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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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## Abstract

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



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


***Key Message***

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

## Introduction

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

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

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

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

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

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

## Materials and methods

### Plasmid construction and tomato plant transformation

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

our previous reports (Hirai et al. 2011a; Hirai et al. 2011b). The 35S-MIR-HSP plasmid was digested with *Sac*I and *Eco*RI, and the digested fragment containing the HSP terminator (252 bp) was isolated and used to replace the NOS terminator between the *Sac*I and *Eco*RI sites of the E8-MIR-NOS plasmid. The resulting binary vector E8-MIR-HSP was then transformed into *Agrobacterium tumefaciens* strain GV2260 (Deblaere et al. 1985) using electroporation. Tomato plants (*Solanum lycopersicum* cv. Micro-Tom) were transformed using *A. tumefaciens* harboring the binary vector E8-MIR-HSP, as described by Sun et al. (2006b). The tomato plants were rooted in root induction medium containing kanamycin, acclimatized on Rockwool cubes and cultivated at 25 ˚C in a growth room under a 16-h light/8-h dark photoperiod with fluorescent lighting at an intensity of 60 μmol/m²/s. The putative transformants were watered with Otsuka-A nutrient solution (Otsuka Chemical Co., Ltd., Osaka, Japan) adjusted to an electrical conductivity (EC) of 1.5 mS/cm. After incubation, the presence of the miraculin gene and the copy number were confirmed by PCR and Southern blot analysis according to the methods described by Sun et al. (2007).

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

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

miraculin accumulation among 17 lines of E8-MIR-NOS transgenic tomatoes, which are homozygous for single copy of miraculin gene and used for controls in this study. The $T_2$ transgenic tomatoes were grown in the same growth room as described above.

**Tissue separation of transgenic tomato fruits**

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

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

The miraculin accumulation levels and patterns in the transgenic tomato fruits were determined using Western blot analysis and ELISA. The separated pericarp tissue isolated from the transgenic tomato fruits, including the exocarp, and mesocarp, stem, leaf, exocarp, mesocarp, and other tissue, was ground into a fine 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 the solution was centrifuged at 15,000 rpm for 20 min at 4 ˚C. The supernatant was used for Western blot analyses and ELISA, as described by Sun et al. (2007) and Kim et al. (2010a), respectively.

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

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

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

## Results

### Transformation of tomato plants with the miraculin gene

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

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

**Miraculin accumulation and miraculin gene expression in transgenic tomato fruits**

To compare the miraculin accumulation level at the green- and red-fruit stages in the transgenic tomato fruits, the 35S-MIR-NOS, E8-MIR-NOS and E8-MIR-HSP transgenic tomatoes were used for immunoblot analyses. The 35S-MIR-NOS and E8-MIR-NOS transgenic strains were produced in a previous work (Hiwasa-Tanase et al. 2011; Hirai et al. 2011a). For the present study, we used $T_2$-generation 35S-MIR-NOS and E8-MIR-NOS plants harboring a homozygous single copy of the miraculin gene. In the 35S-MIR-NOS plants, the Western blot analysis showed that the miraculin level was approximately 60 μg/g fresh weight (FW) in the green fruits and approximately 150 μg/g FW in the red fruits. For the E8-MIR-NOS transgenic plants, miraculin was detected only in the red fruits, at approximately 65 μg/g FW. The miraculin accumulation level in 35S-MIR-NOS and E8-MIR-NOS lines was almost same in our previous works (Hiwasa-Tanase et al. 2011; Hirai et al. 2011a). In the E8-MIR-HSP transgenic tomato, miraculin was only detected at the red-fruit stage, and the miraculin content was 30-250 μg/g FW in lines 2, 6, 10, 18 and 21. However, in lines 3, 5, 7 and 11, miraculin

