

## Gene dosage and genetic background affect miraculin accumulation in transgenic tomato fruits

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**Abstract** Gene dosage and genetic background are factors that influence transgene expression in transgenic plants. In our previous studies, we produced transgenic tomato plants that accumulate miraculin, a taste-modifying protein, in a genetically stable manner. To elucidate the effects of gene dosage and genetic background on miraculin accumulation in transgenic tomato fruits, we generated hybrid tomato lines between the homozygous transgenic line 56B (background cultivar ‘MoneyMaker’) and the pure cultivars ‘Micro-Tom,’ ‘MoneyMaker,’ ‘Ailsa Craig,’ ‘M82,’ ‘Rutgers’ and ‘Aichi-first’ and analyzed them for miraculin mRNA expression and miraculin protein accumulation. The hybrid lines exhibited variation in their fruit structures. Among the hybrid lines heterozygous for the miraculin gene, miraculin accumulation in the fruits varied from 111.0  $\mu\text{g g}^{-1}$  fresh weight (FW) to 159.4  $\mu\text{g g}^{-1}$  FW. Furthermore, the homozygous line 56B showed higher miraculin accumulation and miraculin mRNA expression than the heterozygous line 56B×‘MoneyMaker.’ These results demonstrate the profound effects of gene dosage and genetic background on miraculin accumulation in transgenic tomato fruits.

**Key words:** Gene dosage, genetic background, miraculin, taste-modifying protein.

Miraculin is a taste-modifying protein made by miracle fruit (*Richadella dulcifica*) (Theerasilp and Kurihara 1988) that causes sour tastes to be perceived as sweet (Brouwer et al. 1968; Kurihara and Beidler 1968). Individuals suffering from diseases such as obesity, diabetes, hyperglycemia and caries could use miraculin as a natural low-calorie sweetener (Kant 2005). Nevertheless, commercial production of miraculin is limited because the natural source is a tropical plant that is difficult to cultivate and is not very productive. Efforts to produce miraculin in other organisms such as *Escherichia coli* (Kurihara 1992), yeast (Kurihara and Nirasawa 1997), tobacco (Kurihara and Nirasawa 1997), lettuce (Sun et al. 2006), tomato (Sun et al. 2007), and strawberry (Sugaya et al. 2008) have been going on since 1990. Recently, a research group reattempted to produce miraculin in *E. coli*, but the recombinant protein exhibited lower taste-modifying activity than the native miraculin because the recombinant miraculin was not glycosylated (Matsuyama et al. 2009).

Tomato is a major vegetable crop that is cultivated throughout the world, and its growth requirements are well-understood. Therefore, we decided to produce recombinant miraculin in transgenic tomatoes. We developed transgenic tomato plants that accumulate significant levels of miraculin protein (Sun et al. 2007),

and the protein produced had almost same level of taste-modifying activity as native miraculin from miracle fruits. Furthermore, we characterized the miraculin accumulation pattern in the transgenic tomato fruits in detail and found that miraculin accumulation was highest in the exocarp (Kim et al. 2010). The exocarp is composed of small cells that allow high levels of miraculin protein to accumulate. Among the miraculin-accumulating transgenic tomato lines, a line that we named 56B had a single copy of the miraculin transgene and accumulated miraculin to high levels in the  $T_0$  generation. Line 56B was propagated up to the  $T_5$  generation, which corresponds to six generations (Yano et al. 2010). That study showed that miraculin transgene inheritance and expression were stable through multiple generations in transgenic tomatoes. Taken together, these studies suggest that tomato is a potential platform for the mass production of miraculin.

