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**An E8 promoter-HSP terminator cassette promotes the high-level accumulation of recombinant protein predominantly in transgenic tomato fruits: a case study of miraculin**

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1    **Abstract**

2

3    Strong, tissue-specific transgene expression is a desirable feature in transgenic  
4    plants to allow the production of variable recombinant proteins. The expression  
5    vector is a key tool to control the expression level and site of transgene and  
6    recombinant protein expression in transgenic plants. The combination of the E8  
7    promoter, a fruit-ripening specific promoter, and a heat shock protein (HSP)  
8    terminator, derived from heat shock protein 18.2 of *Arabidopsis thaliana*,  
9    produces the strong and fruit-specific accumulation of recombinant miraculin in  
10   transgenic tomato. Miraculin gene expression was driven by an E8 promoter and  
11   HSP terminator cassette (E8-MIR-HSP) in transgenic tomato plants, and the  
12   miraculin concentration was the highest in the ripening fruits, representing 30-  
13   630µg miraculin of the gram fresh weight. The highest level of miraculin  
14   concentration among the transgenic tomato plant lines containing the E8-MIR-  
15   HSP cassette was approximately four times higher than those observed in a  
16   previous study using a constitutive 35S promoter and NOS terminator cassette  
17   (Hiwasu-Tanase et al. 2011). These results demonstrate that the combination of  
18   the E8 promoter and HSP terminator cassette is a useful tool to increase  
19   markedly the accumulation of recombinant proteins in a ripening fruit-specific  
20   manner.

21

22    **Keywords:** *expression vector, E8 promoter, HSP terminator, miraculin,*  
23    *transgenic tomato plants*

24

25    **Key Message**

26    The E8 promoter-HSP terminator expression cassette is powerful tool for  
27    increasing the accumulation of recombinant protein in a ripening tomato fruit.

## 1 **Introduction**

2

3           Controlling the accumulation level and site in plant tissue is an important  
4 feature for the effective production of heterologous recombinant proteins in  
5 transgenic plants. Because a low recombinant protein content in transgenic  
6 plants is a common challenge in research and development, strong expression is  
7 essential for the economical production of recombinant proteins (Streatfield 2007).  
8 In addition, controlling the target tissue and timing of recombinant protein  
9 accumulation prevents the stress and damage caused by the undesirable  
10 accumulation of recombinant proteins, such as toxic and unnecessary proteins  
11 that interfere with plant metabolism (Cheon et al. 2004; Desai et al. 2010). To  
12 overcome the low accumulation of recombinant proteins, different promoters  
13 (Dhristensen et al. 1992; Outchkourov et al. 2003; Bhattacharyya et al. 2012) and  
14 expression enhancer sequences (Sato et al. 2004; Matsuura et al. 2008; Sugio et  
15 al. 2008) have been tested for recombinant protein production. Conversely, tissue-  
16 and organ-specific promoters have also been tested to overcome the undesirable  
17 accumulation of recombinant proteins (Matsuoka et al. 1993; Dai et al. 2000;  
18 Rasmussen and Donaldson 2006; He et al. 2008).

19           We have studied the effective production of recombinant proteins in  
20 transgenic plants, using miraculin as a case study. Miraculin is a glycoprotein  
21 that converts sour tastes into sweet tastes. This unique protein was discovered in  
22 the red berries of the miracle fruit (*Richadella dulcifica*), a shrub native to  
23 tropical West Africa (Kurihara and Beidler 1968; Theerasilp and Kurihara 1988).  
24 Miraculin itself is not sweet, but the human tongue, once exposed to the protein,  
25 perceives ordinarily sour foods, such as lemons and citrus, as sweet for up to an  
26 hour afterward. Miraculin is an attractive alternative to more traditional  
27 sweeteners, such as sucrose, but miracle fruit is a tropical plant that is difficult  
28 to cultivate outside of its natural environment. Thus, plant genetic engineering

1 technology has become a powerful tool for the production of miraculin, and the  
2 miraculin gene has been introduced into other plants for production. Using the  
3 cauliflower mosaic virus (CaMV) 35S promoter and NOS terminator cassette, we  
4 have succeeded in accumulating recombinant miraculin in transgenic lettuce  
5 (Sun et al. 2006a), tomato (Sun et al. 2007) and strawberry (Sugaya et al. 2008).  
6 Among these species, tomato was found to be the most suitable host for  
7 recombinant miraculin production: the level of miraculin accumulation remained  
8 steady over multiple generations, and the introduced miraculin gene was stably  
9 inherited (Yano et al. 2010).