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

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

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

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

## Discussion

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

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

We selected elite lines that expressed the highest concentrations of recombinant protein among many transgenic plants. In previous studies, the miraculin accumulation levels were very different among transgenic tomatoes transformed with the same expression cassette (Hiwasa-Tanase et al. 2011; Sun et al. 2007; Hirai et al. 2011a). In the present study, 22 lines of E8-MIR-HSP transgenic tomato plants were obtained, and the miraculin accumulation levels in the red fruits varied among the lines. The miraculin accumulation levels in E8-MIR-HSP lines 3, 5, 7, 11, 21 were higher than in the other lines, and the accumulation in line 7 was markedly higher than in any other line. For comparison, we used 35S-MIR-NOS and E8-MIR-NOP plants that were selected based on the high miraculin accumulation in the $T_0$ generation and produced $T_2$ generation plants that were homozygous for a single copy of the miraculin gene. The average miraculin accumulation level in the red fruit of E8-MIR-HSP was higher than that in 35S-MIR-NOS or E8-MIR-NOS. The miraculin accumulation level in the red fruit of E8-MIR-HSP line 7 was approximately 7.7-times higher than that in the E8-MIR-NOS transgenic line. In a previous study, the miraculin gene expression was strongly enhanced and miraculin accumulation level was increased by approximately 7.5-fold using the 35S promoter and HSP terminator

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

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

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

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

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

in 35S-MIR-HSP. Moreover, Kim et al. (2010b) demonstrated that plants homozygous for the miraculin gene exhibited only approximately 1.3 times higher accumulation levels of miraculin than heterozygotes because the gene dosage affected the miraculin gene expression and protein accumulation. The E8-MIR-HSP line 7 we tested was a $T_0$ generation plant heterozygous for the miraculin gene. It is expected that homozygotes derived from subsequent generations of this line will have approximately 1.3 times greater miraculin accumulation levels, reaching the expression levels observed in 35S-MIR-HSP. Overall, the E8-MIR-HSP transgenic tomatoes accumulated uniformly high levels of miraculin in the red fruits, which is desirable for processed tomato production.

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

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## References

Bhattacharyya J, Chowdhury AH, Ray S, Jha JK, Das S, Gayen S, Chakraborty A, Mitra J, Maiti MK, Basu A, Sen SK (2012) Native polyubiquitin promoter of rice provides increased constitutive expression in stable transgenic rice plants. Plant Cell Rep 31:271-279. doi: 10.1007/s00299-011-1161-4

Cheon BY, Kim HJ, Oh KH, Bahn SC, Ahn JH, Choi JW, Ok SH, Bae JM, Shin JS (2004) Overexpression of human erythropoietin (EPO) affects plant morphologies: retarded vegetative growth in tobacco and male sterility in tobacco and Arabidopsis. Transgenic Res 13:541-549. doi: 10.1007/s11248-004-2737-3

Christensen AH, Sharrock RA, Quail PH (1992) Maize polyubiquitin genes-structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18:675-689. doi: 10.1007/bf00020010

Dai ZY, Hooker BS, Anderson DB, Thomas SR (2000) Expression of *Acidothermus*

*cellulolyticus endoglucanase E1* in transgenic tobacco: biochemical characteristics and physiological effects. Transgenic Res 9:43-54. doi: 10.1023/a:1008922404834

Daniell H, Streatfield SJ, Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends in Plant Sci 6:219-226. doi: 10.1016/s1360-1385(01)01922-7

Deblaere R, Bytebier B, De Greve H, Deboeck F, Schell J, van Montagu M, Leemans J (1985) Efficent octopine Ti plasmid derived vectors for *Agrobacterium*-mediated gene transfer in plants. Nucleic Acids Res 13:4777-4785

Desai PN, Shrivastava N, Padh H (2010) Production of heterologous proteins in plants: Strategies for optimal expression. Biotechnol Adv 28:427-435. doi: 10.1016/j.biotechadv.2010.01.005

Hackel A, Schauer N, Carrari F, Fernie AR, Grimm B, Kuhn C (2006) Sucrose transporter LeSUT1 and LeSUT2 inhibition affects tomato fruit development in different ways. Plant J 45:180-192. doi: 10.1111/j.1365-313X.2005.02572.x

He ZM, Jiang XL, Qi Y, Luo DQ (2008) Assessment of the utility of the tomato fruit-specific E8 promoter for driving vaccine antigen expression. Genetica 133:207-214. doi: 10.1007/s10709-007-9201-2

