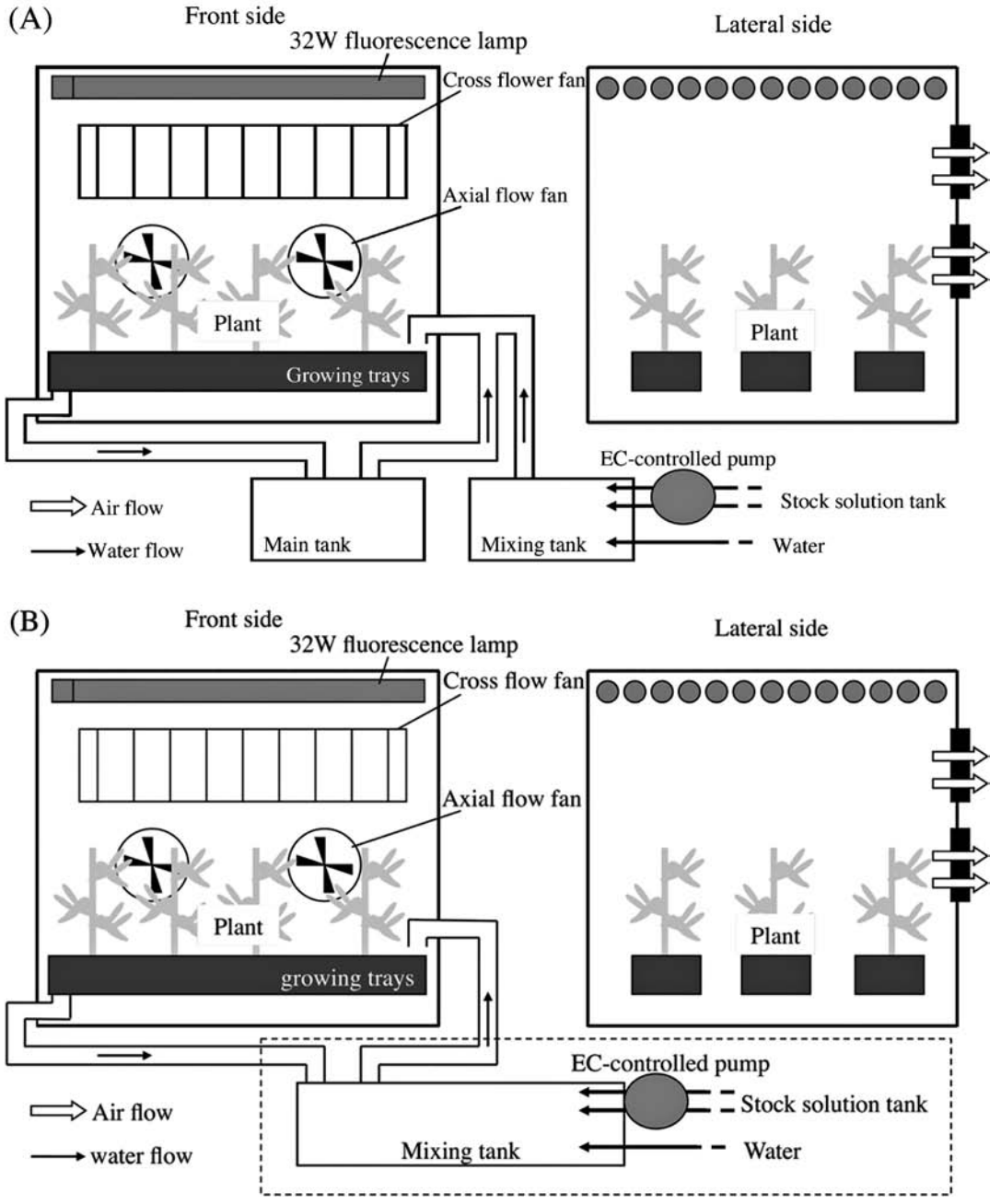

Figure 1. Schematic diagram of lighting and other components of the two-layer cultivation system (**A**) and improved nutrient system (**B**). The main tank was removed, and the mixing tank size was enlarged in the improved nutrient system. The dashed line shows the improved parts.

