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The HSP Terminator of *Arabidopsis thaliana* Induces a High Level of Miraculin Accumulation in Transgenic Tomatoes

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ABSTRACT: High-level accumulation of the target recombinant protein is a significant issue in heterologous protein expression using transgenic plants. Miraculin, a taste-modifying protein, was accumulated in transgenic tomatoes using an expression cassette in which the miraculin gene was expressed by the cauliflower mosaic virus (CaMV) 35S promoter and the heat shock protein (HSP) terminator (MIR-HSP). The HSP terminator was derived from heat shock protein 18.2 in *Arabidopsis thaliana*. Using this HSP-containing cassette, the miraculin concentration in T_0 transgenic tomato lines was 1.4−13.9% of the total soluble protein (TSP), and that in the T_1 transgenic tomato line homozygous for the miraculin gene reached 17.1% of the TSP. The accumulation level of the target protein was comparable to levels observed with chloroplast transformation. The high-level accumulation of miraculin in T_0 transgenic tomato lines achieved by the HSP terminator was maintained in the successive T_1 generation, demonstrating the genetic stability of this accumulation system.

KEYWORDS: high-level expression, HSP terminator, miraculin, transgenic tomato plants, CaMV 35S promoter

INTRODUCTION

Heterologous protein production systems, such as bacteria, fungi, mammalian and insect cell cultures, and transgenic animals and plants, have excellent potential for the production of target proteins. These protein production systems are required to be low cost and must scale up easily. When compared to other systems, transgenic plants have the potential to provide a more efficient and economical protein production system.[1,2] The large-scale cultivation of transgenic plants is relatively simple, and contamination by human pathogenic microorganisms is easily preventable.[3,4] However, to be adaptable to a variety of uses, for example, the production of enzymes for industrial applications,[5,6] edible vaccines,[7] and pharmaceuticals for administration to humans,[1] increased accumulation of the target protein in transgenic plants is required.

Modification of the expression cassette is one strategy that has been used to increase the expression of the target gene and induce a high level of accumulation of the target protein. Generally, the CaMV 35S promoter/NOS terminator expression cassette has been used in dicots, whereas other promoters such as the petE promoter,[8] the rcbS1 promoter,[9] and the polyubiquitin promoter[10] have also been used to improve target protein production. An alternative approach to increasing the expression of the target gene has been to increase the efficiency of target mRNA translation using the 5′ untranslated region (Ω sequence) of the tobacco mosaic virus[11] and the 5′ untranslated region from the tobacco alcohol dehydrogenase gene.[12] The highly efficient heat shock protein (HSP 18.2) terminator from *Arabidopsis thaliana*[13] and the simian virus 40 late polyadenylation site[14] have also been used in expression cassettes. Nagaya et al.[13] isolated six different terminator sequences from *A. thaliana* and tested their ability to support high expression levels of foreign genes. The results demonstrated that the HSP terminator was highly efficient. It is likely that the HSP terminator is more efficient than the NOS terminator at mRNA 3′ end formation, resulting in higher levels of accumulated mRNA. Matsui et al.[15] used the HSP terminator for the production of the B subunit of Shiga toxin 2e (Stx2eB) in transgenic lettuce and analyzed the protein production in the T_0 generation. This transgenic lettuce accumulated 10−80 mg/100 g fresh weight (FW) Stx2eB, which was significantly higher than the levels observed using the NOS terminator.

The taste-modifying protein, miraculin, was first discovered in the red miracle fruit berry (*Richadella dulcifica*), which is a shrub native to tropical western Africa. The miraculin protein is capable of modifying a sour taste into a sweet taste and can be used as a sweetener and as an alternative to sucrose. Because of its low-calorie properties, miraculin has attracted a great deal of attention in the food industry. However, the mass production of miraculin has met with difficulties because of the low productivity of the miracle fruit itself and the difficulties associated with cultivating the fruit outside tropical regions. Attempts to produce recombinant miraculin in heterologous systems such as *Escherichia coli*,[16] yeast, and tobacco[17] have failed to produce a miraculin that has a taste-modifying activity. Matsuyama et al.[18] succeeded in producing active recombinant miraculin by using the transgenic *E. coli*, but the taste-modifying activity was only 16% that of the native miraculin. Recently, the expression of miraculin with a taste-modifying activity was successful in transgenic lettuce,[19] tomatoes,[20] and strawberries.[21] In these transgenic plants, the miraculin gene was expressed using the CaMV 35S promoter and NOS terminator. In miraculin-accumulating lettuce, the accumulation level

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of the protein was high enough to induce taste-modifying activity in the T_0 generation but was lost in T_1 and successive generations. In miraculin-accumulating strawberries, the miraculin accumulated in vegetatively propagated plants, but the levels were low. In miraculin-accumulating tomatoes, the miraculin accumulation level was sufficient to induce the taste-modifying activity, and miraculin accumulation was observed in generations T_0−T_5.[22]

In a previous study, we used the native miraculin terminator to increase miraculin production in transgenic tomatoes.[23] With this terminator, the miraculin accumulation level in the transgenic tomato fruit was 197 μg/g FW, on average, and it was higher than the level obtained (131 μg/g FW) using the NOS terminator. This study indicated that modification of the terminator in the expression cassette induced a high level of miraculin accumulation.