Hirai T, Kim YW, Kato K, Hiwasa-Tanase K, Ezura H (2011a) Uniform accumulation of recombinant miraculin protein in transgenic tomato fruit using a fruit-ripening-specific E8 promoter. Transgenic Res 20:1285-1292. doi: 10.1007/s11248-011-9495-9

Hirai T, Kurokawa N, Duhita N, Hiwasa-Tanase K, Kato K, Ezura H (2011b) The HSP terminator of *Arabidopsis thaliana* induces a high level of miraculin accumulation in transgenic tomatoes. J Agric Food Chem 59:9942-9949. doi: 10.1021/jf202501e

Hiwasa-Tanase K, Nyarubona M, Hirai T, Kato K, Ichikawa T, Ezura H (2011) High-level accumulation of recombinant miraculin protein in transgenic tomatoes expressing a synthetic miraculin gene with optimized codon usage terminated by the native miraculin terminator. Plant Cell Rep 30:113-124. doi: 10.1007/s00299-010-0949-y

Jiang XL, He ZM, Peng ZQ, Qi Y, Chen Q, Yu SY (2007) Cholera toxin B protein in transgenic tomato fruit induces systemic immune response in mice. Transgenic Res 16:169-175. doi: 10.1007/s11248-006-9023-5

Kato K, Yoshida R, Kikuzaki A, Hirai T, Kuroda H, Hiwasa-Tanase K, Takane K, Ezura H, Mizoguchi T (2010) Molecular breeding of tomato lines for mass production of miraculin in a plant factory. J Agric Food Chem 58:9505-9510. doi: 10.1021/jf101874b

Kim YW, Kato K, Hirai T, Hiwasa-Tanase K, Ezura H (2010a) Spatial and developmental profiling of miraculin accumulation in transgenic tomato fruits expressing the miraculin gene constitutively. J Agric Food Chem 58:282-286. doi: 10.1021/jf9030663

Kim YW, Hirai T, Kato K, Hiwasa-Tanase K, Ezura H (2010b) Gene dosage and genetic background affect miraculin accumulation in transgenic tomato fruits. Plant Biotechnol 27:333-338. doi: 10.5511/plantbiotechnology.27.333

Kurihara K, Beidler LM (1968) Taste-modifying protein from miracle fruit. Science 161:1241-1243.

Leclercq J, Ranty B, Sanchez-Ballesta MT, Li ZG, Jones B, Jauneau A, Pech JC, Latche A, Ranjeva R, Bouzayen M (2005) Molecular and biochemical characterization of LeCRK1, a ripening-associated tomato CDPK-related kinase. J Exp Bot 56:25-35. doi: 10.1093/jxb/eri003

Matsuoka M, Tada Y, Fujimura T, Kanomurakami Y (1993) Tissue-specific light-regulated expression directed by the promoter of a C4 gene, mayze pyruvate, orthophosphate dikinase, in a C3 plant, rice. Proc Natl Acad Sci USA 90:9586-9590. doi: 10.1073/pnas.90.20.9586

Matsuura H, Shinmyo A, Kato K (2008) Preferential translation mediated by Hsp81-3 5 '-UTR during heat shock involves ribosome entry at the 5 '-end rather than an internal site in arabidopsis suspension cells. J Biosci Bioeng 105:39-47. doi: 10.1263/jbb.105.39

Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y, Ohashi Y (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. Plant Cell Phyiol. 37:49-59.

Nagaya S, Kawamura K, Shinmyo A, Kato K (2010) The HSP terminator of *Arabidopsis thaliana* increases gene expression in plant cells. Plant and Cell Physiol 51:328-332. doi: 10.1093/pcp/pcp188

Outchkourov NS, Peters J, de Jong J, Rademakers W, Jongsma MA (2003) The promoter-terminator of chrysanthemum rbcS1 directs very high expression levels in plants. Planta 216:1003-1012. doi: 10.1007/s00425-002-0953-8

Rasmussen TB, Donaldson IA (2006) Investigation of the endosperm-specific sucrose synthase promoter from rice using transient expression of reporter genes in guar seed tissue. Plant Cell Rep 25:1035-1042. doi: 10.1007/s00299-006-0158-x