Transgene expression levels and inheritance show wide variation among independently transformed plants carrying the same construct (Peach and Velten 1991; Spencer et al. 1992; Walters et al. 1992). Many factors can contribute to the variation in transgene expression. Studies in rice showed that transgene expression levels were affected by gene dosage (Baruah-Wolff et al. 1999; Duan et al. 1996). Scott et al. (1998) have claimed that

allelic composition and genetic background affect transgene expression and inheritance in white clover. Another study in rice showed that the expression pattern of *Xa3/Xa26*, which is a rice disease resistance gene, was affected by the genetic background (Cao et al. 2007). The full effects of gene dosage and genetic background on transgene expression and translation remain to be elucidated.

In this study, we generated hybrid lines between transgenic line 56B (background cultivar 'Money-maker'), which is homozygous for the miraculin gene, and the pure line cultivars 'Micro-Tom,' 'Moneymaker,' 'Ailsa Craig,' 'M82,' 'Rutgers' and 'Aichi-first.' Characterization of the hybrids' miraculin mRNA expression and miraculin protein accumulation, revealed the effects of gene dosage and genetic background on miraculin accumulation in transgenic tomato fruits, and in addition we discuss the significance of combining molecular breeding with cross-breeding to produce transgenic tomatoes that accumulate miraculin.

## Materials and methods

### Plant material and growth conditions

The transgenic tomato line 56B was produced in our previous work (Sun et al. 2007). In brief miraculin encoding 660-bp DNA fragment was inserted into the *XbaI/SacI* sites of the plant transformation vectors pBI121 (Sun et al. 2006). Line 56B possesses a single copy of the miraculin gene driven by the CaMV 35S promoter.

In the present study, the miraculin-accumulating transgenic tomato line 56B (background cultivars 'Moneymaker') was crossed with six other cultivars: 'Micro-Tom (accession number TOMJPF00001),' 'Moneymaker (accession number TOMJPF00002),' 'Aichi-first (accession number TOMJPF00003),' 'Ailsa Craig (accession number TOMJPF00004),' 'M82 (accession number TOMJPF00005)' and 'Rutgers (accession number TOMJPF00006),' which were provided by the Tomato National BioResource Project (NBRP, <http://tomato.nbrp.jp/>). Pollen from line 56B was used to fertilize flowers of the six cultivars. Seeds from these six hybrid lines and line 56B were sown on rockwool cubes (5×5×5 cm) and grown in a closed cultivation system (Naeterasu) that was commercially developed by Taiyo Kogyo Co., Ltd. (Tokyo, Japan). Plants were grown at 25/20°C (light/dark) with 16 hr of light from a fluorescent lamp at 450 μmol m<sup>-2</sup> s<sup>-1</sup> (photosynthetic photon flux, PPF) and 8 hr of dark. The plants were provided daily with a nutrient solution containing 565.0 mg L<sup>-1</sup> NO<sub>3</sub><sup>-2</sup>, 15.7 mg L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>, 202.2 mg L<sup>-1</sup> PO<sub>3</sub><sup>-2</sup>, 218.4 mg L<sup>-1</sup> K<sup>+</sup>, 19.9 mg L<sup>-1</sup> Mg<sup>2+</sup>, 95.0 mg L<sup>-1</sup> Ca<sup>2+</sup> and micronutrients with an ebb and flood system. After 32 days of tomato growth in the "Naeterasu," all seedlings were transferred to a netted greenhouse and watered with Otsuka-A nutrient solution (EC, 2.0 dS m<sup>-1</sup>) using a nutrient film technique (NFT) system. Each seedling was arranged randomly on the NFT system's growing trays. The tomato plants were pruned, leaving three leaves above the third truss, and the axillary buds were removed during the experiment. The fruits

were harvested when they turned red and were used for subsequent analysis.

### Separation of Fruit Tissue

The harvested fruits from line 56B and the six hybrid lines were separated into seven parts: exocarp, mesocarp, dissepiment, upper placenta, lower placenta, jelly and seeds. The fresh weights of each separated tissue were measured, and the tissue was analyzed for miraculin mRNA expression level and protein content.