10 To produce miraculin in transgenic tomato more effectively, we attempted  
11 to increase the miraculin content and to control the site of miraculin  
12 accumulation within the plant. First, to produce a transgenic tomato with a high  
13 miraculin accumulation, terminators from the miraculin gene and a heat shock  
14 protein (HSP 18.2) from *Arabidopsis thaliana* were combined with the 35S  
15 promoter and used for miraculin gene expression; the HSP terminator supports  
16 high levels of foreign gene expression (Nagaya et al. 2010). The miraculin  
17 accumulation level was 1.5 times higher when using the 35S promoter and  
18 miraculin gene terminator when compared with the 35S promoter and NOS  
19 terminator cassette (35S-MIR-NOS). In addition, miraculin accumulation in  
20 transgenic tomato using the 35S promoter and HSP terminator cassette (35S-  
21 MIR-HSP) was 7.5 times higher than that accumulated using 35S-MIR-NOS.  
22 Second, a fruit-ripening specific promoter, the E8 promoter, was used to confine  
23 miraculin accumulation to the tomato fruits. Miraculin accumulated only in the  
24 red fruits when the miraculin gene was expressed with the E8 promoter and NOS  
25 terminator cassette (E8-MIR-NOS). Although we succeeded in restricting  
26 miraculin accumulation to the fruit, the miraculin content in the E8-MIR-NOS  
27 transgenic tomato fruits was less than one-fifth of that in the 35S-MIR-NOS  
28 transgenic tomato fruits (Hirai et al. 2011a). The miraculin accumulation pattern

1 was characterized in detail in the 35S-MIR-NOS and E8-MIR-NOS transgenic  
2 tomato fruits. Miraculin protein accumulates abundantly in the exocarp tissues  
3 but is less evident in the other tissues, including the mesocarp, dissepiment,  
4 placenta, and jelly tissues, of the red fruit of the 35S-MIR-NOS transgenic  
5 tomato plants (Hirai et al. 2011a; Kim et al. 2010a). In contrast, the miraculin  
6 content was more uniform in the exocarp, mesocarp, and other tissues of the E8-  
7 MIR-NOS transgenic tomato fruits. The uniform accumulation of miraculin is a  
8 desirable feature for processing tomatoes because the exocarp is generally  
9 removed during tomato processing to produce juice, ketchup, puree, paste, and  
10 other tomato products.

11 Previous studies are strongly expected that the combination of E8  
12 promoter and HSP terminator lead to the strong and fruit-specific accumulation  
13 of such heterologous proteins as miraculin. To demonstrate the effectiveness of  
14 the E8 promoter and HSP terminator expression cassette, we chose to use  
15 miraculin in the present study as a test case. We established a transgenic tomato  
16 in which the miraculin gene was expressed using an E8 promoter-HSP  
17 terminator cassette (E8-MIR-HSP), and we analyzed the miraculin accumulation  
18 patterns in the transgenic tomato plants. In addition, we also discuss the  
19 effectiveness of the E8 promoter and HSP terminator cassette for driving strong  
20 transgene expression and recombinant protein accumulation in a fruit-specific  
21 manner.

22

## 23 **Materials and methods**

24

### 25 **Plasmid construction and tomato plant transformation**

26 The binary vector E8-MIR-HSP, in which the miraculin gene is expressed  
27 using the E8 promoter and terminated using the HSP terminator, was created  
28 from the E8-MIR-NOS and 35S-MIR-HSP constructs, which were described in

1 our previous reports (Hirai et al. 2011a; Hirai et al. 2011b). The 35S-MIR-HSP  
2 plasmid was digested with *SacI* and *EcoRI*, and the digested fragment containing  
3 the HSP terminator (252 bp) was isolated and used to replace the NOS  
4 terminator between the *SacI* and *EcoRI* sites of the E8-MIR-NOS plasmid. The  
5 resulting binary vector E8-MIR-HSP was then transformed into *Agrobacterium*  
6 *tumefaciens* strain GV2260 (Deblaere et al. 1985) using electroporation. Tomato  
7 plants (*Solanum lycopersicum* cv. Micro-Tom) were transformed using *A.*  
8 *tumefaciens* harboring the binary vector E8-MIR-HSP, as described by Sun et al.  
9 (2006b). The tomato plants were rooted in root induction medium containing  
10 kanamycin, acclimatized on Rockwool cubes and cultivated at 25 °C in a growth  
11 room under a 16-h light/8-h dark photoperiod with fluorescent lighting at an  
12 intensity of 60  $\mu\text{mol}/\text{m}^2/\text{s}$ . The putative transformants were watered with Otsuka-  
13 A nutrient solution (Otsuka Chemical Co., Ltd., Osaka, Japan) adjusted to an  
14 electrical conductivity (EC) of 1.5 mS/cm. After incubation, the presence of the  
15 miraculin gene and the copy number were confirmed by PCR and Southern blot  
16 analysis according to the methods described by Sun et al. (2007).

17

#### 18 **Plant material used as controls for miraculin accumulation in comparison to E8-** 19 **MIR-HSP**

20 We compared the miraculin accumulation pattern and accumulation level  
21 in the E8-MIR-HSP transgenic plants to those in the 35S-MIR-NOS and E8-MIR-  
22 NOS transgenic tomatoes produced in our previous work (Hiwas-Tanase et al.  
23 2011; Hirai et al. 2011a). Hiwasa-Tanase et al. (2011) produced the 7 lines of 35S-  
24 MIR-NOS transgenic tomatoes, which are homozygous for single copy of  
25 miraculin gene, and the 35S-MIR-NOS lines 3 and 6 were selected as controls in  
26 this study because these lines accumulated the high- and stable-level of  
27 miraculin among the 7 lines of 35S-MIR-NOS transgenic tomatoes. In addition,  
28 E8-MIR-NOS lines 2 and 9 were selected based on the high- and stable-level of

1 miraculin accumulation among 17 lines of E8-MIR-NOS transgenic tomatoes,  
2 which are homozygous for single copy of miraculin gene and used for controls in  
3 this study. The T<sub>2</sub> transgenic tomatoes were grown in the same growth room as  
4 described above.