Sandhu JS, Krasnyanski SF, Domier LL, Korban SS, Osadjan MD, Buetow DE (2000) Oral immunization of mice with transgenic tomato fruit expressing respiratory syncytial virus-F protein induces a systemic immune response. Transgenic Res 9:127-135. doi: 10.1023/a:1008979525909

Satoh J, Kato K, Shinmyo A (2004) The 5 '-untranslated region of the tobacco *Alcohol dehydrogenase* gene functions as an effective translational enhancer in plant. J Biosci Bioeng 98:1-8. doi: 10.1016/s1389-1723(04)70234-0

Streatfield SJ (2007) Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 5:2-15. doi: 10.1111/j.1467-7652.2006.00216.x

Sugaya T, Yano M, Sun HJ, Hirai T, Ezura H (2008) Transgenic strawberry expressing the taste-modifying protein miraculin. Plant Biotechnol 25:329-333. doi: 10.5511/plantbiotechnology.25.329

Sugio T, Satoh J, Matsuura H, Shinmyo A, Kato K (2008) The 5 '-untranslated region of the *Oryza sativa* alcohol dehydrogenase gene functions as a

translational enhancer in monocotyledonous plant cells. J Biosci Bioeng 105:300-302. doi: 10.1263/jbb.105.300

Sun HJ, Cui ML, Ma B, Ezura H (2006a) Functional expression of the taste-modifying protein, miraculin, in transgenic lettuce. FEBS Lett 580:620-626. doi: 10.1016/j.febslet.2005.12.080

Sun HJ, Uchii S, Watanabe S, Ezura H (2006b) A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant and Cell Physiol 47:426-431. doi: 10.1093/pcp/pci251

Sun HJ, Kataoka H, Yano M, Ezura H (2007) Genetically stable expression of functional miraculin, a new type of alternative sweetener, in transgenic tomato plants. Plant Biotechnol J 5:768-777. doi: 10.1111/j.1467-7652.2007.00283.x

Theerasilp S, Kurihara Y (1988) Complete purification and chracterization of the taste-modifying protein, miraculin, from miracle fruit. J Biol Chem 263:11536-11539.

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends in Biotechnol 21:570-578. doi: 10.1016/j.tibtech.2003.10.002

Yano M, Hirai T, Kato K, Hiwasa-Tanase K, Fukuda N, Ezura H (2010) Tomato is a suitable material for producing recombinant miraculin genetically stable manner. Plant Sci 178:469-473. doi: 10.1016/j.plantsci.2010.02.016

## Figure captions

**Fig. 1**

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

**Fig. 2**

Miraculin accumulation was analyzed by Western blotting in the $T_0$ generation of E8-MIR-HSP and $T_2$ generation of 35S-MIR-NOS and E8-MIR-NOS transgenic tomato fruits. (A) The miraculin concentration in the transgenic tomato fruits was measured using an enzyme-linked immunosorbent assay (ELISA). (B) Protein was extracted from mature green fruits (G) and red-ripe fruits (R). Total soluble protein samples (5.3 mg) were separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was hybridized with antibodies against miraculin. The numbers under the columns indicate the independent transgenic tomato lines, identified as described in Figure 1.