### Isolation of mRNA and Quantitative Reverse-Transcriptase PCR (qRT-PCR)

The miraculin mRNA expression levels of 56B×'Moneymaker' and line 56B were determined by qRT-PCR. Total RNA was isolated from the exocarp and mesocarp of 56B×'Moneymaker' and line 56B using an RNeasy plant mini kit (Qiagen, Tokyo, Japan). cDNA was synthesized from 0.8 μg of total RNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). One microliter of cDNA was diluted in 12.5 μL of TE buffer, and 1 μL of diluted cDNA was used for qRT-PCR amplification with SYBR Premixed Ex-Taq (Takara Bio Inc., Otsu, Japan). The qRT-PCR reaction was performed using miraculin-specific primers and ubiquitin3-specific primers. *Ubiquitin3* has been described previously (Hoffman et al. 1991) and was used as a control (Hackel et al. 2006; Leclercq et al. 2005). The primer sequences have also been described previously (Kim et al. 2010).

### Immunoblot Analysis and Enzyme-Linked Immunosorbent Assay (ELISA)

The miraculin content of the whole fruit and separated tissues from line 56B and all of the hybrid lines was detected with immunoblot analysis and ELISA. Total protein was extracted from 0.1 g of sample with 200 μL of extraction buffer consisting of 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 2% polyvinylpyrrolidone (PVPP). The extracts were centrifuged at 15,000×g for 20 min at 4°C. The resulting supernatant was used for immunoblot analysis and ELISA, which were performed according to Sun et al. (2007) and Kim et al. (2010), respectively.

## Results

### Fruit structure of tomato line 56B and its hybrids

Hybrid lines were generated by crossing the transgenic tomato line 56B (background cultivar 'Moneymaker') with the six cultivars 'Aichi-first,' 'Rutgers,' 'Moneymaker,' 'M82,' 'Ailsa Craig' and 'Micro-Tom,' and the resultant fruits are shown in Figure 1. The fruit weight of line 56B was 77.1 g FW, and those of F1 hybrids showed above were 102.4, 99.2, 96.4, 71.5, 68.7 and 32.4 g FW, respectively (Table 1). The fruit weight of 56B×'Micro-Tom' was extremely light compared to the other F1 hybrids. Although lines 56B and 56B×'Moneymaker' had the same genetic background, the fruit weight of line 56B tended to be less than that of 56B×'Moneymaker.' This difference was not significant

because the fruit weights of 56B×‘Moneymaker’ were varying.

The ratios of the individual tissue in relation to total fruit weight were measured in all lines (Table 1). analysis of the ratios of individual tissue weights to whole fruit weight, showed that lines 56B, 56B×‘Aichi-first,’ 56B×‘M82’ and 56B×‘Micro-Tom’ contained over 2% exocarp. In particular, the fruit of 56B×‘Micro-Tom’ comprised of over 3% exocarp. Line 56B×‘M82’ showed the highest percentage (64.0%) of mesocarp. The percentage of whole fruit made up by lower placenta showed no difference among the lines. In 56B×‘Aichi-first,’ the ratios of dissepiment weight and upper placenta weight to whole fruit weight were higher than those of the other lines. The ratio of jelly to whole fruit weight was especially high in 56B×‘Ailsa Craig’ (19.2%) and especially low in 56B×‘M82’ (10.2%). The ratio of seed to fruit weight was highest in 56B×‘Micro-Tom’ (2.7%) and lowest in 56B×‘M82’ (0.8%).

