Title:

Uniform accumulation of recombinant miraculin protein in transgenic tomato fruit using a fruit ripening-specific E8 promoter

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ABSTRACT

The *E8* promoter, a tomato fruit ripening-specific promoter, and the CaMV 35S promoter, a constitutive promoter, were used to express the *miraculin* gene encoding the taste-modifying protein in tomato. The accumulation of miraculin protein and mRNA was compared among transgenic tomatoes expressing the *miraculin* gene driven by these promoters. Recombinant miraculin protein predominantly accumulated in transgenic tomato lines using the *E8* promoter (E8-MIR) only at the red fruit stage. The accumulations were almost uniform among all fruit tissues. When the 35S promoter (35S-MIR) was used, miraculin accumulation in the exocarp was much higher than in other tissues, indicating that the miraculin accumulation pattern can be regulated by using different types of promoters. We also discuss the potential of the E8-MIR lines for practical use.

KEYWORDS: E8 promoter ; miraculin; taste-modifying protein; tomato

Introduction

The promoter is an essential element for endogenous or exogenous gene expression in eukaryotes (Fluhr et al. 1986; Kaulen et al. 1986; Stougaard et al. 1987). For most studies of plant genetic engineering, a constitutive promoter from cauliflower mosaic virus (CaMV), the 35S promoter, has been used because this promoter exhibits a high level of transcriptional activity in a variety of plant tissues (Williamson et al. 1989). Previous studies have indicated that 35S-driven expression and accumulation patterns may vary depending on the transgene or plant species being transformed (Benfey et al. 1990; Sunilkumar et al. 2002; Szwacka et al. 2009). Several fruit ripening-specific promoters, such as *E4*, *E8*, and *2A11*, have been identified in tomato (Van Haaren and Houck 1993; Xu et al. 1996; Deikman et al. 1998). Because the *E8* promoter was first cloned from the cherry tomato (Deikman et al. 1988), this promoter has been widely used to improve fruit quality in tomato plants and to express recombinant pharmaceutical proteins in transgenic tomatoes in a fruit-specific manner (Giovannoni et al. 1989; Good et al. 1994; Sandhu et al. 2000; Lewinsohn et al. 2001; Mehta et al. 2002; De La Garza et al. 2004; Jiang et al. 2007; Ramírez et al. 2007; He et al. 2008).

The *E8* gene driven from the *E8* promoter is transcriptionally activated at the onset of ripening (Lincoln and Fischer 1988). Analyses of *E8* expression in the fruits of wild-type, mutant, and transgenic plants defective for ethylene biosynthesis, and in fruits treated with an inhibitor of ethylene action, indicate that *E8* is controlled in fruits by both ethylene-dependent and ethylene-independent fruit-ripening signals (Lincoln et al. 1987; Lincoln and Fischer 1988a; Lincoln and Fischer 1988b; Dellapenna et al. 1989; Theologis et al. 1993). Analysis of the E8 promoter has revealed that separate *cis*-elements are involved in ethylene-responsive expression and in expression in response to ethylene-independent fruit-ripening signals (Deikman, et al. 1992).

Miraculin is a taste-modifying glycoprotein contained in the fruits of the miracle fruit plant (*Richadella dulcifica*), which is a shrub native to tropical western Africa (Theerasilp and Kurihara 1988). Miraculin has the unique ability to modify a sour taste into a sweet taste although miraculin itself does not taste sweet. Miraculin could be used as an alternative sweetener for diabetics. However, mass production of miraculin is difficult because miracle

fruit is a tropical plant and the fruits that predominantly accumulate miraculin have low productivity.

We have attempted to express in another plant species using transgenic techniques and have succeeded in accumulating recombinant miraculin in transgenic lettuce (Sun et al. 2006a), tomato (Sun et al. 2007) and strawberry (Sugaya et al. 2008) using the CaMV 35S promoter. Furthermore, we showed that tomato is a suitable species for recombinant miraculin production (Yano et al. 2010), and the miraculin accumulation pattern has been characterized in detail in the transgenic tomato fruits (Kim et al. 2010). The profiling of recombinant miraculin accumulation in transgenic tomatoes expressing the *miraculin* gene driven by the CaMV 35S promoter (35S-MIR) has shown that miraculin accumulates gradually during fruit development and reaches its highest level at the over-ripe stage. In red fruit, the miraculin protein accumulates highly in the exocarp tissues and is less evident in the other fruit tissues, such as mesocarp, dissepiment, placenta, and jelly tissues. However, the exocarp is generally removed during tomato processing to produce products such as juice, ketchup, puree, and paste. Therefore, it is necessary to concentrate miraculin in fruit tissues other than the exocarp. We hypothesized that using a different promoter, such as a fruit-specific promoter, the accumulation pattern of miraculin in transgenic tomato fruits can be changed.

In this study, we generated transgenic tomatoes by expressing the *miraculin* gene under control of the *E8* promoter (E8-MIR) and characterized the miraculin accumulation pattern in detail in the transgenic tomato fruits. We also discuss the mechanism of higher miraculin accumulation in the exocarp of the 35S-MIR lines. Finally, we discuss the practical significance of the E8-MIR lines.