5

#### 6 **Tissue separation of transgenic tomato fruits**

7 To measure the miraculin accumulation levels in different parts of the  
8 red-ripe fruit of the transgenic tomatoes, the fruit was separated into three  
9 tissues: exocarp, mesocarp, and other tissues, which included the dissepiment,  
10 placenta, and jelly. The tissues were separated as described by Kim et al. (2010a)  
11 and Kato et al. (2010).

12

#### 13 **Protein extraction, Western blot analysis and enzyme-linked immunosorbent** 14 **assay (ELISA)**

15 The miraculin accumulation levels and patterns in the transgenic tomato  
16 fruits were determined using Western blot analysis and ELISA. The separated  
17 pericarp tissue isolated from the transgenic tomato fruits, including the exocarp,  
18 and mesocarp, stem, leaf, exocarp, mesocarp, and other tissue, was ground into a  
19 fine powder in liquid nitrogen. The powder (0.1 g) was resuspended in 200 µL  
20 extraction buffer consisting of 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 2%  
21 polyvinylpolypyrrolidone, and the solution was centrifuged at 15,000 rpm for 20  
22 min at 4 °C. The supernatant was used for Western blot analyses and ELISA, as  
23 described by Sun et al. (2007) and Kim et al. (2010a), respectively.

24

#### 25 **Isolation of mRNA and quantitative reverse-transcriptase polymerase chain** 26 **reaction (RT-PCR)**

27 The mRNA expression levels of miraculin in the transgenic tomato fruits  
28 were determined by qRT-PCR. Total RNA was isolated from the mature green

1 and red-ripe fruits using the RNeasy plant mini kit (Qiagen, Tokyo, Japan), and  
2 the cDNA was synthesized from 0.75 µg of total RNA using the SuperScript VILO  
3 cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The cDNA (1 µL) was  
4 diluted in 10 µL TE buffer, and 1 µL of this diluted cDNA was used for qRT-PCR  
5 amplification with SYBR Premixed EX-Taq II (Takara Bio Inc., Otsu, Japan). The  
6 PCR thermal cycling conditions were performed as recommended by the  
7 manufacturer. The qRT-PCR reaction was performed using miraculin-specific  
8 primers, and ubiquitin 3-specific primers were used as a control (Leclercq et al.  
9 2005; Hackel et al. 2006). The primer sequences were as follows: miraculin  
10 forward, 5'-CACCCAATCCGGTTCCTTGAC-3'; miraculin reverse, 5'-  
11 GTGGTGGCGGATACTGTAAGG-3'; ubiquitin 3 forward, 5'-  
12 CACCAAGCCAAAGAAGATCA-3'; and ubiquitin 3 reverse, 5'-  
13 TCAGCATTAGGGCACTCCTT-3'.

14

## 15 **Results**

16

### 17 **Transformation of tomato plants with the miraculin gene**

18 To confirm the efficiency of miraculin production using the E8-MIR-HSP  
19 expression cassette, tomato cotyledons were transformed by infection with  
20 *Agrobacterium tumefaciens* strain GV2260 (Deblaere et al. 1985) containing the  
21 E8-MIR-HSP binary vector (Figure 1A). In this construct, miraculin gene  
22 expression was driven by the fruit-specific E8 promoter and terminated by the  
23 HSP terminator. A total of 200 tomato cotyledon segments were infected, and 100  
24 putative transgenic tomato lines were rooted on selection medium. The  
25 integration of the MIR gene was confirmed by Southern blot analysis in 28 plants  
26 after the selection of diploids using the ploidy test and screening for the presence  
27 of the miraculin gene by genomic PCR. Genomic DNA from each plant, including  
28 an untransformed control, was digested with XbaI, which cuts the T-DNA in the



1 E8-MIR-HSP plasmid at a single site outside of the miraculin gene. In most cases,  
2 the number of bands produced by this digestion reflects the transgene copy  
3 number. The hybridization of the restriction-digested genomic DNA to a  
4 miraculin gene probe revealed that the clones represented independent  
5 transformation events and that the number of transgene copies varied among the  
6 different transformed lines. Single bands (i.e., single copies of the miraculin gene)  
7 were detected in 13 transgenic lines (Figure 1B). Among the 13 transgenic lines,  
8 4 lines showed bands at identical positions, suggesting a clonal origin. We used  
9 the remaining 9 independent transgenic lines harboring a single copy of the  
10 miraculin gene for the subsequent experiments.