In the present study, to establish a high level of miraculin accumulation in transgenic tomatoes, we tested a modified expression cassette in which the miraculin gene was expressed using the CaMV 35S promoter and the HSP terminator. We analyzed the miraculin concentration and accumulation patterns in the transgenic tomato fruit and examined the genetic stability of the high level of miraculin accumulation in the T_0 and T_1 generations.

MATERIALS AND METHODS

Plasmid Construction. To construct the miraculin (AB512278) expression binary vector containing the HSP gene (NM125364) terminator, the HSP terminator plasmid, which was a pBI221-based vector (HSP-pBI221),[13] was digested with *Sac*I and *Eco*RI. The digested HSP terminator fragment (252 bp) was isolated and used to replace the NOS terminator in the 35S-MIR-NOS plasmid[19,23] between the *Sac*I and *Eco*RI sites.

Plant Material and Transformation of Tomato Plants. The newly constructed plasmid (MIR-HSP), in which the miraculin gene is expressed using the CaMV 35S promoter and terminated using the HSP terminator, was transferred into *Agrobacterium tumefaciens*, strain GV2260.[24] Using *A. tumefaciens* harboring the binary vector MIR-HSP, tomato (*Solanum lycopersicum* cv. Micro-Tom) plants were transformed as described by Sun et al.[20,25] The kanamycin-resistant tomato plants were acclimatized on Rockwool cubes and cultivated at 25 °C in a growth room under a 16 h light/8 h dark photoperiod of fluorescent light at an intensity of 60 μmol/m^2/s. The putative transformants were watered with Otsuka-A nutrient solution (Otsuka Chemical Co., Ltd., Osaka, Japan). After growth in the growth room, to select the miraculin-accumulating transgenic tomato, the total soluble protein was extracted from the leaves of the putative transformants using a protein extraction buffer [(20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 2% polyvinylpolypyrrolidone), and the solution was centrifuged at 16200*g* for 20 min at 4 °C. The extracted protein was subjected to protein gel blot analysis to identify the miraculin-accumulating transgenic tomato plants. Protein gel blot analyses were performed in accordance with the methods described by Sun et al.[20] Miraculin-accumulating transgenic tomatoes were selected and cultivated in a growth room under the same conditions as described above. The seeds of the successive generation (T_1) were collected from the T_0 generation and grown in a closed cultivation system (Naeterasu, Taiyo Kogyo Co., Ltd., Tokyo, Japan) as described by Hirai et al.[26]

We compared the accumulation levels of miraculin in transgenic tomatoes expressing MIR-HSP to the miraculin accumulation levels in transgenic tomatoes expressing the miraculin gene driven by the CaMV 35S promoter and the NOS terminator (MIR-NOS).[23] For these experiments, seven transgenic lines of MIR-NOS-expressing tomato plants from the T_0 generation (1−7), each containing a single copy of the miraculin gene, and three lines of the T_1 generation of transgenic tomato plants (1, 3, and 6) that were homozygous for the miraculin gene were used.

Southern Blot Analysis of the T_0 Generation of Transgenic Tomato Plants. To confirm the copy number of the miraculin gene in transgenic tomato plants expressing MIR-HSP, genomic DNA was extracted from 0.2 g of fresh young leaves using Maxwell 16 DNA purification kits consistent with the instructions of the manufacturer (Promega, Fitchburg, WI). Isolated genomic DNA (10 μg) was digested with *Xba*I, electrophoresed on an 0.8% agarose gel at 50 V for 3 h, and transferred to a Hydrond-N+ nylon membrane (GE Healthcare, Little Chalfont, U.K.). The membrane was hybridized overnight at 60 °C in high-SDS buffer (50% deionized formamide (v/v), 5× SSC, 7% SDS, 2% blocking reagent (Roche Diagnostics, Mannheim, Germany), 50 mM sodium phosphate (pH 7.0), and 0.1% *N*-lauroylsarcosine sodium salt (w/v)) containing a miraculin-specific DIG-labeled probe at 45 °C. The miraculin-specific probe (663 bp) was generated from 35S-MIR-NOS plasmid by labeled digoxigenin (DIG) using the PCR DIG Probe Synthesis kit (Roche Diagnostics, Mannheim, Germany) and the following set of primers: 5′-ATGAAGGAATTAACAATGC-TCTCTC-3′ and 5′-TTAGAAGTATACGGTTTTGTTGAAC-3′. The hybridization signals were detected using an LAS4000 mini Image Analyzer (Fujifilm Co. Ltd., Tokyo, Japan).

