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ORIGINAL ARTICLE

Production of recombinant miraculin protein in carrot callus via *Agrobacterium*-mediated transformation

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Abstract

Miraculin is a taste-modifying protein that interacts with human sweet-taste receptors and transforms a sour taste into sweet taste. Miraculin is extracted from the miracle fruit (*Synsepalum dulcificum*). Since mass production of miraculin is difficult because of regional and seasonal limitations, several attempts have been made to express miraculin in various cell systems. In this study, a binary vector containing the *miraculin* gene under the control of the *SWPA2* promotor was introduced into carrot (*Daucus carota*) callus via *Agrobacterium*-mediated transformation to synthesize miraculin in carrot cell cultures. After 4 weeks of co-cultivation with *Agrobacterium tumefaciens*, 20 tentative transgenic callus (TC) lines were obtained on kanamycin selection medium. PCR analysis confirmed that 18 of these 20 lines (TC1–TC18) carried the *miraculin* gene, and 4 TC lines with high cell growth and gene expression (determined by RT-qPCR) were selected for further analysis. Protein analysis of these four TC lines by SDS-PAGE and Western blot showed that the miraculin protein was stably produced in TC lines. The cell growth showed no correlation with gene expression levels. The DNA content and G1 phase ratio were negatively correlated, whereas the S and G2/M phase ratios were positively correlated with gene expression. The ratio of cell cycle was determined by counting the number of cells in each step through flow cytometric analysis. These results indicate that gene expression was higher in TC lines with active cell division. Overall, our results demonstrate the feasibility of mass production of recombinant miraculin protein in transgenic cell culture systems.

Key message

This study expressed the miraculin gene in carrot callus via *Agrobacterium*-mediated transformation based on miraculin expression and cell cycle analyses. Our results demonstrate the feasibility of mass production of recombinant miraculin protein in transgenic cell culture systems

Keywords *Agrobacterium*-mediated transformation · Alternative sweetener · Carrot · Miraculin · Plant cell culture · Recombinant protein

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Introduction

Sugar (sucrose) is as important energy source for all living organisms. However, in humans, lifestyle-related diseases such as diabetes, obesity, and hypertension are increasing because of excessive sugar intake (Misaka 2013). Accordingly, a number of studies have been conducted to identify alternative sweeteners that could replace added sugar in foods and beverages (Wel and Loeve 1972; Kant 2005; Ezura and Hiwasa-Tanase 2018). Saccharin and aspartame are low-calorie artificial sweeteners widely used as alternative sweeteners to prevent lifestyle-related diseases (Misaka 2013). However, the use of some artificial sweeteners is related to heart disease, bladder cancer, heart failure, and brain tumor (Kant 2005). Therefore, many studies are being conducted to use sweet protein as an alternative sweetener that does not cause chronic disease and is low in calories.

Sweet proteins include monellin (Wel and Loeve 1972), brazzein (Ming and Hellekant 1994), curculin (Yamashita et al. 1990), and miraculin (Kurihara and Beidler 1968; Ezura and Hiwasa-Tanase 2018). Miraculin is a sweet protein produced from the miracle fruit (*Richadella dulcificum*) native to West Africa, which exhibits taste-modifying activity, as it interacts with human sweet-taste receptors and converts the sour taste into a sweet taste (Kurihara and Beidler 1968). Miraculin contains 191 amino acid residues and has a molecular weight of approximately 28 kDa (Jin et al. 2013). Miraculin is approximately 3000 times sweeter than sucrose (Akter et al. 2016) and is a very stable protein that maintains its taste-modifying activity for more than 6 months if stored at 5 °C (pH 4) (Akter et al. 2016). However, mass production of miraculin from miracle fruit is limited by regional and climatic factors. Therefore, several attempts have been made to produce recombinant miraculin using heterologous systems such as *Escherichia coli* (Matsuyama et al. 2009), yeast (Ito et al. 2010), and plants (Sun et al. 2006, 2007; Hirai et al. 2010; Yano et al. 2010) via transformation-based approaches (Jung and Kang 2018).