**Fig. 3**

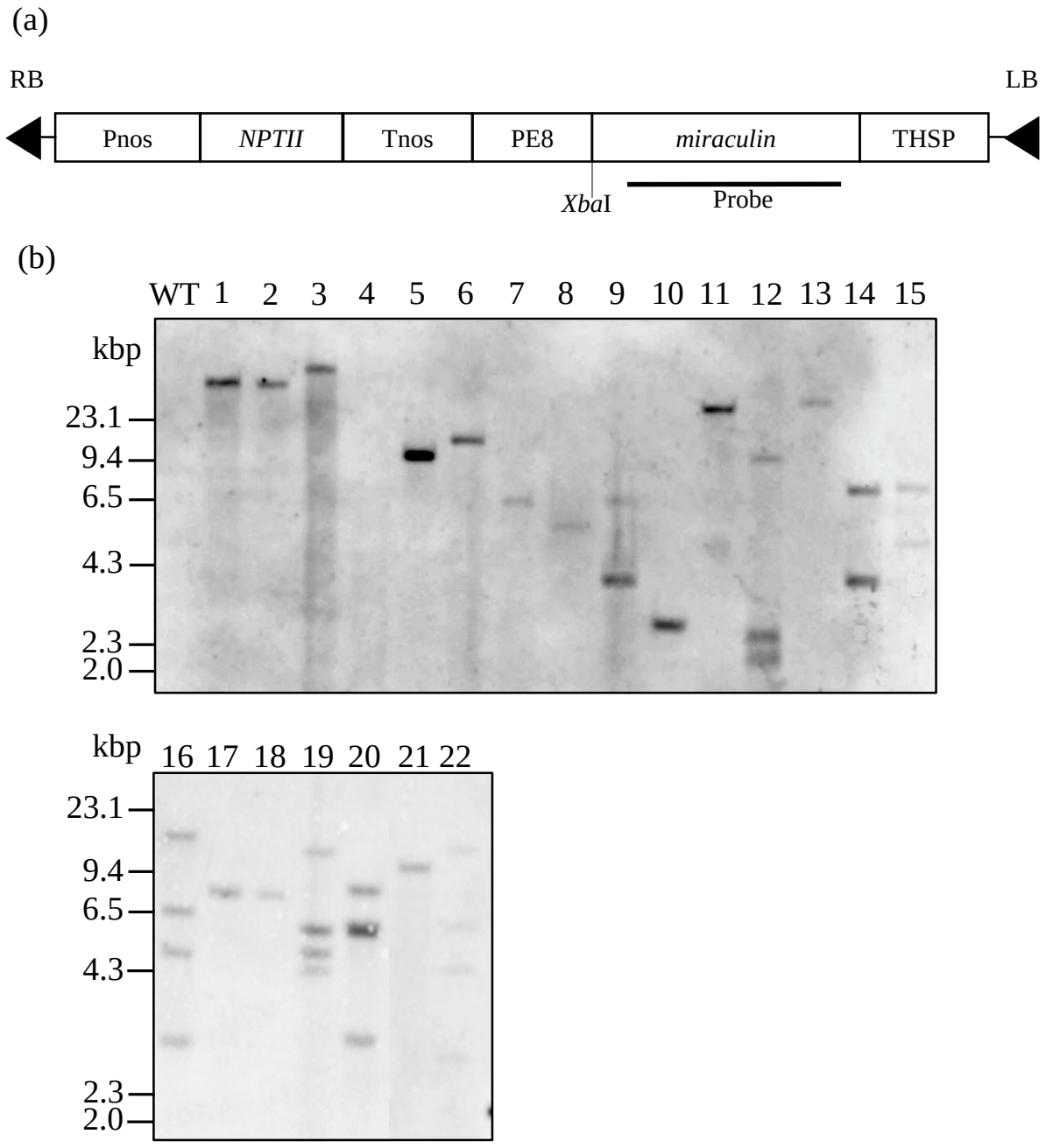
Miraculin mRNA expression level in the mature green fruits (A) and red-ripe fruits (B) of the E8-MIR-HSP ($T_0$), 35S-MIR-NOS ($T_2$) and E8-MIR-NOS ($T_2$) transgenic tomato fruits. The expression levels were determined by quantitative RT-PCR, and the relative quantification was calculated using normalization to

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

Fig. 4

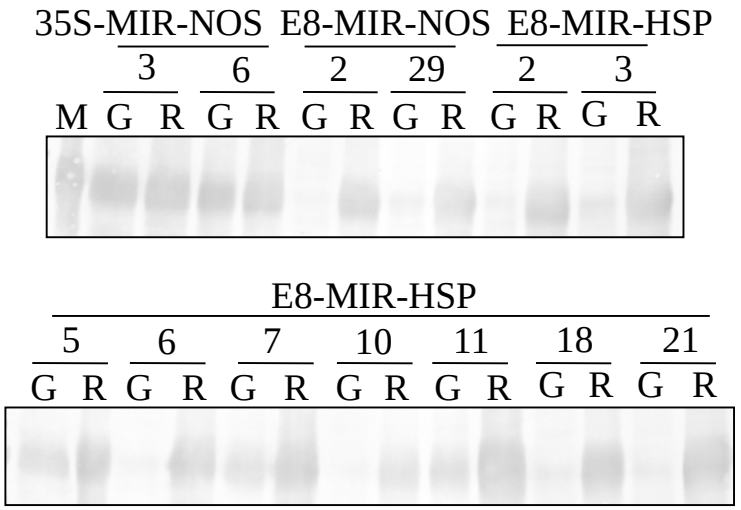
The miraculin concentration in the red-ripe fruit tissue of transgenic tomato lines was analyzed by ELISA. The vertical bars represent the standard deviation for three independent experiments. The numbers under the columns indicate the independent transgenic tomato lines, identified as described in Figure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