MATERIALS AND METHODS

Construction of the *E8*-mir*aculin* vector

The binary vector, E8-MIR, was constructed from pBI121 by inserting 663 bp of the *miraculin* gene (GenBank accession number D38598) between the *Bam*HI and *Sac*I sites, replacing the *GUS* gene, and 2,193 bp of the tomato *E8* promoter (GenBank accession number AF515784) and 5′-untranslated region (UTR) between the *Hind*III and *Bam*HI sites, replacing the CaMV 35S promoter (Supplemental Figure 1A). The highest transcriptional activation of the *E8* gene occurs in the full-length (2,181-bp) *E8* promoter and not in the shorter one (Deikman, et al.

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1992). The full-length *E8* promoter region was amplified by PCR (forward primer, 5′-AAGCTTTCCCTAATGATATTGTTCATGTA-3′; reverse primer, 5′-GGATCCCTTCTTTTGCACTGTGAATGATT-3′). The cloning vector was then mobilized into *Agrobacterium tumefaciens* strain GV2260 (Deblaere 1985) using electroporation.

Plant material and transformation

The tomato (*Solanum lycopersicum*) cultivar Micro-Tom (accession number TOMJPF00001), provided by the University of Tsukuba through the National BioResource Project of MEXT, Japan, was used as the material for the genetic transformation. Transgenic tomato plants with the E8-MIR vector were generated by *Agrobacterium*-mediated transformation according to Sun et al. (2006b, 2007). The kanamycin-resistant tomato plants were acclimatized on Rockwool cubes and grown in a growth room under a 16/8 h (light/dark) photoperiod of fluorescent light at an intensity of 60 μmol/s/m^2 (PPFD) at 25 °C. The transgenic tomatoes were watered with Otsuka-A nutrient solution (Otsuka Chemical Co., Ltd., Osaka, Japan) adjusted to an electronic conductance (EC) of 1.5 mS/cm and a pH of 6.5−7.0.

To compare the miraculin accumulation pattern in the E8-MIR transgenic tomato, the transgenic tomatoes (background Micro-Tom) expressing the *miraculin* gene driven from the CaMV 35S promoter (35S-MIR) produced in our previous work (unpublished data) were used in this study. The five lines, 1, 2, 3, 4, and 5, with a single copy of the *miraculin* gene were used. These T_0 transgenic tomatoes were grown in the same growth room as described above. Moreover, to reveal the relationship of miraculin content in exocarp and mesocarp during the fruit development stage, the transgenic tomato line 56B (background cv. Moneymaker), in which the *miraculin* gene is driven by the CaMV 35S promoter (Sun et al. 2007), was also used. The line 56B tomatoes were selected depending on miraculin accumulation and grown to the T6 generation. They were grown in a netted greenhouse and were supplied with Otsuka-A nutrient solution (Otsuka Chemical Co., Ltd., Osaka, Japan) adjusted to an EC value of 1.8 dS/m using a nutrient film technique system.

Genomic PCR and DNA gel blot analysis

The presence of the *miraculin* gene was confirmed by PCR and DNA gel blot analysis according to the methods described by Sun et al. (2007).

Tissue separation of transgenic tomato fruits

To detect *miraculin* mRNA and recombinant miraculin accumulation in different parts of transgenic tomatoes, the fruits from the T_0 generation were separated into three tissues: exocarp, mesocarp, and others, including dissepiment, placenta, and jelly, according to the methods described by Kim et al. (2010) and Kato et al. (2010).

Immunoblot analysis and enzyme-linked immunosorbent assay (ELISA)

The accumulation of recombinant miraculin in transgenic tomatoes was determined by immunoblot analysis. Separated fruit tissues were ground to a powder in liquid nitrogen. The powder (0.1 g) was resuspended in 200 µL extraction buffer consisting of 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 2% polyvinylpolypyrrolidone, and then the solution was centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatant was used for immunoblot analyses and ELISA. The immunoblot analysis and ELISA were conducted according to Sun et al. (2007) and Kim et al. (2010), respectively.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

The *miraculin* mRNA expression levels in fruit tissues from transgenic tomato plants were measured using qRT-PCR. Total RNA was isolated from the exocarp and mesocarp of tomato fruit and treated with DNase using an RNeasy Plant Mini kit (Qiagen, Tokyo, Japan). cDNA was synthesized from 0.5 µg of total RNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Synthesized cDNA was used for qRT-PCR following Kim et al. (2010).

RESULTS

Selection of transgenic tomato plants

Tomato plants were transformed by infection with *A. tumefaciens* strain GV2260 (Deblaere et al. 1985) harboring the binary vector E8-MIR. Putative transgenic lines were screened by rooting in medium containing kanamycin. The rooted plants were subjected to genomic DNA gel blot analysis. The T-DNA region is described in Supplementary Figure 1A. The genomic DNA of each independent plant was digested with *Bam*HI endonuclease, which cut the T-DNA in plasmid E8-MIR only once outside the *miraculin* gene, so the number of bands, in most cases, should reflect the number of transgenes. Among the plants evaluated, transgenic plants with one or multiple copies of transgene were observed (Supplementary Figure 1B). The genomes of 17 of the plants (lines no. 1–6, 9–11, 13, 14, 16, 17, 22, 29, 30) carried one copy

of the *miraculin* gene, and the other plants contained at least two copies. Five lines of E8-MIR transgenic tomatoes (2, 11, 16, 29, 30) were selected and used for subsequent experiments.