Analysis of Zygosity in the T_1 Generation of Transgenic Tomato Plants. To select transgenic tomato plants in the T_1 generation that were homozygous for MIR-HSP or MIR-NOS, the zygosity of transgenes in transgenic tomato plants was analyzed by real-time PCR using genomic DNA as template according to the methods of Manson et al.[27] and German et al.[28] Genomic DNA was extracted from T_1 transgenic tomato plant leaves as described above. Isolated genomic DNA (30 ng) was used as a template for quantitative real-time PCR (RT-PCR) amplification using the Thermal Cycler Dice Real Time System TP800 (Takara-Bio Inc. Otsu, Japan) using SYBR Premix Ex Taq (Takara-Bio Inc.). The cycling parameters were at 95 °C for 30 s to denature, followed by 40 cycles of 5 s of denaturation at 95 °C and 30 s of annealing/extension at 60 °C. The RT-PCR reaction was performed using miraculin-specific primers. Ubiquitin 3-specific primers, which are known to amplify a single copy of the ubiquitin gene,[29] were used as an internal control to calibrate the miraculin gene copy number. The primer sequences were as follows: miraculin forward, 5′-CACCCAATCCGGT-TCTTGAC-3′; miraculin reverse, 5′-GTGGTGGCGGATACTGTAA-GG-3′; ubiquitin 3 forward, 5′-CACCAAGCCAAAGAAGATCA-3′; and ubiquitin 3 reverse, 5′-TCAGCATTAGGGCACTCCTT-3′.

Tissue Separation of Transgenic Tomato Fruits. To measure miraculin accumulation levels in different parts of the red-ripe fruit of transgenic tomatoes, fruit from transgenic tomato plants homozygotically expressing the miraculin gene was separated into three tissues: exocarp tissue, mesocarp tissue, and other tissue that included dissepiment, placenta, and jelly. Tissues were separated using methods described by Kato et al.[30] and Kim et al.[31]

Protein Extraction, Protein Gel Blot Analysis, and Enzyme-Linked Immunosorbent Assay (ELISA). Miraculin accumulation levels in transgenic tomato fruits were determined by protein gel blot analysis and ELISA. Separated pericarp tissue isolated from transgenic tomato fruits, including the exocarp and the mesocarp, was ground into a fine powder in liquid nitrogen. The powder (0.1 g) was resuspended in 200 μL of 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 16200*g* for 20 min at 4 °C. The supernatant was used for protein gel blot analysis and ELISA. The extracted proteins (1.6 mg of fresh weight equivalents per lane) were separated by SDS−polyacrylamide gel electrophoresis (PAGE) and transferred onto Hybond-P polyvinylidene fluoride membranes (GE Healthcare Ltd., Amersham, Buckinghamshire, U.K.). The blots were reacted with affinity-purified anti-miraculin antibody,[20]

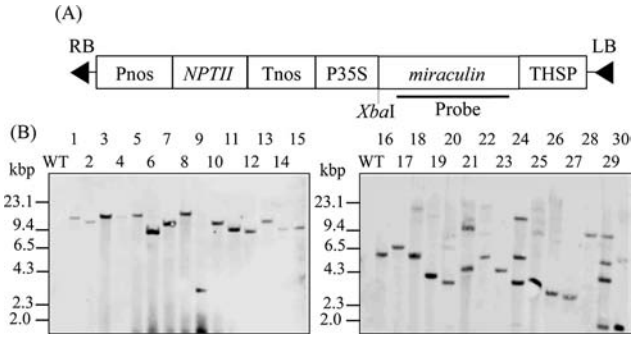

Figure 1. Map of the T-DNA region in the binary vectors (A) and DNA gel blot analysis of the tomato transformants (B). RB, right border of the T-DNA; LB, left border of the T-DNA; Pnos, nopaline synthase gene promoter; NPTII, neomycin phosphotransferase gene; Tnos, nopaline synthase gene terminator; P35S, CaMV 35S promoter; THSP, heat shock protein terminator; miraculin, miraculin gene. Genomic DNA from 30 transgenic lines and a wild-type tomato plant (WT) was used for DNA gel blot analysis. The miraculin coding sequences were used as probes.