# Figure 1

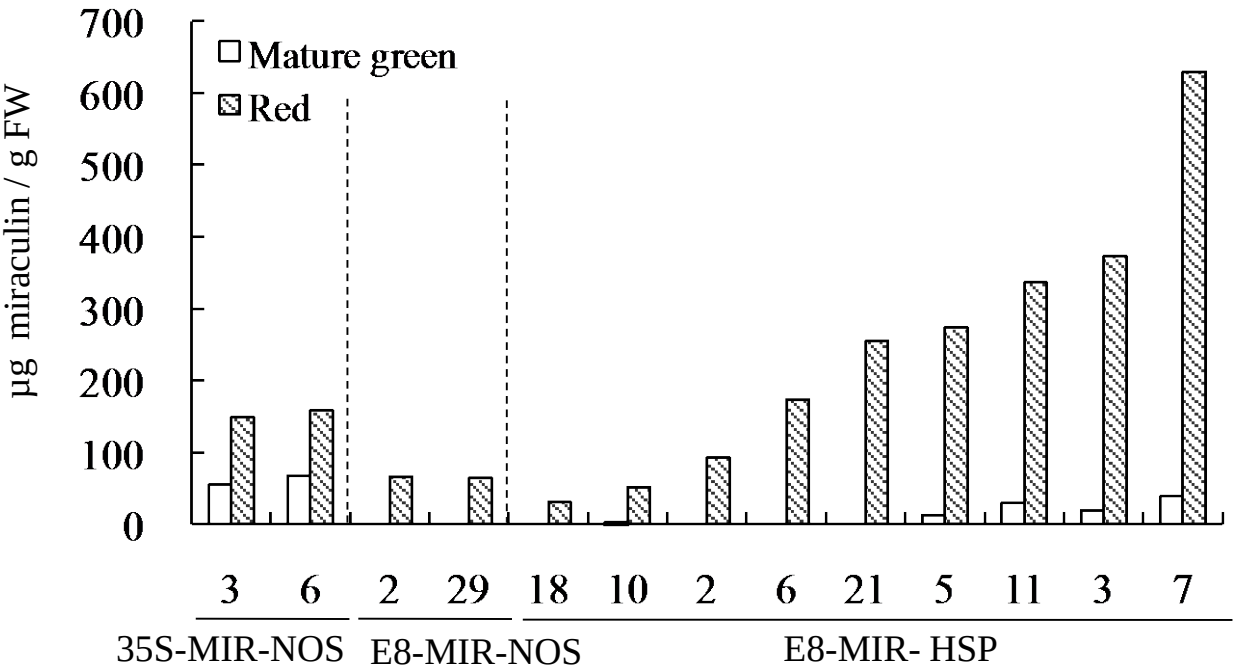

(a)
RB
LB
Pnos
NPTII
Tnos
PE8
miraculin
THSP
XbaI
Probe
(b)
WT 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
kbp
23.1
9.4
6.5
4.3
2.3
2.0
kbp
16 17 18 19 20 21 22
23.1
9.4
6.5
4.3
2.3
2.0

# Figure 2

(a)