Profiling of *miraculin* mRNA expression and protein accumulation in E8-MIR transgenic tomato

To confirm the miraculin accumulation pattern at the fruit stages of the E8-MIR and 35S-MIR transgenic tomato lines, immunoblot analyses were performed. In the 35S-MIR lines, miraculin was detected at the green and red fruit stages in all transgenic lines tested (Figure 1A). However, in the E8-MIR lines, miraculin could not be reproducibly detected at the green fruit stage and was only detected at the red fruit stage in all transgenic lines tested (Figure 1B).

Furthermore, to reveal the spatial accumulation of miraculin of both the E8-MIR and the 35S-MIR lines in the red fruit, the presence and accumulation level of miraculin were examined in different tissues of the red fruit by western blot and ELISA. In the red fruit of the 35S-MIR lines, miraculin was detected in all tissues tested (Supplementary Figure 2A) and the miraculin content in the exocarp was more than twice as high as in other tissues (Figure 2). In contrast, in the E8-MIR lines, miraculin was detected in all tissues of red fruit (Supplementary Figure 2B) and miraculin contents were similar for each tissue (Figure 2). Miraculin contents in all fruit tissues of the 35S-MIR lines were higher than those of the E8-MIR lines.

Miraculin mRNA expression levels were detected by qRT-PCR. The mRNA expression level in the exocarp and mesocarp was similar for the 35S-MIR and E8-MIR lines, respectively. However, the mRNA expression levels in the exocarp and mesocarp of the 35S-MIR lines were significantly higher than those of the E8-MIR lines (Figure 3).

Miraculin accumulation pattern in the exocarp and mesocarp during fruit development in transgenic tomato line 56B (background Moneymaker)

In the present experiments, the 35S-MIR lines showed a different pattern of miraculin accumulation at the red fruit stage compared to the E8-MIR lines; miraculin accumulation in the 35S-MIR exocarp tissue was much higher than in the other tissues. These results are similar to our previous work with transgenic tomato line 56B (background Moneymaker) using the 35S promoter (kim et al. 2010). To explore the effect of constitutive mRNA expression on miraculin accumulation during fruit development, a line 56B was used to measure miraculin contents in the exocarp and mesocarp tissues during fruit development (Figure 4). Miraculin content in the exocarp increased with fruit development, especially from the orange (460 μg/g fresh weight

(FW)) to the red stage (800 µg/gFW). However, miraculin contents in the mesocarp were not significant different between the green (80 µg/gFW) and red stages (122 µg/gFW).

DISCUSSION

Spatial and developmental profiling of miraculin accumulation in transgenic tomato fruit with an *E8* promoter-driven *miraculin* gene

In this study, we demonstrated the use of the *E8* promoter to express the *miraculin* gene in tomato fruits, and the miraculin accumulated in a red fruit-specific manner using the *E8* promoter (Figure 1). Miraculin contents in all tissues of the transgenic tomato fruits of the E8-MIR lines at the red fruit stage were lower than those of the 35S-MIR lines. The lower miraculin content in the E8-MIR fruit was caused by a low expression level of *miraculin* mRNA. These results indicate that the transcriptional activity in tomato is higher with the CaMV 35S promoter than with the *E8* promoter and that high expression of the *miraculin* gene is crucial for high miraculin accumulation in transgenic tomato fruits.

In the 35S-MIR transgenic tomatoes, the content of miraculin in the exocarp was much higher than in the other tissues (Figures 3, 4), and this result is similar to our previous findings (Kim et al. 2010). However, the difference in miraculin contents between the exocarp and mesocarp was smaller when using Micro-Tom as the background in this study compared to a previous study in which Moneymaker was used as the background (Kim et al. 2010). However, in the E8-MIR transgenic tomato fruits, the content of miraculin in the exocarp was almost the same as in the mesocarp (Figure 2, Supplementary Figure 2). To reveal the mechanism of high miraculin accumulation in the exocarp tissues in the 35S-MIR lines, miraculin content was measured during fruit development in the transgenic tomato line 56B (background Moneymaker) expressing the *miraculin* gene driven by the 35S promoter. Moneymaker produces larger fruits than Micro-Tom. We speculated that miraculin was highly accumulated in the exocarp because cell size in the exocarp is smaller than in other tissues (Kim et al. 2010, Kato et al. 2010). The amount of intercellular-layer space per fresh weight is important for miraculin content because miraculin is secreted and accumulates in intercellular spaces in miracle fruit and transgenic tomato (Hirai et al. 2010). In fact, miraculin accumulation in the mesocarp of crossed lines between line 56B and Micro-Tom is higher than that of line 56B, and the cell sizes in the mesocarp in the crossed-line fruits are smaller than those in the line 56B fruits

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(Kato et al. 2010). In this study, miraculin content in the exocarp of line 56B was dramatically increased during fruit development. Thus, it is possible that miraculin is moved from inner tissues to the exocarp and that the miraculin content in the exocarp increased. If the miraculin protein does not move from mesocarp to exocarp, the rate of increase of miraculin in the exocarp should be similar to that in the mesocarp. Therefore, a large-fruit cultivar is better to observe which phenomenon occurs. In the transgenic tomato line 56B, miraculin content in the exocarp was dramatically increased during fruit development (Figure 4). This dramatic increase of miraculin in the exocarp during fruit development indicates that miraculin was moved from the mesocarp to the exocarp. In fact, the significant difference in miraculin accumulation was not detected between the exocarp and mesocarp of the E8-MIR fruits (Figure 2). Production of miraculin started at the turning stage in the E8-MIR fruits. Therefore, this short period of miraculin production might not allow enough time for miraculin accumulation and movement from mesocarp to exocarp. In addition, miraculin contents in the mesocarp and exocarp were uniform in the E8-MIR fruit, without the differences in cell size between the mesocarp and exocarp. E8-driven *GUS* expression is detected in the mesocarp vascular system during the cell expansion stage, and *GUS* is fully expressed throughout the whole tomato fruit at the ripening stage (Deikman et al 1992; Estornell et al. 2007). Thus, this time lag of *miraculin* expression appears to have led to the uniform accumulation in the E8-MIR tomato fruit. Kim et al. (2010) speculated that the excessive miraculin accumulation in the exocarp is primarily caused by the absolute number of cells and intercellular-layer space per unit of fresh weight because exocarp cell size is smaller than in other fruit tissues. Our present results suggest an additional reason why miraculin excessively accumulates in the exocarp of 35S-MIR fruit.