11

### 12 **Miraculin accumulation and miraculin gene expression in transgenic tomato** 13 **fruits**

14 To compare the miraculin accumulation level at the green- and red-fruit  
15 stages in the transgenic tomato fruits, the 35S-MIR-NOS, E8-MIR-NOS and E8-  
16 MIR-HSP transgenic tomatoes were used for immunoblot analyses. The 35S-  
17 MIR-NOS and E8-MIR-NOS transgenic strains were produced in a previous work  
18 (Hiwasa-Tanase et al. 2011; Hirai et al. 2011a). For the present study, we used  
19 T<sub>2</sub>-generation 35S-MIR-NOS and E8-MIR-NOS plants harboring a homozygous  
20 single copy of the miraculin gene. In the 35S-MIR-NOS plants, the Western blot  
21 analysis showed that the miraculin level was approximately 60 µg/g fresh weight  
22 (FW) in the green fruits and approximately 150 µg/g FW in the red fruits. For the  
23 E8-MIR-NOS transgenic plants, miraculin was detected only in the red fruits, at  
24 approximately 65 µg/g FW. The miraculin accumulation level in 35S-MIR-NOS  
25 and E8-MIR-NOS lines was almost same in our previous works (Hiwasa-Tanase  
26 et al. 2011; Hirai et al. 2011a). In the E8-MIR-HSP transgenic tomato, miraculin  
27 was only detected at the red-fruit stage, and the miraculin content was 30-250  
28 µg/g FW in lines 2, 6, 10, 18 and 21. However, in lines 3, 5, 7 and 11, miraculin

1 was detected in both the green- and red-stage fruits, at 12-38 µg/g FW and 270-  
2 630 µg/g FW, respectively.

3 The miraculin gene expression level in each transgenic tomato fruit was  
4 measured by qRT-PCR. At the mature green-fruit stage, the miraculin gene  
5 expression was detected in 35S-MIR-NOS lines 3 and 6 and E8-MIR-HSP lines 3,  
6 5, 7, 11 and 21, and the miraculin gene expression levels were higher in 35S-  
7 MIR-NOS than E8-MIR-HSP. The miraculin gene was expressed in all of the  
8 transgenic tomatoes at the red-fruit stage. The miraculin expression levels in all  
9 of the E8-MIR-NOS lines and in E8-MIR-HSP lines 2, 20 and 18 were lower than  
10 those of the 35S-MIR-NOS transgenic tomatoes. However, the miraculin  
11 expression levels in E8-MIR-HSP lines 3, 5, 6, 7, 11 and 21 were equivalent to or  
12 higher than those in the 35S-MIR-NOS transgenic tomatoes.

13

#### 14 **Miraculin accumulation level in various tissues of transgenic tomatoes**

15 ELISA was used to evaluate the miraculin accumulation level in the  
16 different tissues of the red fruits of two lines of 35S-MIR-NOS, two lines of E8-  
17 MIR-NOS and lines 3, 6, 11 and 21 of E8-MIR-HSP, which had the highest  
18 miraculin accumulation levels of all of the E8-MIR-HSP transgenic tomatoes  
19 tested. In 35S-MIR-NOS, the miraculin content in the exocarp was approximately  
20 260 µg/g FW and was less than half as strong in other tissues. In contrast, the  
21 miraculin contents were similar for each tissue in the E8-MIR-NOS and E8-MIR-  
22 HSP plants. However, the miraculin contents of all of the tissues were markedly  
23 higher in the E8-MIR-HSP transgenic tomatoes than in the 35S-MIR-NOS and  
24 E8-MIR-NOS tomatoes.

25

#### 26 **Discussion**

27 The production of plant-derived recombinant proteins has been studied  
28 over the past decade (Twyman et al. 2003), and recombinant systems using

1 plants have many potential advantages over microorganisms and, especially,  
2 animal cell systems, particularly in terms of the production costs (Daniell et al.  
3 2001). However, a better control of the heterologous protein expression is  
4 necessary; specifically, the accumulation level of heterologous proteins must be  
5 increased, and the heterologous proteins must be reliably expressed in a tissue-  
6 specific manner. In the present study, we aimed to solve these problems using the  
7 fruit-ripening specific E8 promoter and the HSP terminator to drive the  
8 expression of a foreign gene in transgenic tomato. Miraculin was used as the  
9 heterologous protein to demonstrate the effectiveness of our new expression  
10 cassette.

11 We selected elite lines that expressed the highest concentrations of  
12 recombinant protein among many transgenic plants. In previous studies, the  
13 miraculin accumulation levels were very different among transgenic tomatoes  
14 transformed with the same expression cassette (Hiwasa-Tanase et al. 2011; Sun  
15 et al. 2007; Hirai et al. 2011a). In the present study, 22 lines of E8-MIR-HSP  
16 transgenic tomato plants were obtained, and the miraculin accumulation levels  
17 in the red fruits varied among the lines. The miraculin accumulation levels in  
18 E8-MIR-HSP lines 3, 5, 7, 11, 21 were higher than in the other lines, and the  
19 accumulation in line 7 was markedly higher than in any other line. For  
20 comparison, we used 35S-MIR-NOS and E8-MIR-NOP plants that were selected  
21 based on the high miraculin accumulation in the T<sub>0</sub> generation and produced T<sub>2</sub>  
22 generation plants that were homozygous for a single copy of the miraculin gene.  
23 The average miraculin accumulation level in the red fruit of E8-MIR-HSP was  
24 higher than that in 35S-MIR-NOS or E8-MIR-NOS. The miraculin accumulation  
25 level in the red fruit of E8-MIR-HSP line 7 was approximately 7.7-times higher  
26 than that in the E8-MIR-NOS transgenic line. In a previous study, the miraculin  
27 gene expression was strongly enhanced and miraculin accumulation level was  
28 increased by approximately 7.5-fold using the 35S promoter and HSP terminator

1 cassette compared with using the 35S promoter and NOS terminator cassette  
2 (35S-MIR-HSP). These results indicated that the HSP terminator increases the  
3 miraculin accumulation level by up to 7 times in the transgenic tomato,  
4 regardless of the promoter used. Thus, the HSP terminator could be a powerful  
5 tool for the enhancement of heterologous protein accumulation in transgenic  
6 plants.