**Miraculin accumulation in transgenic tomato fruits of different genetic backgrounds**

Miraculin protein was detected in the leaves of all hybrid lines by immunoblot analysis (data not shown). The size of the detected band was 47 kDa, corresponding to the size of the miraculin dimer (Igeta et al. 1991). These

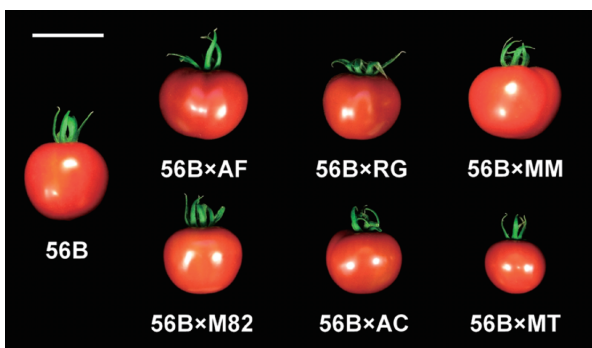


Figure 1. Images of the fruits of line 56B and the six hybrids. The hybrids were obtained from crosses between 56B and six different cultivars: AF, Aichi-first; RG, Rutgers; MM, Moneymaker; AC, Ailsa Craig; MT, Micro-Tom. Bars represent 5 cm.

results indicate that the miraculin gene was expressed and correctly translated in the hybrid lines. The miraculin accumulation level in whole red fruits of all lines was measured by ELISA, and these results are

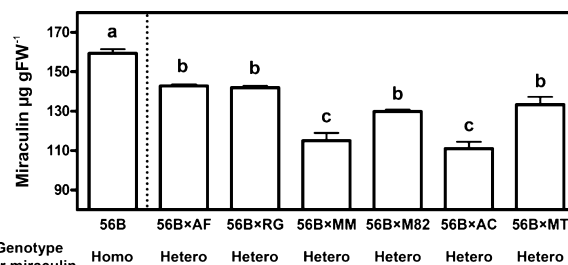


Figure 2. Miraculin accumulation in whole red fruits of line 56B and the six hybrid lines. The miraculin content was measured by ELISA. The data presented are the average of three independent fruits, and vertical bars show the standard error. AF, Aichi-first; RG, Rutgers; MM, Moneymaker; AC, Ailsa Craig; MT, Micro-Tom. Statistically significant differences (P<0.05) between lines 56B and the hybrid lines were determined using Tukey’s Multiple Comparison Test to compare all possible pairs of columns. FW, fresh weight.

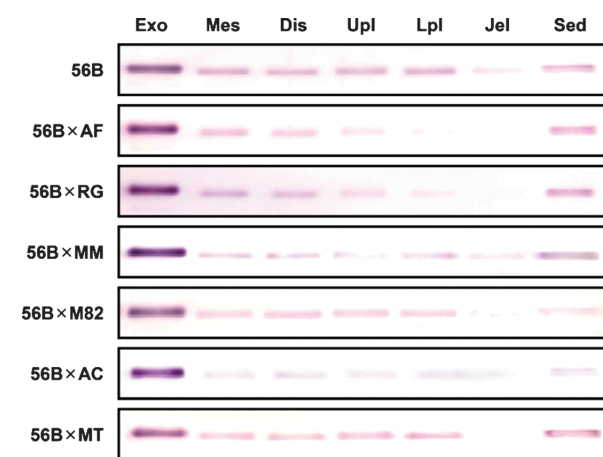


Figure 3. Immunoblot analysis of miraculin protein in extracts from fruit tissues of line 56B and the six hybrid lines. Protein samples were extracted from 0.1 mg fresh weight samples, separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was hybridized with antibodies to miraculin. Exo, exocarp; Mes, mesocarp; Dis, dissepiment; Upl, upper placenta; Lpl, lower placenta; Jel, jelly; Sed, seed.