Utilization of transgenic tomato fruit with *miraculin* driven by the *E8* promoter

Generally, when producing tomato juice, ketchup, puree, paste, and other such products, exocarp tissues are removed. Therefore, using the 35S promoter to express the *miraculin* gene is inefficient for processing because miraculin accumulation is extremely high in the exocarp. This accumulation is about 2–3 times higher than in the other fruit tissues (Figure 2). Thus, it would be advantageous to develop plants that accumulate miraculin in other tissues. A possible strategy for changing the accumulation pattern of miraculin is to choose another promoter. In this study, we used the *E8* promoter from tomato, which can drive transgene expression in a ripening-specific manner. The transgenic tomato plant showed a different

pattern of miraculin accumulation in the fruits: miraculin accumulation in the fruit tissues was more uniform than when using the 35S promoter (Figures 3, 4). However, the miraculin accumulation level in the E8-MIR lines was lower than in the 35S-MIR lines. To create a uniform distribution of miraculin in the transgenic tomato fruit, we need to highly express the *miraculin* gene only in the ripening stage of the tomato fruit. Many efforts have sought to increase the expression of a foreign gene effectively, and some expression and translation enhancers have been identified. To increase the amount of protein translated per unit mRNA, the 5'-UTR is known to play an important role. Satoh et al. (2004) reported the 5'-UTR of the NtADH sequence functions as an effective translational enhancer in plant cells. The leader sequence of the tobacco mosaic virus (Ω sequence) is also an effective translational enhancer (Sato et al. 2004). Moreover, the terminator of heat shock protein 18.2 from *Arabidopsis thaliana* is able to increase the mRNA accumulation level compared with transcripts driven by the terminator of nopaline synthase gene (Nagaya et al. 2010). If those enhancers are used with the *E8* promoter, *miraculin* expression might be higher and specific to the fruit-ripening stage, and the miraculin protein accumulation level might be higher and be uniform in each tissue of the tomato. Therefore, the *E8* promoter has potential value for facilitating uniform miraculin accumulation in transgenic tomato fruits in a manner suitable for use in processed tomato products.

ACKNOWLEDGMENTS

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Figure Captions

Figure 1

Miraculin protein accumulation in the green and red fruits of the 35S-MIR (A) and E8-MIR (B) transgenic tomato lines (background cv. Micto-Tom). Green (G) and red (R) fruits were used for the immunoblot analysis. Seven-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.

Figure 2

Miraculin protein contents in red fruit tissues of the 35S-MIR and E8-MIR transgenic tomato lines (background cv. Micto-Tom). Miraculin content was analyzed by ELISA

Figure 3

Miraculin mRNA expression level in red fruit tissues of the 35S-MIR and E8-MIR transgenic tomato lines (background cv. Micto-Tom). The expression levels were determined by quantitative RT-PCR in the exocarp and mesocarp of red fruit, and the relative quantification was calculated by normalization to *S. lycopersicum ubi3* gene (accession number X58253) expression. Vertical bars show the standard deviation from three independent experiments.