Plant cell culture is an important system for the production of valuable foreign proteins (Huang and McDonald 2012). Unlike whole-plant production systems, the plant cell culture system is performed under environmentally controlled conditions previously established for a given species (Corbin et al. 2016). In addition, the plant cell culture system is suitable for mass production of recombinant proteins because of advantages such as rapid cell proliferation, easy genetic transformation, and high-level protein expression (Corbin et al. 2016).

To produce recombinant protein in a heterologous plant system, the gene-of-interest is introduced into the host plant via transformation (Habibi et al. 2017). Different methods are used for plant transformation, such as *Agrobacterium*-mediated transformation and electroporation. Among these methods, *Agrobacterium*-mediated transformation is used widely because of excellent transformation efficiency (Gleba et al. 2005; Chung et al. 2009). In this method, the host plant is infected with *Agrobacterium* at a wound site through which the transfer-DNA (T-DNA) portion of the tumor-inducing (Ti) plasmid of *Agrobacterium* is inserted into the plant genome (Hiei et al. 1997; Gelvin 2003). The *Agrobacterium*-mediated transformation method has several advantages; for example, the protocol is relatively simple and inexpensive (Hansen and Wright 1999; Zagorskaya and Deineko 2017).

In this study, we used *Agrobacterium*-mediated transformation to express the *miraculin* gene in carrot (*Daucus carota*) callus under the control of the sweet potato *SWPA2* promoter; because the *SWPA2* gene is strongly induced by oxidative stress, its promoter is widely used to produce recombinant proteins under stress conditions (Kim et al. 2003). We then determined the correlation between cell proliferation and miraculin protein yield to infer the role of nutrient concentration in recombinant protein biosynthesis in host cells.

Materials and methods

Plant materials

Carrot (*Daucus carota*) cv. Dream 7 was used for callus induction. To induce callus formation, carrots were peeled, sterilized in 70% ethanol for 30 s, and then surface sterilized in a 2% NaOCl for 15 min. The surface-sterilized carrots were rinsed 3 times with sterilized water, and then cut into 0.5×0.5 cm sections containing the cambium layer under clean bench and cultured on Murashige and Skoog (MS) medium (Callus Induction medium [CI medium]: 0.25 g L^{-1} casein hydrolysate; 0.22 mg L^{-1} BA; 1 mg L^{-1} 2,4-D; 30 g L^{-1} sucrose; and 2.4 g L^{-1} gelrite) at 24 ± 1 °C in the dark for 4 weeks. The induced callus was transferred to fresh CI medium and cultured for 4 weeks. When a sufficient amount of callus was obtained, it was used for *Agrobacterium*-mediated transformation.

Agrobacterium-mediated transformation

Transgenic *Agrobacterium tumefaciens* strain LBA4404 containing the pCAMBI2400 vector, which carried the miraculin gene under the control of the oxidative stress-inducible *SWPA2* promoter, was used to transform carrot callus (Fig. 1a). The kanamycin resistance gene (*KanR*) was inserted in the T-DNA for the selection of transgenic