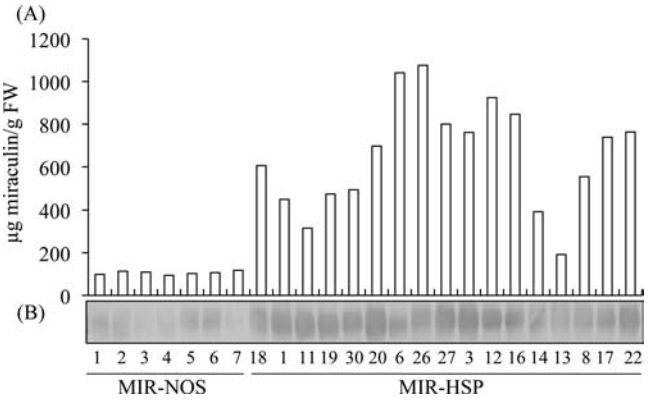

Figure 2. Miraculin concentration of T_0 transgenic tomato fruits measured using an enzyme-linked immunosorbent assay (ELISA). (A) Miraculin accumulation was analyzed by protein gel blotting in the T_0 transgenic tomato fruit. (B) Sample protein was extracted from 1.6 mg of fresh red-ripe fruit of T_0 transgenic tomatoes. The samples were separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was hybridized with antibodies to miraculin. The numbers under the columns show the independent transgenic tomato lines identified as described in Figure 1.

followed by incubation with anti-rabbit immunoglobulin G (IgG) coupled to horseradish peroxidase. Immunoreactive signals were detected using a Peroxidase Stain Kit for Immuno-blotting (Nakalai Tesque, Inc., Kyoto, Japan), according to the manufacturer's instructions. The concentration of miraculin was determined by ELISA, as described by Kim et al.[31]

Isolation of mRNA and Quantitative Reverse-Transcriptase Polymerase Chain Reaction (Real-Time PCR). The mRNA expression levels of miraculin in transgenic tomato fruits were determined by RT-PCR. Total RNA was isolated from the red-ripe fruit using an RNeasy plant mini kit (Qiagen, Hilden, Germany). cDNA was synthesized from 0.8 μg of total RNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). cDNA (1 μL) was diluted in 10 μL of TE buffer, and 1 μL of this diluted cDNA was used for RT-PCR amplification with the Thermal Cycler Dice Real Time System TP800 (Takara-Bio Inc.) using SYBR Premix Ex Taq (Takara-Bio Inc.). The cycling parameters were at 95 °C for 10 min to denature, followed by 40 cycles at 95 °C for 30 s to denature, followed by 40 cycles of 5 s of denaturation at 95 °C and 30 s of annealing/extension at 60 °C. The RT-PCR reaction was performed using miraculin-specific primers, and ubiquitin 3-specific primers were used as a control.[32,33] Primer sequences were described above.

Purification of Miraculin from Transgenic Tomato Fruits Expressing MIR-NOS and MIR-HSP. For the purification of miraculin, transgenic tomato fruits from the T_1 generation were used. Transgenic tomato fruit pericarps (5 g) were ground into a fine powder in liquid nitrogen, and this powder was homogenized in 20 mL of distilled water for washing of soluble proteins and centrifuged at 13400*g* for 20 min. The supernatant was discarded, and the washing process was repeated. The pellet was resuspended with 10 mL of extraction buffer (0.5 M NaCl, 20 mM Tris-HCl, pH 7.2). After centrifugation at 12000 rpm for 20 min, the supernatant was purified using an immobilized metal-affinity chromatography (IMAC) column (bed volume, 1 mL; HiTrap IMAC HP, GE Healthcare) and ion exchange chromatography column (bed volume, 1 mL; HiTrap CM FF, GE Healthcare) according to the method of Duhita et al.[34,35]

RESULTS

Transforming Tomato Plants with the Miraculin Gene. Tomato cotyledons were transformed by infection with the *A. tumefaciens* strain GV2260[24] containing the binary vector MIR-HSP (Figure 1A). In this construct, miraculin gene expression is driven by the constitutive CaMV 35S promoter and terminated by the HSP terminator. Putative transgenic tomato lines (115) were rooted on selection medium and examined for miraculin accumulation in the leaves by protein gel blot analysis; 30 transgenic lines that tested positive for accumulating miraculin were selected. The transgene copy number in these 30 lines was determined by Southern blot analysis (Figure 1B). Genomic DNA from each plant, including an untransformed control, was digested with *Xba*I endonuclease, which cuts the T-DNA in the MIR-HSP plasmid at a single site outside the miraculin gene. In most cases, the number of bands reflects the number of transgenes. The hybridization of restricted genomic DNA for the miraculin gene revealed that the clones represented independent transformation events, and the number of transgene copies varied from one to several among different transformed lines. Multiple copies of the miraculin gene were detected in 4 transgenic lines, and 26 transgenic lines harbored a single copy of miraculin gene. Among the 26 transgenic lines, 9 lines (lines 1, 3, 4, 5, and 8 were cloned, lines 7 and 10 were cloned, and lines 6 and 11 were cloned) showed a band at the same position as in other lines, indicating a clonal origin. Finally, 17 independent transgenic tomato lines were selected and used for the experiments (Figure 1B).