Figure 4

Changes in miraculin protein accumulation in exocarp and mesocarp of transgenic tomato line 56B (background cv. Moneymaker) (*29*) during fruit development and ripening. (A) Immunoblot analysis of the miraculin protein in fruit tissues at different developing stages. Five-milligram fresh-weight equivalents of tomato fruit were separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was hybridized with antibody to miraculin. (B) The miraculin contents of fruit tissues at different stages of development were analyzed by ELISA. Vertical bars show the standard error from three independent experiments. The fruits were harvested at the stages of development as follows: green (G), immature green (IMG), mature green (MG), orange (OG), and red (RE).
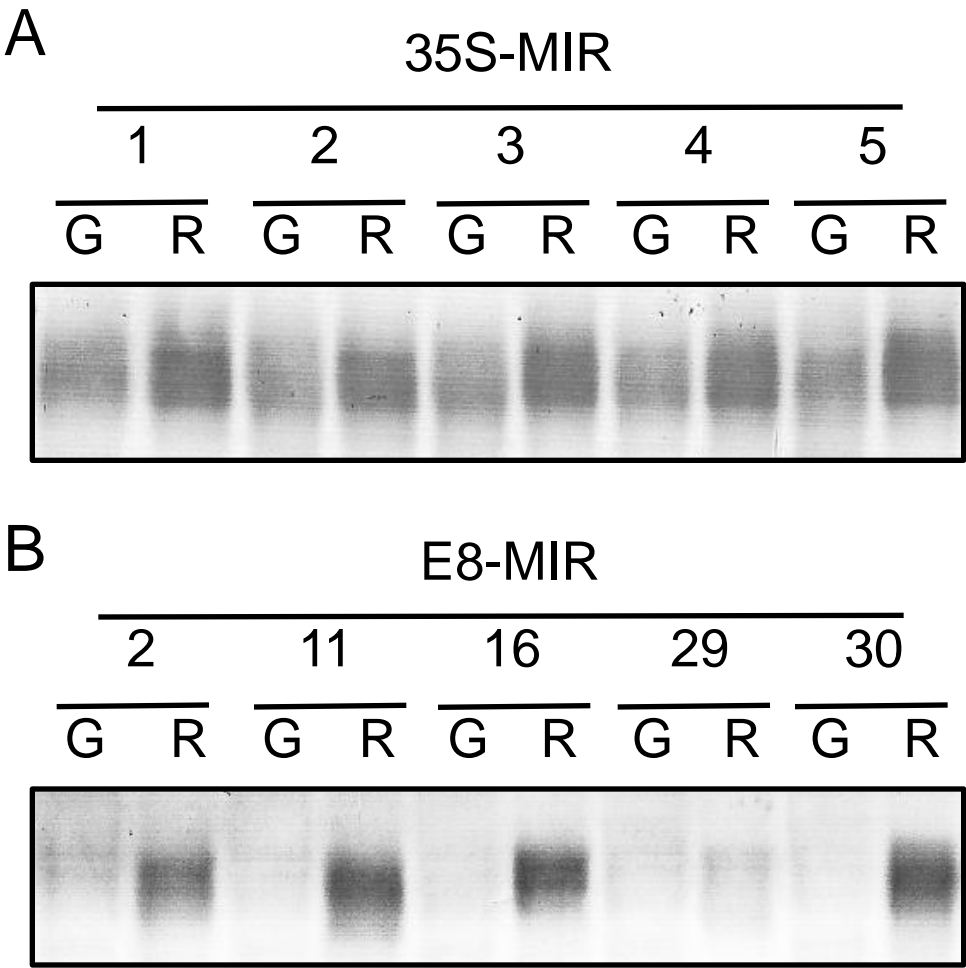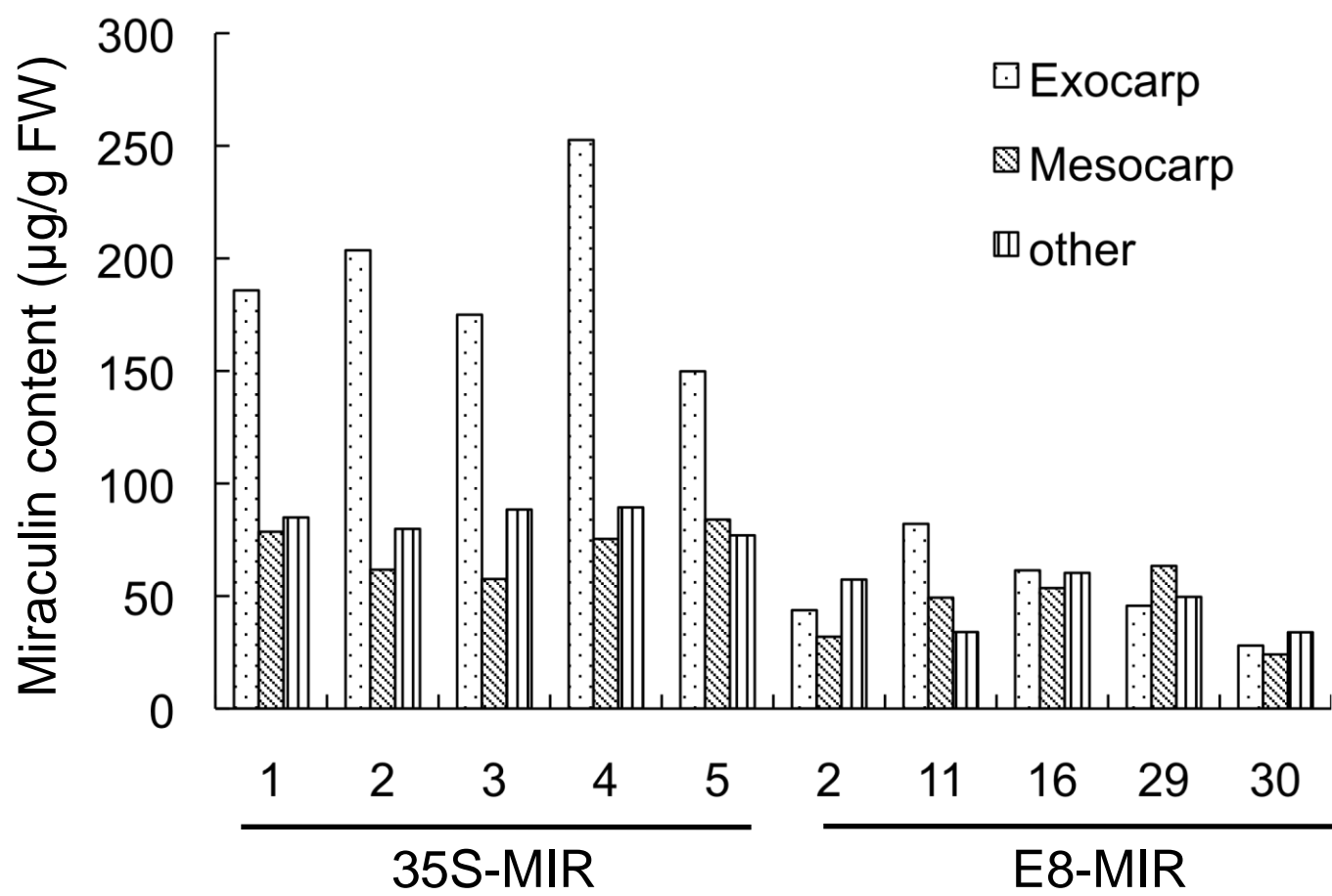
Fig. 1