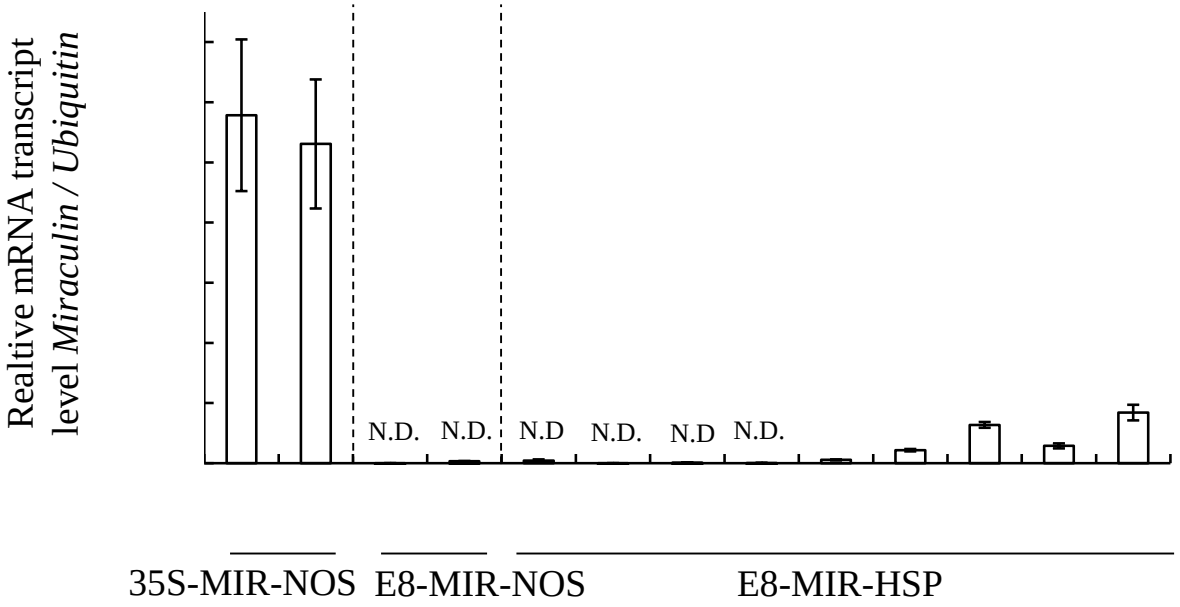

35S-MIR-NOS
E8-MIR-NOS
E8-MIR-HSP
3
6
2
29
2
3
M G R G R G R G R G R G R
E8-MIR-HSP
5
6
7
10
11
18
21
G R G R G R G R G R G R G R

(b)


700
600
500
400
300
200
100
0
μg miraculin / g FW
Mature green
Red
3 6 2 29 18 10 2 6 21 5 11 3 7
35S-MIR-NOS
E8-MIR-NOS
E8-MIR- HSP

# Figure 3

(a)


Realtive mRNA transcript level *Miraculin / Ubiquitin*
N.D.
N.D.
N.D
N.D.
N.D
N.D.
35S-MIR-NOS
E8-MIR-NOS
E8-MIR-HSP

(b)