Miraculin Accumulation in T_0 Transgenic Tomato Fruits. The 17 transgenic lines selected in the Southern blot experiments were grown in a cultivation room. These transgenic tomatoes showed the normal growth phenotype in appearance compared with the wild type tomato. To detect the miraculin accumulation, the pericarp from the red fruit of each transgenic tomato line was analyzed by protein gel blot analysis. The bands observed in the transgenic plants using the HSP terminator (MIR-HSP) were much stronger than those in the control line using the NOS terminator (MIR-NOS). The concentration of recombinant miraculin was measured using ELISA (Figure 2A). The results of the protein gel blot analysis reflected those of the ELISA with respect to the miraculin levels. The miraculin concentration in tomato fruit of MIR-HSP lines ranged from 191.5 to 1075.7 μg/g FW, which was equivalent to 1.4−13.9% of total soluble protein

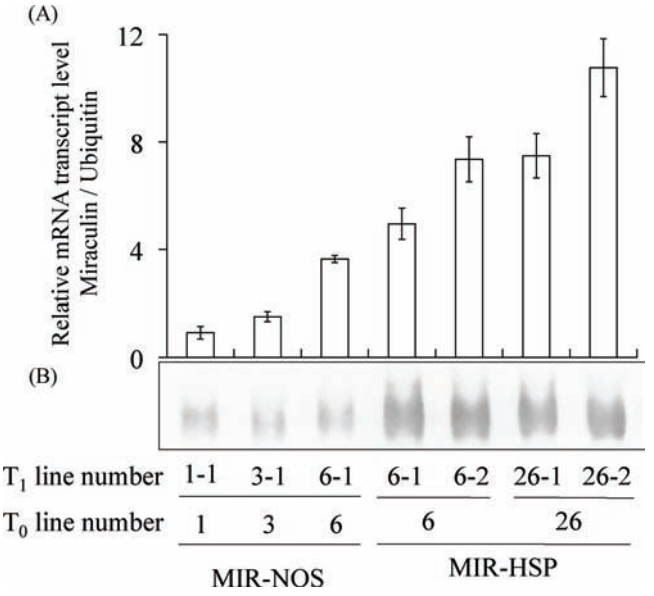

Figure 3. Miraculin mRNA expression in red-ripe fruit from the T_1 generation of transgenic plants measured using real-time quantitative PCR analysis (A). Vertical bars show the standard deviation from three independent experiments. Miraculin accumulation was analyzed by protein gel blotting (B). The sample protein was extracted from 1.6 mg of fresh red-ripe fruit of T_1 transgenic tomatoes. The samples were separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was hybridized with antibodies to miraculin. The numbers under the columns show the independent transgenic tomato lines identified as described in Figure 1.

(TSP), and that of MIR-NOS lines ranged from 93.8 to 117.5 μg/g FW, which was equivalent to 0.8−1.5% of TSP. The average concentration of miraculin in MIR-HSP expressing plants (654 μg/g FW) was 6.5-fold higher than that in the MIR-NOS expressing plants (106 μg/g FW). The maximum accumulation observed was 1075.7 μg/g FW of miraculin in MIR-HSP transgenic tomato line 26.

Miraculin Gene Expression and Protein Accumulation in T_1 Transgenic Tomato Fruits. To analyze the stability of miraculin accumulation in successive generations, we chose MIR-HSP lines 6 and 26. Both lines accumulated high levels of miraculin, and T_1 generation seeds were harvested from the T_0 generation plant. We also harvested T_1 generation seeds from the MIR-NOS lines 1, 3, and 6. In the T_1 generation of the transgenic tomato plants, 12 seeds from each transgenic line were sown and cultivated. In all transgenic lines, transgenic tomato plants homozygously expressing the miraculin gene were selected using genomic qRT-PCR and used for the following experiments.