MATERIALS AND METHODS

Construction of the Tomato Cultivation System. The tomato cultivation system was set up in a temperature- and CO_2 concentration-controlled room. The cultivation system consisted of four growth chambers (94 cm (W) × 86 cm (L) × 83 cm (H)) (**Figure 1A**), which had double layers for cultivation.

In this cultivation scheme, the tomatoes were watered by a nutrient film technique (NFT) system. Each growth chamber was equipped with three growing trays (88 cm (W) × 16 cm (L) × 9 cm (H)), which were provided with a nutrient solution from the mixing tank (45 L) or main tank (45 L) by an electrical pump controlled by a timer. The nutrient solution was made of two stock solutions in a mixing tank. The stock solutions contained high concentrations of nutrient components prepared in two tanks. Equal volumes of the stock solutions were injected via two pumps into the mixing tank and were diluted with water by an EC-controlled pump. When the main tank held enough nutrient solution, the plants were watered from the main tank; however, if the main tank did not contain enough nutrient solution, a new nutrient solution was made in the mixing tank to water the plant. Any leftover nutrient solution was collected by the main tank. When the main tank held enough nutrient solution, watering from the mixing tank was stopped.

Each chamber was equipped with 12 cool white fluorescence lamps as the sole light source. The light sources were set at the top of the growth chambers, and the intensity level of these sources could be adjusted from 350 to 600 $\mu mol/m^2/s^1$ (photosynthetic photon flux, PPF). The photoperiod was controlled by a timer. Each growth chamber was equipped with three air circulation fans to exhaust the heated air from the fluorescence lamps and promote air exchange (**Figure 1**).

Improvement of the Tomato Culture System. To stabilize the EC value of the nutrient solution, the system providing the nutrient solution was improved. Each growth chamber was equipped with three growing trays, which were provided with a nutrient solution from the mixing tank (100 L) by an electrical pump controlled by a timer. The nutrient solution was made of two stock solutions in a mixing tank. The stock solutions

Table 1. Fruit Quality and Fruit Yield of Tomatoes Grown in a Prototype Closed Plant Cultivation System[a]

light intensity (μmol/m^2/s)	light period (h/day)	marketable fruit (fruit/plant)	unmarketable fruit (fruit/plant)	marketable fruit weight (gFW/fruit)	marketable fruit yield (gFW/plant)	days to harvest of all tomatoes (days after sowing)
450	16	1.7 ± 0.3	5.5 ± 0.4	49.6 ± 4.0	82.7 ± 25.2	103.0 ± 1.0
600	16	2.0 ± 0.3	5.1 ± 0.4	53.4 ± 3.2	97.7 ± 18.0	99.5 ± 2.5
t test		N.S.	N.S.	**	**	**

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

contained high concentrations of the nutrient components and were prepared in two small tanks. Equal volumes of these stock solutions were injected via two pumps into a mixing tank and were diluted with water by an EC-controlled pump (**Figure 1B**).

Growth Conditions of Miraculin-Expressing Tomatoes in the Cultivation System. Transgenic tomatoes (Line 56B, uplight type cv. Moneymaker) that accumulate high concentrations of miraculin have been previously produced and evaluated for their miraculin content (*12*, *20*). By adopting this system, the expression of the miraculin gene within transgenic tomatoes was driven by the CaMV 35S promoter, leading to recombinant miraculin accumulation in the whole plant. Seeds at the T_7 generation were germinated on a Petri dish covered with moist filter paper at 25 °C in 16 h of light and 8 h of dark. Four days after sowing, the seedlings were transplanted to rockwool cubes (5 × 5 × 5 cm) and grown in a closed cultivation system (Naeterasu), which was developed commercially by Taiyo Kogyo Co., Ltd. (Tokyo, Japan). This system supplies a nutrient solution for seedlings by an ebb and flood system. Plants were grown at 25/20 °C (light/dark), with 16 h of light from a fluorescent lamp at 450 μmol/m^2/s^1 (PPF) and 8 h of dark. 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. After 42 days of tomato growth in the Naeterrace, 48 plants were transferred to the above-described tomato cultivation system constructed for this study.