A

35S-MIR

1	2	3	4	5
G R	G R	G R	G R	G R

B

E8-MIR

2	11	16	29	30
G R	G R	G R	G R	G R

Fig. 2

Fig. 3

Fig. 4

A

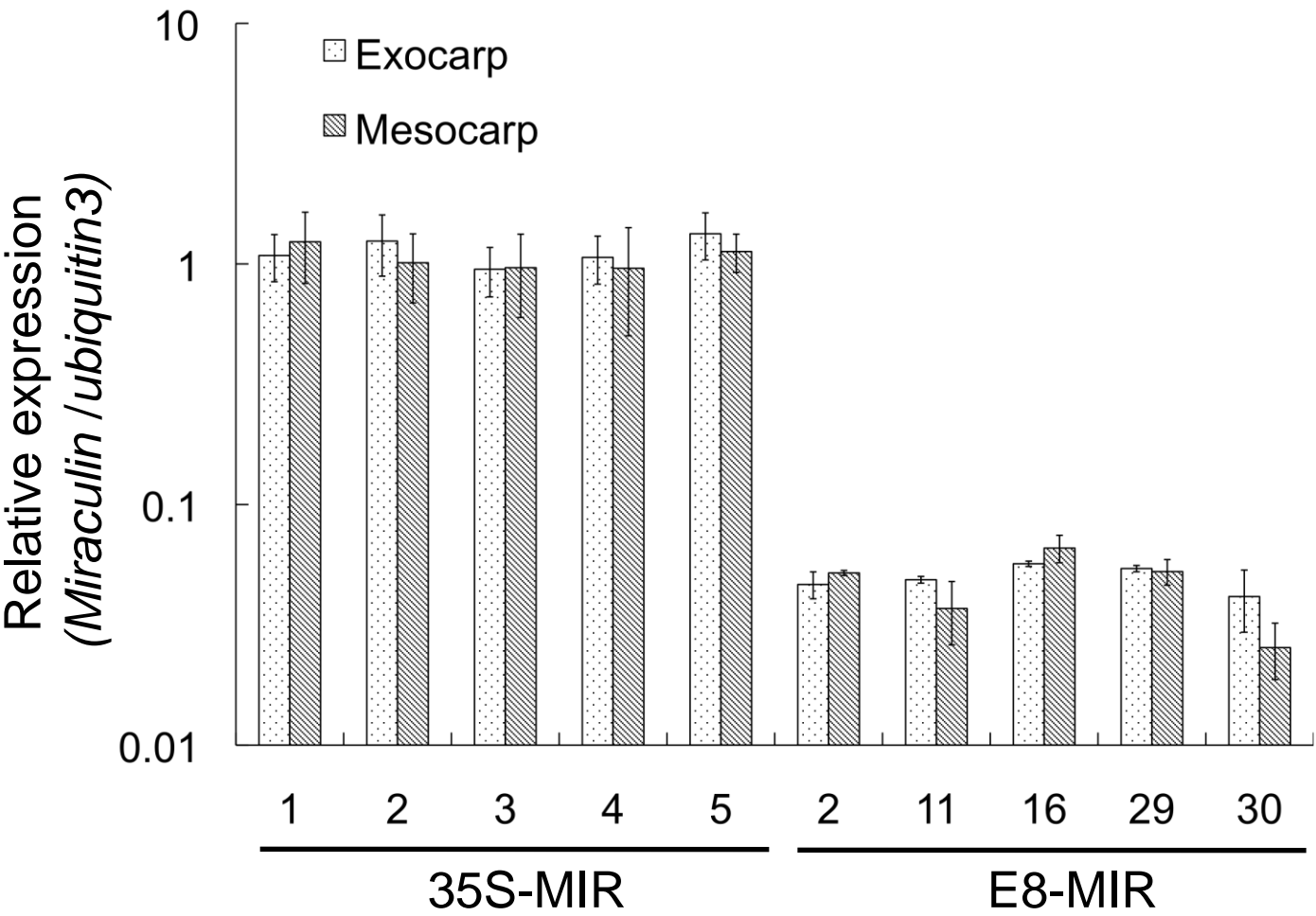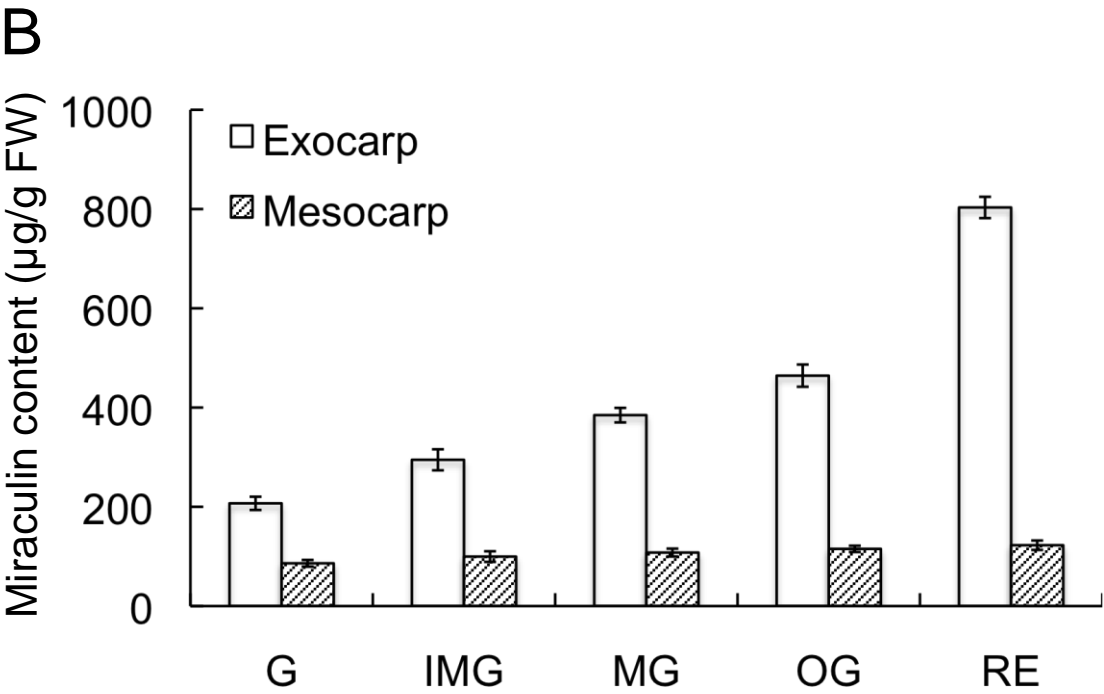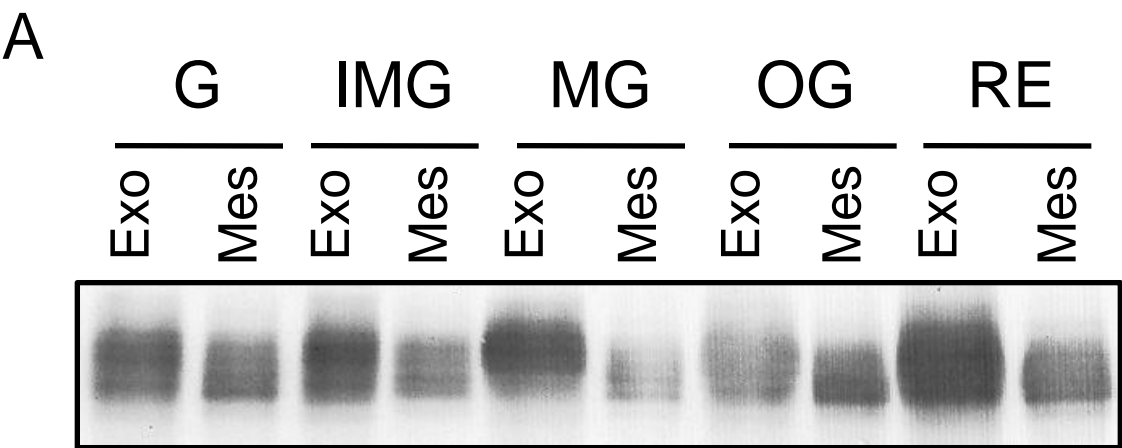| G	IMG	MG	OG	RE
Exo Mes	Exo Mes	Exo Mes	Exo Mes	Exo Mes