7 Tissue-specific promoters can overcome the potential stress and damage  
8 caused by the undesirable accumulation of heterologous protein using a  
9 constitutive expression promoter, such as the 35S promoter. Many reports have  
10 demonstrated that heterologous protein accumulation in the red fruits of  
11 transgenic tomatoes can be driven by the E8 promoter (Sandhu et al. 2000; Jiang  
12 et al. 2007). Miraculin was not detected in the green fruits of any of the E8-MIR-  
13 NOS lines or E8-MIR-HSP lines 2, 6, 10, 18, or 21; however, a low miraculin  
14 accumulation in the green fruits was detected in E8-MIR-HSP lines 3, 5, 7, and  
15 11. These lines also exhibited higher miraculin accumulation in the red fruits. In  
16 general, the miraculin gene expression level in the green fruits was very low  
17 when the miraculin gene was driven by the E8 promoter, but the HSP terminator  
18 strongly enhanced the gene expression in E8-MIR-HSP lines 3, 5, 7, and 11 such  
19 that miraculin was detected in these transgenic tomatoes. These results  
20 indicated that a strong expression enhancer, such as the HSP terminator, can  
21 disrupt the tissue-specificity of expression under a tissue-specific promoter, such  
22 as E8.

23 To effectively accumulate a heterologous protein in target tissues of the  
24 transgenic plants, it is important to increase the transgene expression in the tissue-specific  
25 manner. Many promoters have been used to improve the transgene expression  
26 (Dhristensen et al. 1992; Outchkourov et al. 2003; Bhattacharyya et al. 2012). Gene  
27 expression enhancer sequences such as  $\Omega$  sequence of the tobacco mosaic virus and  
28 5' untranslated region from the tobacco alcohol dehydrogenase gene were also tested to

1 increase the transgene expression (Mitsuhara et al. 1996; Satoh et al. 2004), especially  
2 combined with constitutive promoters. However, alteration of promoters and combined-  
3 use with enhancer sequences sometimes cause the change of transgene expression pattern.  
4 By contrast, terminator cannot change for gene expression pattern because terminator acts  
5 mainly for 3'-tail formation. This study is the first report to demonstrate that HSP  
6 terminator is able to increase the transgene expression and the heterologous protein  
7 accumulation in a tissue specific manner, and provide an idea of using the HSP terminator  
8 with various tissue/organ specific promoters for commercial transgenic plants.

9         To produce tomato juice, ketchup, puree, or paste, the exocarp is removed  
10 during processing. For this reason, the uniform accumulation of miraculin in  
11 fruit tissue, particularly non-exocarp tissue, is desirable. In our previous study,  
12 we analyzed the miraculin accumulation pattern in the fruit tissue of miraculin-  
13 accumulating 35S-MIR-NOS, 35S-MIR-HSP, and E8-MIR-NOS tomatoes. The  
14 35S promoter generated stronger miraculin accumulation levels in the exocarp  
15 than in the other tissues. In E8-MIR-NOS, the miraculin protein accumulated  
16 more uniformly in the red fruits but at lower levels than those observed in 35S-  
17 MIR-NOS. In the present study, we showed that the miraculin contents in E8-  
18 MIR-HSP were both uniformly distributed and higher than those in the E8-MIR-  
19 NOS tomatoes. Kim et al. (2010a) measured the ratio of fruit tissues to fruit  
20 weight for various tomato cultivars and showed that the ratio of exocarp to whole  
21 fruit did not exceed 5%. This suggests that the efficiency of miraculin production  
22 in E8-MIR-HSP was mostly attributable to the accumulation of miraculin in the  
23 mesocarp and other tissues. Hirai et al. (2011b) showed that the miraculin  
24 accumulation level in the mesocarp and other tissues of 35S-MIR-HSP, in which  
25 the miraculin gene was expressed as a homozygous single copy, were  
26 approximately 600 and 700  $\mu\text{g/g}$  FW. In the present study, the miraculin  
27 accumulation levels in the mesocarp and other tissues in E8-MIR-HSP line 7  
28 were approximately 450 and 600  $\mu\text{g/g}$  FW, respectively, lower than those observed

1 in 35S-MIR-HSP. Moreover, Kim et al. (2010b) demonstrated that plants  
2 homozygous for the miraculin gene exhibited only approximately 1.3 times higher  
3 accumulation levels of miraculin than heterozygotes because the gene dosage  
4 affected the miraculin gene expression and protein accumulation. The E8-MIR-  
5 HSP line 7 we tested was a T<sub>0</sub> generation plant heterozygous for the miraculin  
6 gene. It is expected that homozygotes derived from subsequent generations of  
7 this line will have approximately 1.3 times greater miraculin accumulation levels,  
8 reaching the expression levels observed in 35S-MIR-HSP. Overall, the E8-MIR-  
9 HSP transgenic tomatoes accumulated uniformly high levels of miraculin in the  
10 red fruits, which is desirable for processed tomato production.