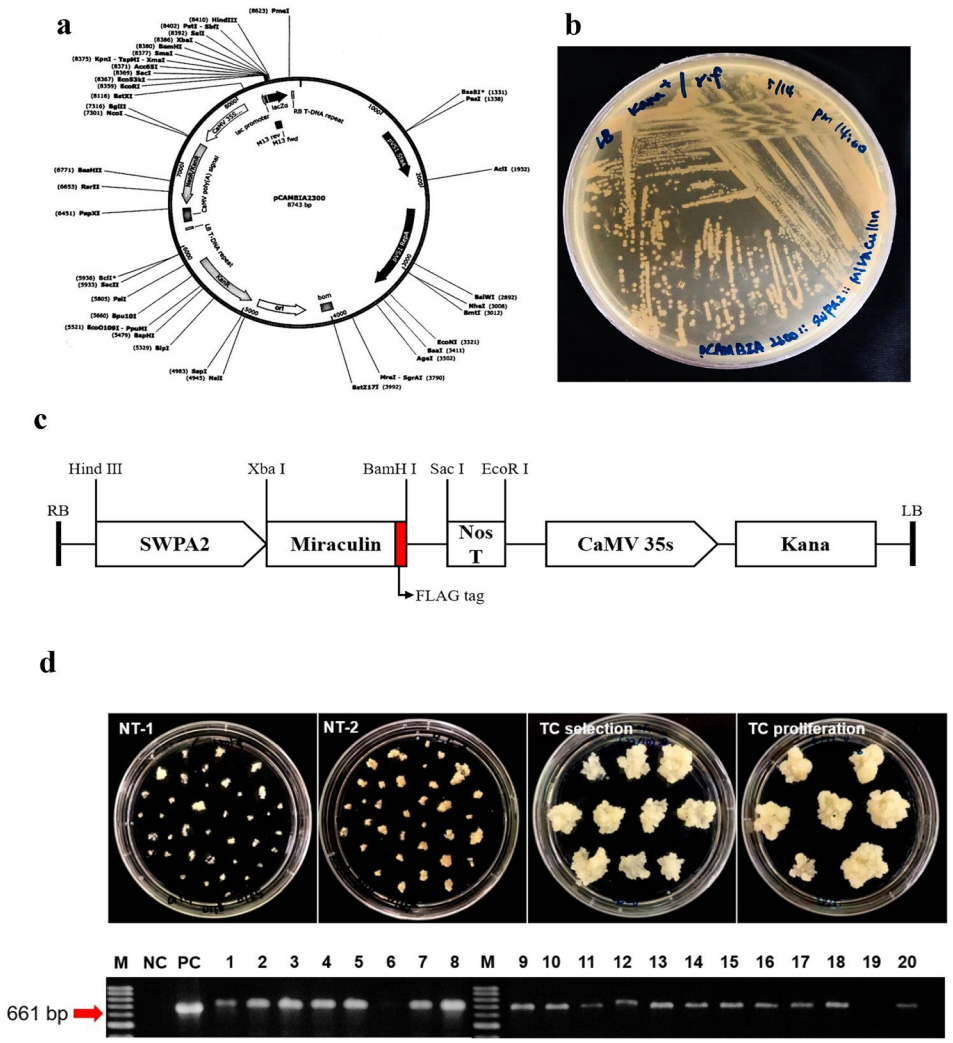

Fig. 1 Vector map (**a**) and T-DNA (**c**) of *Agrobacterium tumefaciens* for miraculin transformation (**b**). Selection of transgenic callus (TC) on 50 µg/ml Kanamycin medium and confirmation of miraculin gene insertion by PCR (**d**)

calli (TCs) (Fig. 1b). Prior to transformation, transgenic *A. tumefaciens* cells were grown on solid LB medium in an incubator at 28 ± 1 °C for 2 days (Fig. 1c). Subsequently, 1–2 colonies of *A. tumefaciens* were inoculated in liquid LB medium containing kanamycin, and grown at 28 ± 1 °C with 150 rpm for 2 days. To acclimate *A. tumefaciens* to the plant medium, 1–2 mL *Agrobacterium* suspension was inoculated in MS medium containing 100 µM acetosyringone and grown for 2 days. To conduct transformation, 2 g carrot callus was immersed in the *Agrobacterium* suspension (OD_{600} = 0.1) for 30 min. Then, the *Agrobacterium* suspension was removed onto a sterilized filter paper. The infected calli were placed on CI medium and co-cultured at 24 ± 1 °C in the dark for 3 days. Subsequently, the infected calli were cultured in CI medium containing 500 µg mL^{-1} carbenicillin and 250 µg mL^{-1} cefotaxime for 2 days to remove residual *A. tumefaciens*, and were then transferred to the solid CI medium containing 100 µg mL^{-1} kanamycin to select transgenic callus (TC). Subcultures were performed weekly. Each TC line was frozen in liquid nitrogen, and the insertion of the miraculin gene was confirmed by PCR analysis. The TC lines were stored at − 70 °C until analysis. The selected TC lines were cultured in CI medium for the next experiment.