With the initial cultivation system (Figure**1A**), tomatoes were grown under a light intensity of 450 and 600 μmol/m^2/s^1 with a 16-h light and 8-h dark photoperiod. In each light condition, plants were grown at 25/20 °C (light/dark) and were supplied with Otsuka-A nutrient solution (Otsuka Chemical Co., Ltd., Osaka, Japan) adjusted to an EC value of 1.8 dS/m. The tomatoes were pruned, leaving three leaves above the flower truss, and the axillary buds were removed during the experiment. In both the Naeterrace and the constructed tomato cultivation systems, the CO_2 concentration was maintained at 600 ppm.

Using the improved cultivation system, we subjected the tomatoes to three light conditions. The tomatoes were grown in 450 μmol/m^2/s^1 light intensity for a 16-h light per day, 600 μmol/m^2/s^1 light intensity for a 12-h light per day, or 600 μmol/m^2/s^1 light intensity for a 16-h light per day. For each light condition, plants were grown at 25/20 °C (light/dark). The nutrient solution and CO_2 concentration were the same for both the initial and improved cultivation systems.

Analysis of Tomato Quality, Yield, and Miraculin Content. Fruits were harvested once every other day, and the harvest days were recorded. The harvest was classified according to marketable and unmarketable fruits (due to symptoms of blossom-end rot, etc.), and then the weight of each fruit was measured. The accumulation and accumulation level of recombinant miraculin were determined by immunoblot analyses and enzyme-linked immunosorbent assays (ELISAs), respectively. The harvested fruits were ground to a fine powder in liquid nitrogen, and 100 mg of the fine powder was homogenized in 200 μL of extraction buffer consisting of 20 mM Tris−HCl (pH 8.0), 500 mM NaCl, and 2% polyvinylpolypyrrolidone (PVPP). The extracts were centrifuged at 15,000 rpm for 20 min at 4 °C, and the resulting supernatant was used for immunoblot analyses and ELISAs, which were performed according to Sun et al. (*12*) and Kim et al. (*20*), respectively.

Cultivation of Miraculin Gene-Expressing Tomatoes in a Netted Greenhouse. To study the stability of the miraculin content in the transgenic tomatoes, the miraculin content of fruits grown in a netted greenhouse was compared to that of fruits grown in the closed cultivation system. Tomatoes expressing the miraculin gene were grown in a netted greenhouse from February 21, 2008 to June 6, 2008 and March 4, 2009 to June 25, 2009.

Transgenic tomato seeds (Line 56B) were germinated on a Petri dish covered with moist filter paper at 25 °C for 16 h of light and 8 h of dark. Four days after sowing, they were transplanted to rockwool cubes (5 × 5 × 5 cm) and grown in the Naeterrace system. Forty days after sowing, the tomato seedlings were transplanted to a netted greenhouse. In the netted greenhouse, the tomato seedlings were watered with Otsuka-A nutrient solution (EC, 1.8 dS/m) by an NFT system. The tomato plants were pruned, leaving three leaves above the third truss, and the axillary buds were removed during the experiment. The tomatoes were harvested when they turned red and were then analyzed for miraculin content.

RESULTS AND DISCUSSION

Plant Growth, Fruit Quality, and Fruit Yield of Transgenic Tomatoes Grown in the Cultivation System. To achieve stable production of recombinant miraculin using transgenic tomato plants expressing the miraculin gene in a closed plant factory, normal plant growth and stable harvest of fruits are necessary. This study is the first report of tomatoes cultured using artificial light as the sole light source in a closed environment. Transgenic tomatoes accumulating miraculin protein were cultured with different light intensities (450 and 600 μmol/m^2/s^1) using the constructed cultivation system. The vegetative growth of the tomatoes was normal in the closed cultivation system, but the tomato fruits showed abnormal growth. The harvested tomato quality and yield are shown in **Table 1**. In each light condition, there was no significant difference in the numbers of marketable and unmarketable (affected by blossom-end rot) fruits per plant between each light condition. These results indicated that these numbers were not affected by light intensity. Light intensity had little influence on fruit numbers, but environmental factors such as the EC value of nutrient solution might have a greater influence on fruit numbers in this cultivation. The average number of marketable fruit per plant was about 2, while the number of unmarketable fruit was about 5.5. A similar tendency was observed in wild type tomato (up-light cultivar of line 56B, cv. Moneymaker); a higher number of unmarketable fruit was induced in this cultivation system (data not shown). Therefore, this higher number of unmarketable fruits was not specific for transgenic tomato fruits. Blossom-end rot is induced by an imbalance in the nutrient solution, such as Ca^{2+} deficiency (*21*, *22*), K^+ deficiency (*23*), ammonium-to-nitrate ratio (*24*), and high EC values (*25*). The EC value of the nutrient solution was very unstable and dramatically increased throughout the cultivation time course (**Figure 2A**), resulting from the tomatoes not evenly absorbing salt from the new nutrient solution, even though the same volume was added. Therefore, salt accumulated in the nutrient solution, and the EC value increased. Normally, we did not manually adjust the EC value of the nutrient solution during cultivation; instead, we replaced the high EC nutrient solution with a new solution every week. The averages marketable fruit weight per plant and total fruit weight were higher using a light intensity of 600 μmol/m^2/s^1 than using a light intensity of 450 μmol/m^2/s^1. The duration of harvesting all of the tomatoes on the day after sowing was shorter with the light intensity set at 600 μmol/m^2/s^1.