11 In conclusion, to demonstrate the effectiveness of the E8 promoter and  
12 HSP terminator expression cassette for the strong and tissue-specific  
13 accumulation of recombinant proteins, we chose to use miraculin as a test case  
14 and generated E8-MIR-HSP transgenic tomatoes. E8-MIR-HSP resulted in the  
15 greater accumulation of miraculin in the red-ripe fruits than the other transgenic  
16 constructs. The accumulation of a recombinant protein is dependent on the  
17 properties of that protein; thus, it is unclear whether the E8 promoter and HSP  
18 terminator expression cassette would produce transgenic tomatoes expressing  
19 similarly high levels of other recombinant proteins. However, gene expression is  
20 not dependent on the properties of the recombinant protein, and it is clear that  
21 the E8 promoter and HSP terminator expression cassette drives strong gene  
22 expression in the red-ripe fruits of transgenic tomato plants. The E8 promoter  
23 and HSP terminator expression cassette was remarkably effective for inducing  
24 the strong and red-ripe fruit-specific expression of the recombinant miraculin  
25 protein gene in transgenic tomato plants. This is the first report on  
26 demonstrating the effectiveness of the HSP terminator even for a tissue specific  
27 promoter.

28

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2

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## **Figure captions**

3

### **Fig. 1**

4 Map of the T-DNA region in the binary vector (A) and Southern blot analysis of  
5 the tomato transformants (B). RB, right border of the T-DNA; LB, left border of  
6 the T-DNA; Pnos, nopaline synthase gene promoter; NPTII, neomycin  
7 phosphotransferase gene; Tnos, nopaline synthase gene terminator; PE8, tomato  
8 E8 gene promoter; THSP, heat shock protein terminator; miraculin, miraculin  
9 gene. Genomic DNA from 22 transgenic lines and a wild-type tomato plant (WT)  
10 was subjected to Southern blot analysis. Miraculin coding sequences were used  
11 as the probes.  
12

13

### **Fig. 2**

14 Miraculin accumulation was analyzed by Western blotting in the T<sub>0</sub> generation of  
15 E8-MIR-HSP and T<sub>2</sub> generation of 35S-MIR-NOS and E8-MIR-NOS transgenic  
16 tomato fruits. (A) The miraculin concentration in the transgenic tomato fruits  
17 was measured using an enzyme-linked immunosorbent assay (ELISA). (B)  
18 Protein was extracted from mature green fruits (G) and red-ripe fruits (R). Total  
19 soluble protein samples (5.3 mg) were separated by SDS-PAGE and blotted onto a  
20 PVDF membrane. The membrane was hybridized with antibodies against  
21 miraculin. The numbers under the columns indicate the independent transgenic  
22 tomato lines, identified as described in Figure 1.  
23

24

### **Fig. 3**

25 Miraculin mRNA expression level in the mature green fruits (A) and red-ripe  
26 fruits (B) of the E8-MIR-HSP (T<sub>0</sub>), 35S-MIR-NOS (T<sub>2</sub>) and E8-MIR-NOS (T<sub>2</sub>)  
27 transgenic tomato fruits. The expression levels were determined by quantitative  
28 RT-PCR, and the relative quantification was calculated using normalization to  
29

1 the expression of the *S. lycopersicum ubi3* gene (accession number X58253). The  
2 vertical bars represent the standard deviation for three independent experiments.  
3 The numbers under the columns indicate the independent transgenic tomato  
4 lines, identified as described in Figure 1. N.D., not detected.

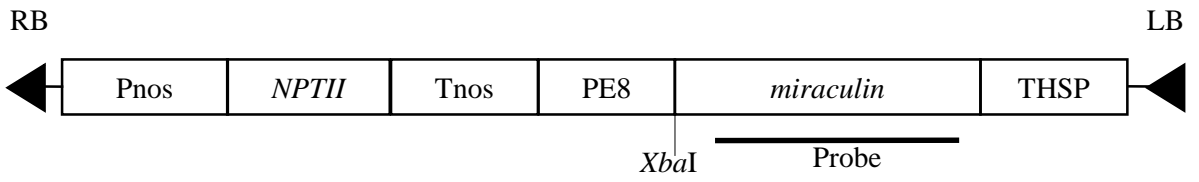
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6 Fig. 4