Table 1. Ratio of individual fruit tissue weight to total fruit weight in line 56B and hybrid lines

Line	Fruit weight (g)	Tissue per fruit weight (%) <sup>a</sup>							n
		Exocarp	Mesocarp	Dissepiment	Upper placenta	Lower placenta	Jelly	Seed	
56B	77.1 ± 4.7bc	2.3 ± 0.1b	59.7 ± 1.0b	11.3 ± 0.4b	6.7 ± 0.3ab	5.4 ± 0.2a	12.1 ± 1.4cd	0.9 ± 0.1bc	14
56B×‘Aichi-first’	102.4 ± 6.7a	2.2 ± 0.1b	57.5 ± 0.7bc	13.1 ± 0.5a	7.1 ± 0.3a	5.5 ± 0.2a	13.6 ± 0.6bc	1.0 ± 0.1bc	20
56B×‘Rutgers’	99.2 ± 3.8a	2.0 ± 0.1b	58.0 ± 0.9bc	11.3 ± 0.4b	6.7 ± 0.4ab	4.9 ± 0.3a	15.8 ± 0.8abc	1.5 ± 0.1bc	14
56B×‘Moneymaker’	96.4 ± 4.8ab	1.8 ± 0.1b	58.5 ± 0.6b	10.7 ± 0.3bc	6.6 ± 0.3ab	5.1 ± 0.2a	16.3 ± 0.3ab	1.1 ± 0.1bc	16
56B×‘M82’	71.5 ± 2.6c	2.0 ± 0.1b	64.0 ± 0.6a	10.6 ± 0.3bc	6.5 ± 0.2ab	6.0 ± 0.3a	10.2 ± 0.6d	0.8 ± 0.1c	18
56B×‘Ailsa Craig’	68.7 ± 2.9c	1.8 ± 0.1b	55.8 ± 0.8c	10.0 ± 0.3bc	5.7 ± 0.2b	5.3 ± 0.3a	19.2 ± 0.8a	2.3 ± 0.2a	20
56B×‘Micro-Tom’	32.4 ± 2.2d	3.5 ± 0.4a	56.3 ± 1.6bc	9.3 ± 0.6c	6.3 ± 0.3ab	6.0 ± 0.4a	15.9 ± 1.2abc	2.7 ± 0.3a	13

The different letters within the columns show a significant difference between each lines by Tukey’s Multiple Comparison Test at p<0.05.

<sup>a</sup> Average percentages (±SE) of tissues determined from each tissue weight per fruit weight.

shown in Figure 2. Line 56B, which is homozygous for the miraculin gene, showed the highest level of miraculin accumulation. In contrast, in all of the heterozygous hybrid lines, the miraculin accumulation level was significantly lower than that of line 56B. The lines were separated into high and low accumulation groups. The high accumulation group, containing 56B×‘Aichi-first,’ 56B×‘Rutgers,’ 56B×‘M82’ and 56B×‘Micro-Tom,’ contained more than  $130 \mu\text{g g}^{-1}$  FW of miraculin protein. The low accumulation group, consisting of 56B×‘Moneymaker’ and 56B×‘Ailsa Craig,’ accumulated about  $110 \mu\text{g g}^{-1}$  FW of miraculin protein.

To compare miraculin accumulation patterns between line 56B and the hybrid lines, their separated fruit tissues were subjected to immunoblot analysis (Figure 3). Miraculin protein was detected in all separated tissues in all lines. In line 56B, the miraculin accumulation level was highest in the exocarp. The mesocarp, dissepiment, upper placenta, lower placenta, and seeds showed almost the same accumulation levels. The jelly showed the lowest accumulation levels, which corroborates our previous report (Kim et al. 2010). Despite the different genetic backgrounds, the accumulation pattern of miraculin in the hybrid lines was similar to that of line 56B.

#### Comparison of miraculin protein accumulation and mRNA expression in the exocarp and mesocarp of fruits from lines that are homozygous and heterozygous for the miraculin gene

To precisely determine the difference in miraculin accumulation levels between the homozygous line 56B and the heterozygous hybrid lines, the miraculin levels in the exocarp and mesocarp were measured by ELISA (Figure 4). The miraculin levels in the exocarp and mesocarp of line 56B were  $1171.7 \mu\text{g g}^{-1}$  FW and  $87.44 \mu\text{g g}^{-1}$  FW, respectively, indicating a profound effect of tissue type on miraculin accumulation. A similar tendency was observed in all hybrid lines, in which miraculin highly accumulated in exocarps. In the exocarp of the hybrid lines, the miraculin levels were lower than that of line 56B but quite variable among the different hybrids. In the mesocarp, the miraculin levels in the hybrid lines except 56B×‘Micro-Tom’ were significantly lower than that of line 56B; 56B×‘Micro-Tom’ had levels similar to line 56B.