B

Supplementary Material

Title:

Uniform accumulation of recombinant miraculin protein in transgenic tomato fruit using a fruit ripening-specific E8 promoter

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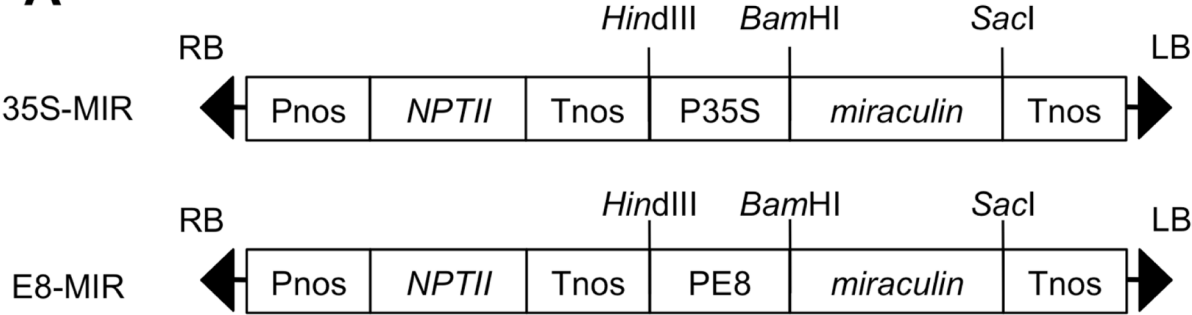
Supplementary Figure 1