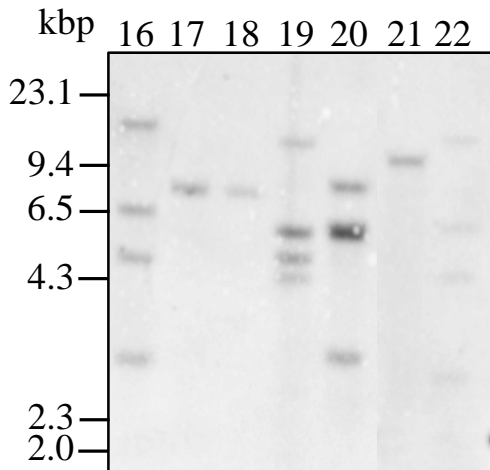
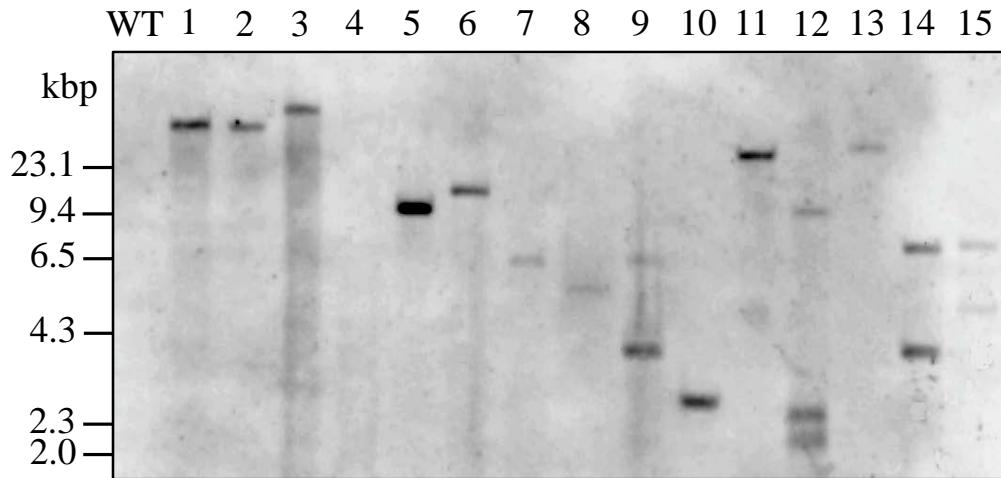
7 The miraculin concentration in the red-ripe fruit tissue of transgenic tomato lines  
8 was analyzed by ELISA. The vertical bars represent the standard deviation for  
9 three independent experiments. The numbers under the columns indicate the  
10 independent transgenic tomato lines, identified as described in Figure 1. Exo,  
11 exocarp; Mes, mesocarp; Other, dissepiment, placenta and jelly tissue.

# Figure 1

(a)

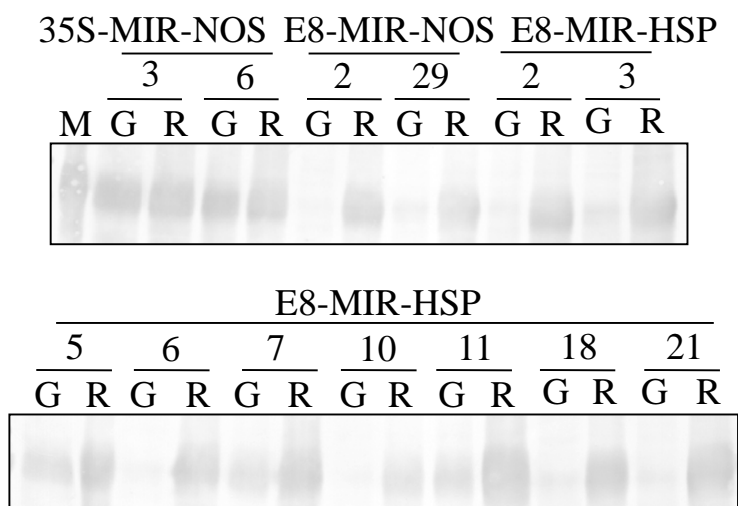


(b)

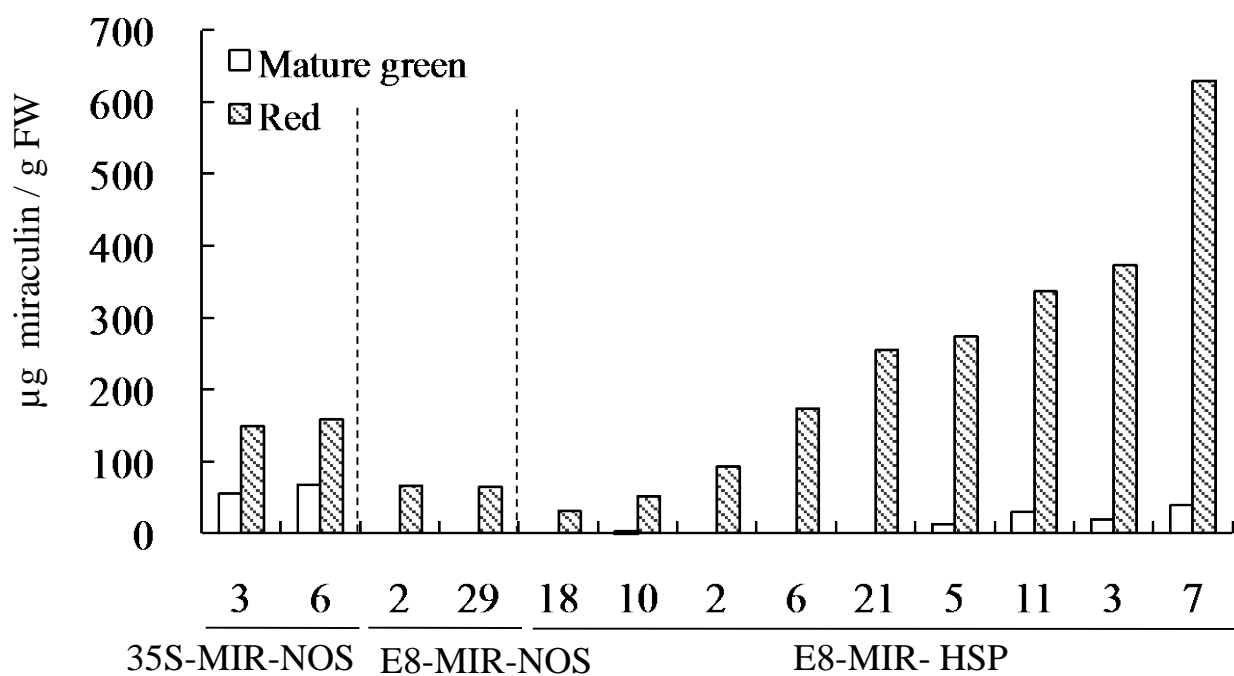


# Figure 2

(a)