Miraculin gene expression and protein accumulation in pericarp from T_1 transgenic tomatoes that expressed each promoter−terminator cassette were measured by RT-PCR and protein gel blot analysis, respectively (Figure 3). The miraculin expression levels in the MIR-HSP lines were higher than those in the MIR-NOS lines (Figure 3A). A band that represented miraculin was detected in all of the transgenic lines; however, the band was clearer in the MIR-HSP transgenic line than in the MIR-NOS transgenic line (Figure 3B). Miraculin accumulation in the T_1 generation of each transgenic line was measured by ELISA. The miraculin concentration in the MIR-HSP lines (955.0−1725.8 μg/g FW) was higher than that in the MIR-NOS lines (102.4−176.4 μg/g FW) (Table 1). The average concentration of miraculin in MIR-HSP expressing plants (1236.0 μg/g FW) was 8.4-fold higher than that in the MIR-NOS expressing plants (146.3 μg/g FW) (Table 1). The total soluble protein (TSP) concentration in the T_1 plants was similar to each transgenic line. That is, the miraculin concentration in the MIR-HSP tomato lines ranged from 7.07 to 17.11% of the TSP and that in the MIR-NOS tomato lines ranged from 1.02 to 1.59% of the TSP.

Spatial Profiling of Miraculin Accumulation in Transgenic Tomato Fruit. In our previous studies, the miraculin accumulation pattern of each tissue in tomato fruit expressing the MIR-NOS cassette was analyzed, and the miraculin accumulation level in the exocarp was found to be much higher than that in other tissues.[31,36] Using the MIR-HSP expression cassette, miraculin accumulation levels in the pericarp increased compared with the MIR-NOS expression cassette (Figure 2 and Table 1). To measure miraculin accumulation levels in different parts of the red-ripe fruit in MIR-HSP-expressing tomatoes, the fruit was separated into three parts: exocarp, mesocarp, and other tissues that included dissepiment, placenta, and jelly. Each tissue was analyzed for miraculin accumulation using ELISA. In transgenic tomato lines expressing HSP-MIR, the miraculin concentration in the exocarp (approximately 1200 μg/gFW) was higher than that in mesocarp (approximately 500 μg/gFW) and in other tissues (approximately 700 μg/gFW) (Figure 4). In transgenic tomato lines expressing MIR-NOS, the miraculin accumulation patterns in the three tissue types were similar to those in the MIR-HSP expressing lines; however, the miraculin accumulation level was very low (Figure 4).

Purification of Recombinant Miraculin from Transgenic Tomato Fruits. To compare the production of purified recombinant miraculin from transgenic tomato plant lines expressing MIR-NOS and MIR-HSP, recombinant miraculin was purified from the pericarp of tomato fruits from each transgenic line (Table 2). Prior to being purified with a nickel column, the miraculin concentration was determined by ELISA using the supernatant after centrifugation during the extraction steps, wherein the highest miraculin concentration (173.8 μg/mL) was obtained using MIR-HSP line 6. After purification on a nickel column, the miraculin concentration was 76.2 μg/mL and was still highest in MIR-HSP transgenic line 6. The recovery rate was calculated by comparing the miraculin concentration in the sample before and after extraction on the nickel column, and it was found to be highest (62%) in MIR-HSP transgenic line 26. These results demonstrate that the purification of miraculin using a nickel column improved the recovery rate of miraculin in transgenic tomato cell lines exhibiting high levels of miraculin accumulation.

DISCUSSION

Effective selection of transgenic plants accumulating a target protein is a necessary technique for the commercial production of transgenic plants. In general, putative transgenic plants rooted in selection medium are first subjected to genomic PCR selection, but the genomic PCR cannot distinguish the copy number in the transgenic plants. In this study, first we obtained 115 plants after rooting on selection medium and selected 30 transgenic tomato plants expressing miraculin by protein gel blot analysis. Among these plants, 26 transgenic tomato plant lines had a single copy of the transgene. The ratio (26/30) of transgenic lines containing a single copy of the gene to all transgenic lines produced was much higher than that observed in previous studies.[20] Previous

Table 1. Concentration of Miraculin in T_1 Transgenic Tomatoes with MIR-NOS and MIR-HSP

construct:	MIR-NOS			MIR-HSP			
T_0 line:	1	3	6	6		26	
T_1 line:	1-1	3-1	6-1	6-1	6-2	26-1	26-2
μg miraculin/g fresh weight[a]	102.4	160.2	176.4	1068.8	955.0	1725.8	1194.5
mg total soluble protein/g fresh weight[b]	10.1	10.0	13.8	10.6	13.5	10.1	12.3
μg miraculin/mg total soluble protein	10.2	15.9	12.8	101.0	70.7	171.1	96.8

[a] Miraculin concentration in protein extracts obtained from red-ripe transgenic tomato fruit with MIR-NOS and MIR-HSP determined using ELISA. [b] Total soluble protein concentrations determined using a BCA Protein Assay Kit (Pierce, Rockford, IL).