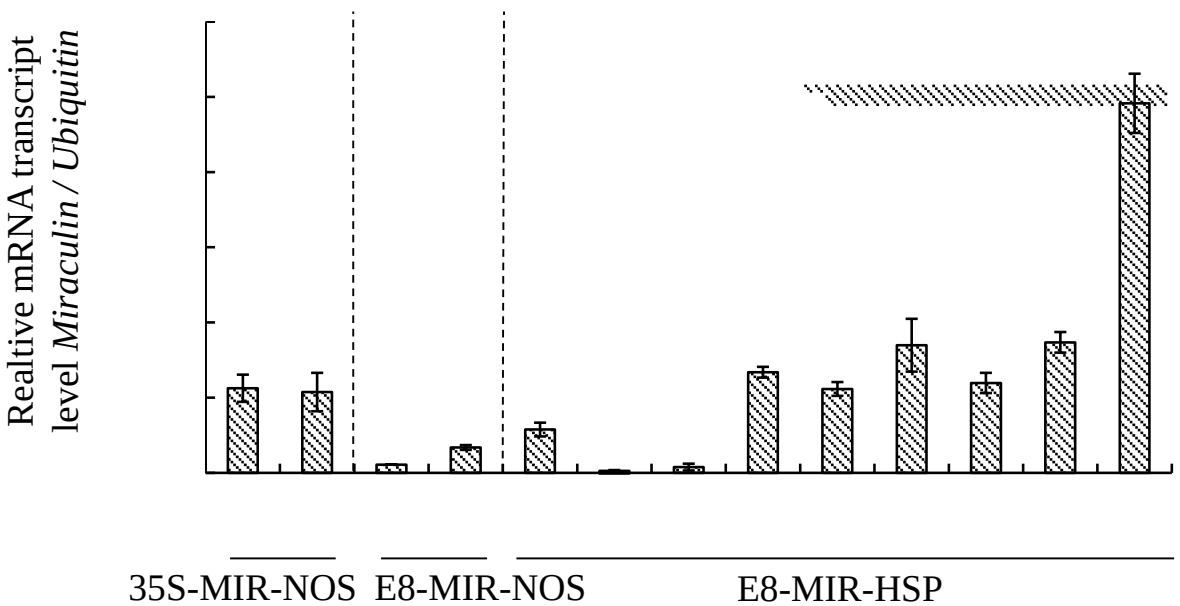
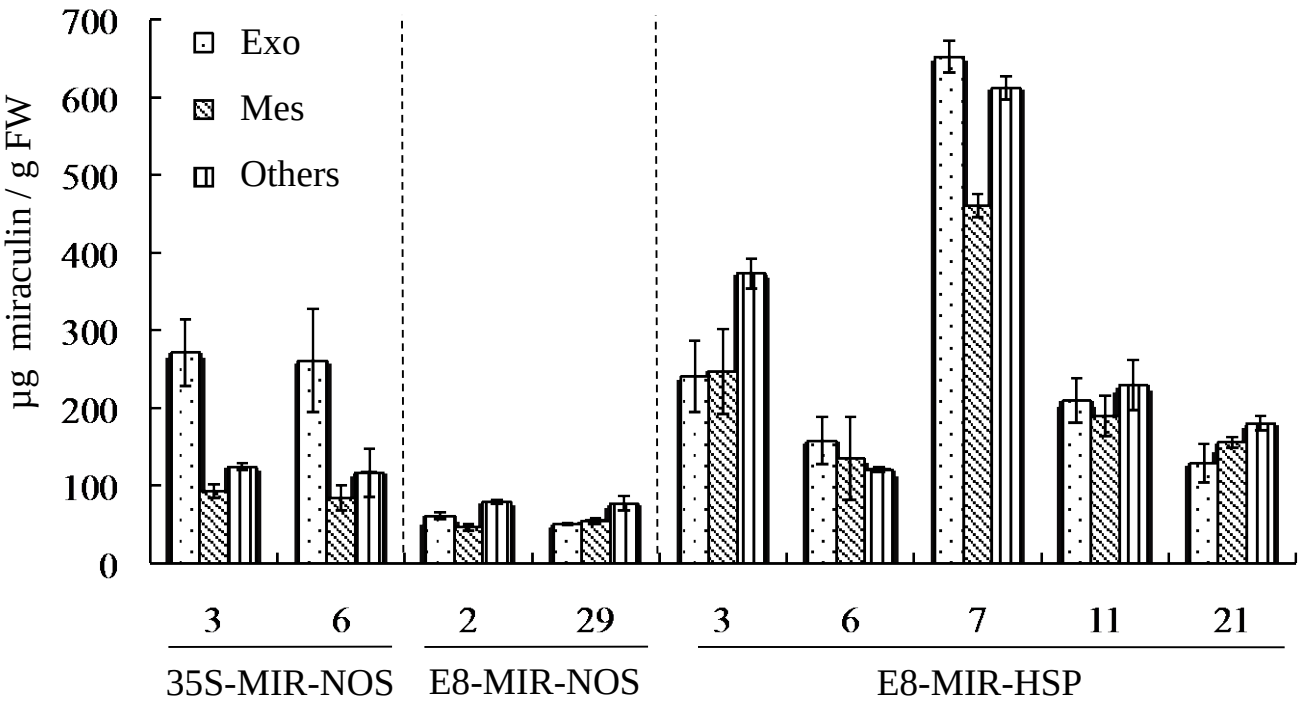

Realtive mRNA transcript level *Miraculin / Ubiquitin*
35S-MIR-NOS
E8-MIR-NOS
E8-MIR-HSP

Figure 4


700
600
500
400
300
200
100
0
µg miraculin / g FW
Exo
Mes
Others
3
6
2
29
3
6
7
11
21
35S-MIR-NOS
E8-MIR-NOS
E8-MIR-HSP