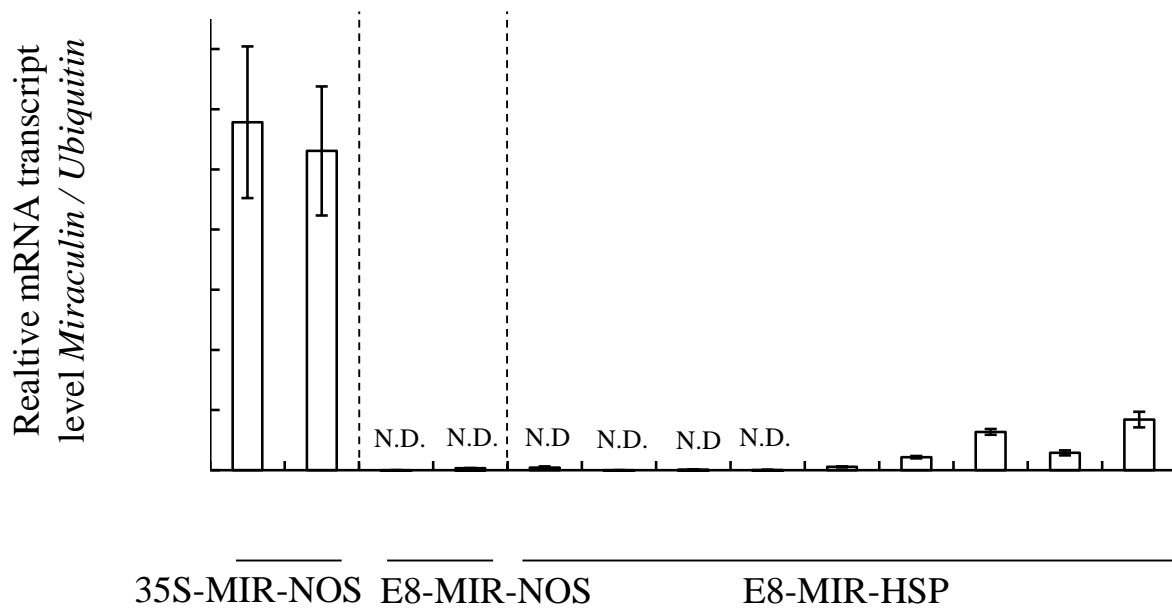
(b)





# Figure 3

(a)



(b)

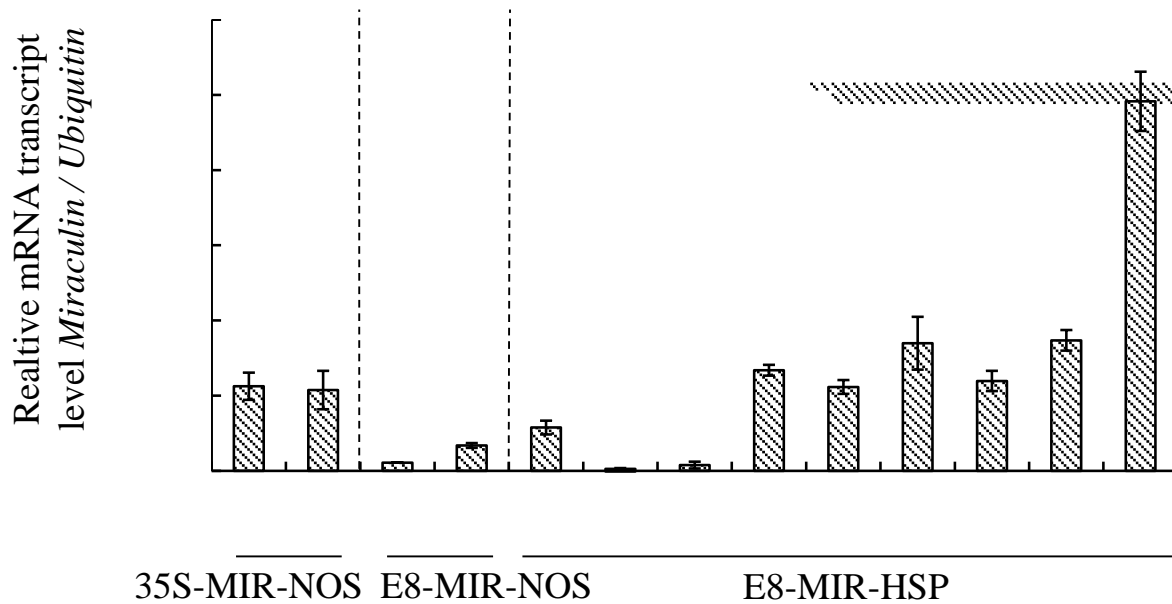


Figure 4