Plant Growth, Fruit Quality, and Fruit Yield of Transgenic Tomatoes Grown in the Improved Cultivation System. In the first constructed cultivation system, we could not obtain sufficient yields of transgenic tomatoes because blossom-end rot was frequently found in all of the treatment conditions. We hypothesized that blossom-end rot is caused by the instability of the EC value of the nutrient solution, and therefore, we improved the system by providing a nutrient solution. Afterward, transgenic tomatoes were grown under different conditions of light intensity and photoperiod using the cultivation system and an improved system for providing the nutrient solution. This improved cultivation system provided water into the mixing tank to stabilize the EC value of the nutrient solution (**Figure 1B**). This improvement resulted in a stable EC value during cultivation (**Figure 2B**), and the number of fruits with blossom-end rot was significantly reduced. The harvested tomato quality and yield are shown in **Table 2**. For all light conditions, the number of marketable fruits per plant, the number of unmarketable fruits per plant, and the marketable fruit weight per fruit were not significantly different. In addition, the number of marketable fruits was higher than that of unmarketable fruits. The total weight of marketable fruit per plant was higher with 600 μmol/m^2/s^1 light intensity and 12-h light per day or 16-h light per day conditions than in the 450 μmol/m^2/s^1 light intensity and 16-h light per day conditions. The time to harvest of all tomatoes (in days) was shortest in the 600 μmol/m^2/s^1 for 16 h per day light condition. We succeeded in generating high fruit production with reduced incidence of blossom-end rot in the improved cultivation system, even though some blossom-end rot was still present. Logendra et al. (*26*) used various sizes of rockwool cubes for tomato cultivation to restrict the root environment, showing that blossom-end rot occurs frequently in a root-restricted environments. Their results indicate that blossom-end rot is caused by poor root growth, leading to inefficient and insufficient absorption of calcium and water from the nutrient solution. In our study, small rockwool cubes were used; therefore, it may be possible to produce a higher fruit yield by incorporating larger rockwool cubes in this cultivation system.

The installation area of the cultivation system was about 1.7 m^2, and 48 tomato plants could be grown in this system. The total duration of the tomato harvest was 105 days, which consisted of 42 days of growth in the Naeterrace for seedling culture followed by 63 days in the cultivation system. Tomatoes can be harvested six times per year in this system, when cultivation in the Naeterrace and constructed cultivation systems is used at the same time. Therefore, by estimation from the tomato productivity described in these results, up-scaling the cultivation system would allow for harvesting 45 kgFW/1,000m^2/year of tomatoes.

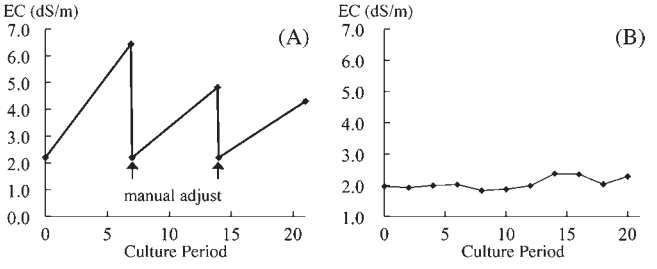

Figure 2. Changes in EC value during tomato cultivation with the initial nutrient system (**A**) and with the improved nutrient system (**B**). In each nutrient system, the EC value was adjusted by an EC-controlled pump. The EC value was adjusted manually once a week in the initial nutrient system (**A**).