PCR analysis

To confirm the presence of the *miraculin* gene, 100 mg of each putative TC line was pulverized using TissueLyser II (QIAGEN, Germany) and centrifuged, and the supernatant was transferred to a new tube. Genomic DNA (gDNA) was extracted using the 5 min Plant DNA Extraction Kit (BioFactories, Korea), according to the manufacturer's protocol. The concentration and purity of gDNA were determined using the Nanodrop (DeNovix, USA). To conduct PCR, gDNA was diluted with sterile distilled water to a final concentration of 10 pmol $µl^{-1}$. PCR was carried out on the CFX96 Touch™ Real-Time PCR System (Bio-Rad, California) in a 20 µl volume reaction containing 3 µl of diluted gDNA, 2 µl of each forward and reverse primer (10 pmol $µl^{-1}$; Table 1), 10 µl of the Prime *Taq* Premix (2X) (GeNet Bio, Korea), and

Table 1 List of primers used in this study

Primer name	Sequence (5′→3′)	Amplicon size (bp)	Purpose
SWPA2 Fw	CCAATTAAAGTCCCCAACCA	661	PCR
Miraculin Rv	ACGGTGGGACAGAAA ACAAG		
Miraculin-RT Fw	CATCAATTTCTCGGCGTTCAT	218	RT-qPCR
Miraculin-RT Rv	AAACCACTACCACAAAACTCCT		
DcActin Fw	GCGGGAAATTGTTCGTGATATG	241	
DcActin Rv	TAGATGGCTGGAAGAGGACTT		

3 µl of sterile distilled water. Thermal cycling involved 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 40 s, followed by a final extension at 72 °C for 7 min. The PCR products were mixed with Dyne LoadingSTAR (DYNEBIO, Korea) in a 1:4 ratio and were electrophoresed on 1.5% agarose gel at 100 V for 30 min using the Mupid-exU system (ADVANCE, Japan).

Miraculin gene expression

To analyze miraculin expression, 100 mg of each TC line cryopreserved in liquid nitrogen was pulverized with TissueLyser II (QIAGEN, Germany), and total RNA was extracted using the NucleoSpin® RNA Plant and Fungi Kit (MN, Germany), according to the manufacturer's instructions. The extracted total RNA was converted into cDNA using the ReverTra Ace® qPCR Master Mix (TOYOBO, Japan). Reverse transcription quantitative real-time PCR (RT-qPCR) was performed on the CFX96 Touch™ Real-Time PCR System (Bio-Rad, California) in a 20 µl reaction containing 10 µl TB Green Premix Ex *Taq* (Takara, Japan), 0.5 µl of each forward and reverse primer (Table 1, Jung and Kwang 2018), 2 µl cDNA (100 ng•µL^{-1}), and 7 µl sterile distilled water. The *DcActin* gene was used as an internal reference for data normalization.

SDS-PAGE

To extract the total soluble protein (TSP), 200 mg TC was pulverized with 400 µl protein extraction buffer (500 µl of 3 M NaCl, 200 µl of 1 M Tris [pH 7.5], 20 µl of 0.5 M EDTA, 1 mL of 10% Triton-X 100, 100 µl of 10% SDS, 10 µl of 1 M dithiothreitol [DTT], and 10 µl of protease inhibitor cocktail) using TissueLyser II (QIAGEN, Germany). The pulverized sample was centrifuged at 13,500 rpm, and the supernatant was transferred to a new tube. The concentration of TSP was analyzed using the Bradford assay (Bradford 1976). The extracted TSP was diluted with sterile water to a final concentration of 30 µg µl^{-1}. Then, 24 µl of the diluted total protein was mixed with 6 µl of 5X protein sample buffer (ELPIS, Korea), and the mixture was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using ExpressPlusTM PAGE Gels (GenScript, USA) containing Bis–Tris 10% MOPS buffer. SDS-PAGE was performed on a Mini-Slab Chamber (ATTO, Japan) at 140 V for 55 min. BrightBAND (ZEPTO, Taiwan) was used as a protein marker. After electrophoresis, the gel was stained with EzStainAQua (ATTO, Japan) for 1 h and washed with distilled water three times for 30 min each to observe the protein bands.