Among hybrid lines, the miraculin level in exocarp of 56B×‘Moneymaker’ was significantly lower than the other hybrid lines except 56B×‘Micro-Tom,’ and that of 56B×‘Micro-Tom’ was similar level. The miraculin levels in mesocarp of 56B×‘Ailsa Craig’ and 56B×‘Micro-Tom’ were significantly higher than that of 56B×‘Moneymaker.’ These results demonstrated the significance of gene dosage and genetic background on

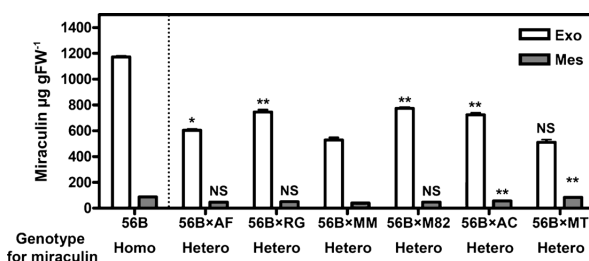


Figure 4. Miraculin accumulation in the exocarp and mesocarp tissues of line 56B and the hybrid lines. The miraculin levels were measured by ELISA. The data presented are the average of three independent fruits, and vertical bars show the standard error. The miraculin genotypes of these tomatoes are shown at the bottom of the graph. Asterisks indicate significant difference between 56B×‘Moneymaker’ (control column) and the other hybrid lines at the 1% ( $P < 0.01$ , double) and 5% ( $P < 0.05$ , single) significance level using Dunnett’s Multiple Comparison Test. NS means not significant. Exo, exocarp; Mes, mesocarp; AF, Aichi-first; RG, Rutgers; MM, Moneymaker; AC, Ailsa Craig; MT, Micro-Tom.

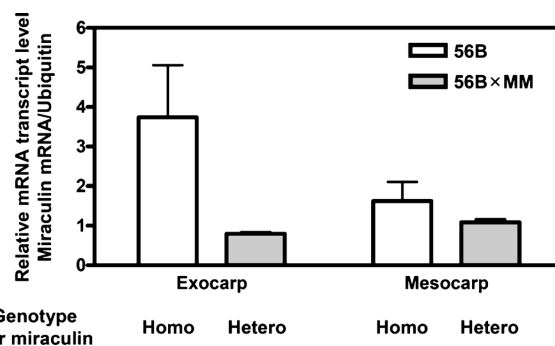


Figure 5. Miraculin mRNA accumulation in the exocarp and mesocarp of lines 56B and 56B×Moneymaker. Relative expression levels of the miraculin gene were normalized to the *ubi3* expression level as an internal reference. The Y-axis values reflect the means and standard errors of three independent fruits.

miraculin accumulation in fruit tissues.

To assess the correlation between mRNA expression and miraculin protein accumulation, mRNA expression in the exocarp and the mesocarp was compared between the homozygous line 56B and the heterozygous line 56B×‘Moneymaker’ by qRT-PCR (Figure 5). The miraculin mRNA expression level in the exocarp of line 56B was 4.7 times higher than that of line 56B×‘Moneymaker,’ which correlated with miraculin protein accumulation (Figure 4). In the mesocarp, the miraculin expression level of line 56B was 1.5 times higher than that of line 56B×‘Moneymaker,’ which also correlated with miraculin accumulation (Figure 4). These results indicated that mRNA expression reflected miraculin protein accumulation in the transgenic tomato fruits, and the mRNA expression was affected by gene dosage.