Comparison of Recombinant Miraculin Production in Transgenic Tomatoes Grown in a Cultivation System and a Netted Greenhouse. The systematic production and stable accumulation of recombinant protein from plants are important for food and industrial uses. In the field and in greenhouses, plants are exposed to environmental stresses that vary from year to year; therefore, suitable production of recombinant protein is difficult. By contrast, in a closed plant factory, the environment (including temperature, CO_2 concentration, and nutrient solution) can be strictly controlled so that each plant is exposed to the same environmental stresses during each round of cultivation. Therefore, plants grown in a closed plant factory show uniform growth and recombinant protein production each time they are cultivated.

Recombinant miraculin in tomatoes grown in the improved cultivation system (**Figure 3**) and in other conditions (data not shown) were detected by immunoblot analysis, allowing for the analysis of the recombinant miraculin productivity of transgenic tomatoes grown in different environments. The recombinant miraculin protein was purified from the fruits grown in all growth conditions, and the taste-modifying properties were confirmed. The recombinant miraculin content of tomatoes grown in the improved cultivation system was measured by ELISA, and the fruit yield was highest in the improved cultivation system. Transgenic tomatoes grown in a netted greenhouse were also assayed for their recombinant miraculin content. In each cultivation condition, eight fruits were measured, and the results of these tests are shown as a box plot in **Figure 4**. In the improved cultivation system, the median miraculin contents of tomatoes grown under a light intensity of 450 μmol/m^2/s^1 for 16 h per day or 600 μmol/m^2/s^1 for 12 h per day or 16 h per day were 81, 82, and 87 μg/gFW, respectively. When the plants were grown in a netted greenhouse in 2008 and 2009, the median miraculin contents were 94 and 65 μg/gFW, respectively. The differences in the medians from the various growth conditions in the culture system were smaller than the change between the 2008 and 2009 plants grown

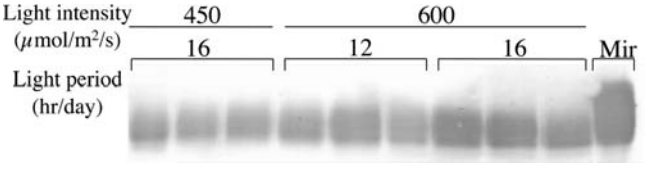

Figure 3. Accumulation of recombinant miraculin protein in transgenic tomatoes grown in the closed plant cultivation system. Recombinant miraculin and native miraculin were detected by Western blot analysis. Crude protein (2 μg) extracted from tomatoes grown in various light conditions was applied. Three fruits were used for the analysis of each light condition. Mir indicates purified native miraculin protein.

Table 2. Fruit Quality and Fruit Yield of Tomatoes Grown in an Enhanced Closed Plant Cultivation System[a]

light intensity (μmol/m^2/s)	light period (h/day)	marketable fruit (fruit/plant)	unmarketable fruit (fruit/plant)	marketable fruit weight (gFW/fruit)	marketable fruit yield (gFW/plant)	days to harvest of all tomatoes (days after sowing)
450	16	3.6 ± 0.3 a	1.6 ± 0.3 a	60.6 ± 2.3 a	209.7 ± 14.9 b	109.0 ± 0.0 c
600	12	4.8 ± 0.4 a	1.4 ± 0.4 a	62.7 ± 3.9 a	273.7 ± 25.3 a	105.8 ± 0.7 b
600	16	4.3 ± 0.3 a	2.5 ± 0.6 a	66.6 ± 3.2 a	260.8 ± 20.9 a	104.0 ± 0.4 a

[a] The data presented are the means ± standard errors of eight plants. Means followed by different letters are significantly different ($P < 0.05$).