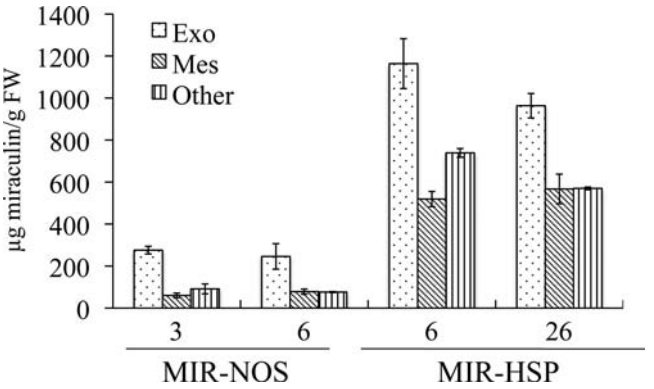

Figure 4. Miraculin concentration in the fruit tissue of transgenic tomato lines as analyzed by ELISA. Vertical bars show the standard error from three independent experiments. Exo, exocarp; Mes, mesocarp; Other, dissepiment, placenta, and jelly tissue.

studies produced many transformants containing multiple copies of the transgene, and these multiple-copy transformants demonstrated low levels of transgene expression or showed silencing of the transgene.[37,38] Single-copy transgenic plants tend to exhibit more stable expression of the foreign gene than plants expressing multiple copies of the gene.[39] We obtained a high ratio of single-copy transgenic tomato plant lines using protein gel blot analysis to select miraculin-accumulating transgenic tomato plants, and this selection method increased the likelihood of obtaining single-copy transgenic tomato plants as confirmed by Southern blot analysis. If an antibody against the target protein is available, a first round of selection by protein gel blotting is a practical method for improving the selection of transgenic lines that successfully accumulate the target protein.

In this study, the miraculin concentration in the MIR-HSP transgenic tomato lines was 71−171 μg miraculin/mg total soluble protein (7−17% TSP). This accumulation level of recombinant protein is much higher than that generally observed in the production of recombinant proteins using nuclear transformation.[1] In previous studies, the concentration of recombinant protein obtained using nuclear transformation did not exceed 1−3% (TSP),[40−42] whereas that obtained using chloroplast transformation was 5−25% (TSP).[1,42,43] In this study, we used nuclear transformation to generate the miraculin-accumulating transgenic tomato plants, and the miraculin concentration obtained in the transgenic tomato plants using the MIR-HSP cassette was similar to that obtained via chloroplast transformation. The miraculin concentration in transgenic tomato plants when using the MIR-NOS cassette was 1−1.5% (TSP), and this concentration was the same as that observed in previous studies.[40−42] Our results indicate that the HSP terminator increases miraculin gene expression and protein accumulation to levels comparable to that of chloroplast transformation. The stability of the mRNA expressed from the foreign gene is important for promoting high expression of the gene.[44] Nagaya et al.[13] concluded that the HSP terminator may be more efficient than the NOS terminator for the formation of the 3′ end of the mRNA, and this may contribute to the accumulation of higher levels of mRNA. The higher miraculin concentration observed in transgenic tomato using the MIR-HSP cassette was achieved because of the accuracy of mRNA 3′ end formation induced by the HSP terminator.

Subcellular localization of the foreign protein is important for high-level accumulation of recombinant proteins in transgenic plants. In previous studies, many types of foreign protein were targeted to different subcellular sites (e.g., vacuole, cell surface, endoplasmic reticulum, nucleus, plastids and cytoplasm),[45−47] but high protein accumulation at each site was different for each foreign protein. Because the chemical environments (e.g., pH) of subcellular sites differ and the chemical properties (e.g., PI) of foreign proteins also differ, the relationship between the chemical environment of the subcellular site and the chemical properties of a foreign protein may play a crucial role in the high-level accumulation of foreign proteins. Miraculin is secreted, and it accumulates in intercellular spaces in the miracle fruit and in transgenic tomatoes.[48] The native miraculin concentration in the pericarp of the miracle fruit is approximately 400 μg/g FW[30] and approximately 10% of the TSP (data not shown). The miraculin concentration in transgenic tomatoes expressing the MIR-HSP cassette was almost the same as the concentration found in the miracle fruit. Therefore, the intercellular space is a suitable environment for miraculin accumulation in transgenic tomatoes.