## Discussion

Cross-breeding is a powerful technique for crop

improvement. Once elite transgenic line is obtained, the elite line can be improved by crossing with other lines conferring superior traits. Previously, we reported the ratio of individual tissue weights to whole fruit weight in pure cultivar lines (Kim et al. 2010). The fruits weights of pure cultivar lines, 'Aichi-first' and 'Micro-Tom' used in this study were 263.4 and 3.66 g FW, respectively. The whole fruit weight of line 56B×'Aichi-first' was heavier than that of line 56B (77.1 g FW). Line 56B×'Micro-Tom' (32.4 g FW) weighed less than that of line 56B. The increased fruit weight observed in the hybrids is in agreement with Larson and Currence (1944), who reported larger tomato fruit size from those inbred lines having larger fruits. The ratio of individual tissue weights to whole fruit weight were altered by crossing the line 56B with these pure cultivar lines. For instance, the exocarp ratio of line 56B×'Micro-Tom' (3.5%) was significantly higher than that of line 56B (2.3%), while dissepiment ratio of line 56B (11.3%) was significantly higher than that of line 56B×'Aichi-first' (13.1%). These results indicate that the overall fruit weight and the ratio of individual fruit tissues to total fruit weight can be altered by cross-breeding.

We previously reported that the miraculin protein accumulates to high levels in the exocarp and mesocarp of line 56B (Kim et al. 2010). In this study, we used hybrid lines to demonstrate that the miraculin accumulation pattern in tomato fruit tissue is unchanged in different genetic backgrounds, while the ratio of individual fruit tissue weight to total fruit weight does change. These results indicate that the miraculin productivity in transgenic tomato fruit can be altered by changing the ratio of exocarp and mesocarp in fruit through cross-breeding. For example, fruits with a higher percentage of exocarp and mesocarp should be able to accumulate higher amounts of miraculin in the whole fruit. In fact, the miraculin content of whole fruits was higher in 56B×'Aichi-first,' 56B×'Rutgers,' 56B×'M82' and 56B×'Micro-Tom,' and these fruits also had higher ratios of exocarp and mesocarp.

A potential problem with breeding transgenic cultivars is that instability of the transgene is not apparent until the second or third generation (Srivastava et al. 1996). Ideally, an introduced gene should be expressed in every genotype of a transgenic cultivar. This end can be achieved by maintaining the transgene in a homozygous state, thereby ensuring that all progeny of the next generation will inherit the transgenic trait. In this study, line 56B was homozygous for the miraculin gene, while the hybrid lines were heterozygous for the miraculin gene because the pure cultivars crossed with line 56B did not possess the transgene. We compared the miraculin content of lines 56B and 56B×'Moneymaker,' which have the same genetic background. The miraculin levels in the whole fruit (Figure 2), exocarp and mesocarp

(Figure 4) of line 56B were higher than those in 56B×'Moneymaker.' Importantly, we showed that the higher levels of miraculin in the exocarp and mesocarp of line 56B were due to higher miraculin gene expression in each tissue. These results indicate that gene dosage is reflected in mRNA expression and miraculin protein accumulation in the transgenic tomato fruits. Therefore, to produce a transgenic tomato line that accumulates high levels of miraculin in its fruits, a line that is homozygous for the miraculin gene will be required.

In conclusion, gene dosage impacts miraculin mRNA expression and miraculin protein accumulation in transgenic tomato fruits. This result clearly indicates that to produce hybrid tomato cultivars that accumulate miraculin to high levels, both pure parental lines should have a miraculin gene in their genomes. Genetic background affects miraculin protein accumulation in transgenic tomato fruits via fruit structure. This result suggests that it is possible to alter the level of miraculin accumulation by crossing a transgenic line expressing the miraculin gene to other tomato lines with different genetic backgrounds. Finally, in this study, we propose that molecular breeding combined with cross-breeding is an effective means to improve the production level of recombinant protein using transgenic plants.

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