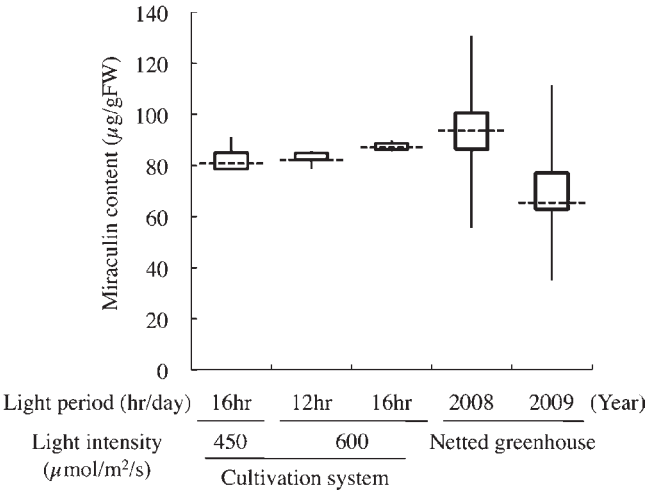

Figure 4. Box plot representation showing the miraculin content of fruit grown in the closed cultivation system and netted greenhouse. ELISA was used to measure the miraculin content of eight fruits under each culture condition. Box plots show median (dotted line through the box) and second and third quartiles, which indicate the dispersion of 50% of the data points (boxes); bars show the data range. These experiments were repeated twice with similar results.

in a netted greenhouse. The difference between the highest and lowest contents in the cultivation system was smaller than the corresponding values from the netted greenhouse. In the greenhouse, the stress of tomatoes from the environment such as light intensity, temperature, and humidity, etc. not only varies with the time of day but also differs between each individual tomato fruit. These differences induce unstable miraculin content in each transgenic tomato. However, in a closed cultivation system, these environmental factors are controlled, and each plant is exposed to the same environmental stress. This controlled environment induces stable miraculin content in each tomato fruit.

In the improved cultivation system, the miraculin contents of tomatoes grown under each light condition were almost the same. By contrast, in the netted greenhouse, the miraculin contents were significantly different between the two years. These results demonstrate that cultivation in a closed plant factory is ideal for stable miraculin production using transgenic tomatoes.

Initially, we will use the miraculin accumulating tomatoes for processing. Moreover subsequently, the miraculin accumulating tomatoes will be targeted for the fresh fruit material. To show a taste-modifying activity, transgenic tomato fruits should contain more than a certain amount of miraculin.

The miraculin content of transgenic tomatoes grown in the cultivation system was 90 μg/gFW, a value similar to that reported by Sun et al. (*12*). According to the estimated transgenic tomato yields and the miraculin content of tomatoes, the miraculin yield could reach 4 kgFW/1,000m^2/year if this cultivation system is up-scaled. To induce the taste-modifying activity, 50 μg of miraculin is needed. Consequently, we could produce 800 million times enough miraculin for use in the taste-modify trial in 1,000 m^2 of this cultivation system over the course of 1 year.

The closed cultivation system used in this study was a prototype model in a small scale. However, the high running cost in this cultivation system compared to that of the netted greenhouse was mainly due to electric powered artificial lighting and air conditioning.

The production of fruits in this system was 45 kgFW/1,000m^2/year. This production was higher than that in other countries including Japan other than The Netherlands, etc. However, the high running cost and fruit productivity could be improved by up-scaling this cultivation system. In the next step, we will demonstrate the mass production of recombinant miraculin using transgenic tomatoes in the up-scaled cultivation system.

In conclusion, we constructed a cultivation system in a closed environment for the stable production of recombinant miraculin using transgenic tomatoes expressing the miraculin gene. We succeeded in cultivating tomatoes and harvesting fruit using this constructed cultivation system and showed that the recombinant miraculin content of the tomatoes grown in the cultivation system is more stable than that present in tomatoes from a netted greenhouse. Using this cultivation system, 45 kg fresh weight fruit/10a/year of tomatoes and 4 kg/10a/year of recombinant miraculin could be produced. Further improvement of the cultivation system led to a higher yield of recombinant miraculin. Increased miraculin production using an up-scaled facility will be required.

ACKNOWLEDGMENT

We thank members of the Ezura laboratory for helpful discussions.

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Received for review February 1, 2010. Revised manuscript received April 15, 2010. Accepted April 19, 2010. This study was supported by a project from the Ministry of Economy, Trade, and Industry of Japan, "Development of Fundamental Technologies for Production of High-value Materials Using Transgenic Plants" (to H.E.).