Gene silencing is a serious problem that hinders the production of heterologous proteins in transgenic plants. Gene silencing may be positively correlated with the level of expression of the transgene; that is, transgenes expressed by strong constitutive promoters are more likely to be silenced. The use of tissue-specific promoters or weak constitutive promoters can reduce the problem of gene silencing.[4] In this study, the miraculin concentration was very high in transgenic tomato plants expressing the MIR-HSP cassette in the T_0 generation. We promoted the generation of transgenic tomato plants and analyzed the stability of miraculin gene expression and protein accumulation in the T_1 generation. The miraculin gene was expressed in all transgenic lines in the T_1 generation, and the miraculin concentration was slightly higher in the T_1 generation than in the T_0 generation. Although very high miraculin gene expression was detected in transgenic tomato plants expressing the MIR-HSP

Table 2. Miraculin Purification from Miraculin Accumulating Tomato Fruit Expressing MIR-NOS and MIR-HSP[a]

transgenic line		before purification[b] (μg/mL)		after purification[c] (μg/mL)		recovery rate[d] (%)
		total soluble protein	miraculin	total soluble protein	miraculin	
MIR-NOS	3	197.5	34.9	13.9	10.2	29%
	6	206.3	31.2	12.6	7.4	24%
MIR-HSP	6	255.4	173.8	81.9	76.2	45%
	26	208.8	113.3	70.4	69.6	62%

[a] Miraculin concentrations determined using the enzyme-linked immunosorbent assay (ELISA). [b] Miraculin concentration in protein extracts from transgenic tomato fruit pericarps before purification using an immobilized metal-affinity chromatography (IMAC) column. [c] Miraculin concentration after purification using an immobilized metal-affinity chromatography (IMAC) column and ion exchange chromatography. [d] Recovery rate calculated from the data in footnotes *b* and *c*.

cassette, gene silencing did not occur in the T_1 generation. Kim et al.[49] demonstrated that homozygotes of the miraculin gene exhibited higher accumulation of miraculin than heterozygotes because gene dosage affected miraculin gene expression and accumulation of miraculin. The results of this study are similar to those of a previous study[49] in that miraculin accumulation levels increased in the T_1 homozygous transgenic lines compared with the T_0 transgenic lines.

The exocarp is removed during processing of tomatoes into products such as tomato juice, ketchup, puree, and paste. For this reason, the uniform accumulation of miraculin in fruit tissue is desirable. In a previous study, we analyzed the miraculin accumulation pattern in the fruit tissue of a miraculin-accumulating tomato line (56B, background Moneymaker) expressing the MIR-NOS cassette and showed that miraculin accumulation levels in the exocarp were higher than in other tissues.[31] In addition, we analyzed the miraculin accumulation pattern in the fruit tissue from a miraculin-accumulating tomato plant (background Micro-Tom) that contained a CaMV 35S promoter cassette (MIR-NOS) and an E8 promoter cassette (E8 is a fruit-ripening-specific promoter).[36] Miraculin-accumulating tomatoes with the CaMV 35S promoter (MIR-NOS) showed the same miraculin accumulation pattern in the fruit of line 56B, whereas tomatoes with the E8 promoter showed a different accumulation pattern in the fruit of line 56B; that is, the E8 promoter conferred a uniform accumulation, but the miraculin concentration was lower than that in line 56B. The results indicate that miraculin accumulation patterns can be altered by modifying the expression cassette. In this study, the miraculin accumulation pattern in the fruit tissue of MIR-NOS and MIR-HSP transgenic tomato plant was measured. Miraculin accumulation in the exocarp was approximately 1150 and 250 μg/gFW in MIR-HSP and MIR-NOS cassette expressing lines, respectively. In each transgenic tomato, the concentrations of miraculin in the exocarp were 2 or 3 times higher than in the mesocarp and other tissues. In the MIR-HSP transgenic tomato, the miraculin accumulation pattern was similar to that observed in our previous study.[36] Therefore, exchanging the NOS terminator for the HSP terminator does not change the miraculin accumulation pattern in fruit tissues of transgenic tomatoes. However, the HSP terminator can increase miraculin accumulation levels dramatically. It is likely that a transgenic tomato uniformly expressing high levels of miraculin can be generated using the E8 promoter combined with the HSP terminator.

Miraculin-accumulating tomatoes will be developed for the purification of miraculin for use as a food additive and as a chemical for research. We have developed a simple, efficient,and effective purification method for the recovery of recombinant miraculin from miraculin-accumulating tomato plants. His residues in the miraculin were used as a tag for nickel-immobilized affinity chromatography and ion exchange chromatography, which enabled us to obtain highly purified miraculin that was not denatured.[34,35] A high concentration of the target protein in the source materials improves miraculin recovery rates during the purification process. We subjected the transgenic tomatoes expressing MIR-NOS and MIR-HSP to miraculin purification. The results of the miraculin purification from transgenic tomatoes expressing MIR-HSP and MIR-NOS are shown in Table 1. The recovery rate was calculated by comparing the miraculin concentration in the sample before and after extraction using the nickel column. We demonstrate that the recovery rate of miraculin is higher in transgenic tomatoes expressing MIR-HSP than in tomatoes expressing MIR-NOS. Our results indicate that increased miraculin accumulation in transgenic tomatoes may improve the recovery rate of recombinant miraculin from transgenic tomato plants.

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