Map of the T-DNA region of binary vectors 35S-MIR and E8-MIR (A) and DNA gel blot analysis of transgenic tomato (background cv. Micto-Tom) (B). RB, right border of T-DNA; LB, left border of T-DNA; Pnos, nopaline synthase gene promoter; *NPTII*, neomycin phosphotransferase gene; Tnos, nopaline synthase gene terminator; P35S, CaMV 35S promoter; PE8, *E8* promoter. The genomic DNA from 30 transgenic tomato plants (1–30) and a wild-type tomato plant (WT) were used for DNA gel blot analysis.
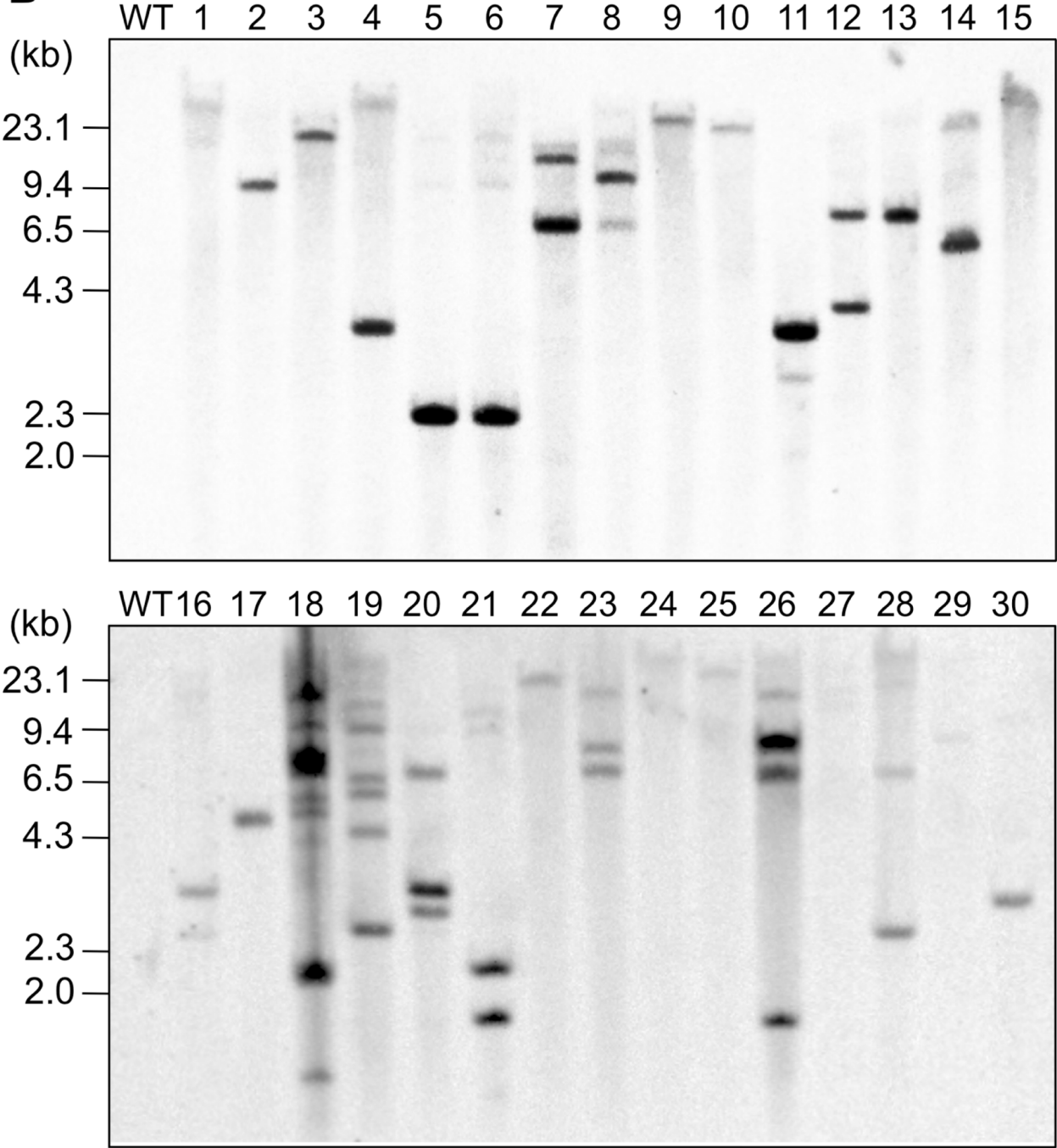

Supplementary Figure 2

Miraculin protein accumulation of different tissues in the red fruit of the 35S-MIR (A) and E8-MIR (B) transgenic tomato lines (background cv. Micto-Tom). Exocarp (Exo), mesocarp (Mes), and other tissues (Other) in the red fruits were used for immunoblot analysis. Seven-milligram fresh-weight equivalents of fruit tissue were separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was hybridized with antibody to miraculin.

Supplementary Figure 1

A

35S-MIR

RB →◄ | Pnos | *NPTII* | Tnos | P35S | *miraculin* | Tnos | ► LB

*Hin*dIII   *Bam*HI   *Sac*I

E8-MIR

RB →◄ | Pnos | *NPTII* | Tnos | PE8 | *miraculin* | Tnos | ► LB

*Hin*dIII   *Bam*HI   *Sac*I

B

Supplementary Figure 2

A   35S-MIR

| 1 | 2 | 3 | 4 | 5 |
| Exo | Mes | Other | Exo | Mes | Other | Exo | Mes | Other | Exo | Mes | Other | Exo | Mes | Other |

B   E8-MIR

| 2 | 11 | 16 | 29 | 30 |
| Exo | Mes | Other | Exo | Mes | Other | Exo | Mes | Other | Exo | Mes | Other | Exo | Mes | Other |