Western blot analysis

TSP extracted from TC17 line was analyzed by western blot analysis. Proteins from the gel were transferred onto Clear Blot membrane (ATTO, Japan), which was pre-activated in methanol for 1 min and washed in transfer buffer (EzFast-Blot, ATTO, Japan) for 30 min. Additionally, three sheets of filter paper (Absorbent paper, ATTO, Japan) were pre-soaked in transfer buffer. A filter paper–membrane–gel–filter paper sandwich was prepared, and western blotting was performed using the HorizeBLOT 2 M (ATTO, Japan) at 20 V for 20 min. After transfer, the membrane was blocked with 10 ml of EzBlockChemi (ATTO, Japan) for 30 min. ANTI-FLAG M2-Peroxidase (HRP) (Sigma, USA) was diluted with 1X TBST solution (10X TBST Buffer, ELPIS, Korea) at a concentration of 1:50,000 (v/v) and reacted with the membrane for 1 h. Then, the membrane was washed with distilled water for 30 min. Subsequently, the two luminous reagents provided in EzWestLumi plus (ATTO, Japan) were mixed in a 1:1 ratio, and 1 mL of the mix was applied to the membrane for several seconds. The membrane was then inserted into a transparent film, and luminescent bands were detected using LuminoGraph (ATTO, Japan).

Enzyme-linked immunosorbent assay (ELISA)

To quantify the yield of miraculin protein, ELISA was performed with the anti-FLAG antibody using the DYKDDDDK-Tag Detection ELISA Kit (Cayman, Germany), according to the manufacturer's instructions. Absorbance was measured at 450 nm using Optizen POP (Mecasys Co., Korea).

Flow cytometric analysis

The DNA content and cell cycle of each TC line was analyzed by flow cytometry. To extract nuclei, 200 µl of extraction buffer was added to 100 mg of the TC placed in a Petri dish. The mixture was then filtered through a 50 µm nylon mesh filter. Then, 800 µl of staining solution (containing staining buffer, Propidium iodide solution, and RNase) was added to the filtrate. Samples were fluorescent stained for 20 min in the dark and then analyzed by CytoFLEX (Beckman Caulter, US). At least 20,000 cells per sample were analyzed, and data were measured using the Cytexpert program (Beckman Coulter, US). Potato (*Solanum tuberosum* cv. Sumi) was used as an internal standard for DNA content analysis.

Statistical analysis

Statistical analysis of mean values was performed using one-way analysis of variance (ANOVA). Statistical significance of the difference between mean values was then assessed by Duncan's multiple range test (DMRT) at P-value < 0.05. All data were analyzed using the SAS program (version 9.4; SAS Institute Inc. Cary, NC, USA).

Results and discussion

Identification of TC lines

In this study, we used carrot callus as the heterologous system for the production of miraculin protein. Carrot callus was transformed with *A. tumefaciens* carrying the *miraculin* gene under the control of the *SWPA2* promoter. The transformed calli were grown in selection medium containing 100 µg mL^{-1} kanamycin for 3 weeks by sub-culturing at weekly intervals (Fig. 2a). Calli with high cell proliferation rates compared with non-transformed callus were selected. After three selection cycles, 20 putative TC lines with the highest cell proliferation rates were selected among 100 lines and cultured for 2 weeks. To confirm the presence of the transgene, we performed PCR analysis of genomic DNA extracted from these 20 lines. The presence of miraculin gene was confirmed in 18 out of 20 lines (TC1–TC18) (Fig. 2b). Thus, 18% transformation efficiency was achieved in carrot callus (data not shown).

Agrobacterium-mediated transformation has been used to produce recombinant proteins in plant systems such as tobacco, rice, and citrus (Bachchu et al. 2011; Pham et al. 2012; Corbin et al. 2016). In rice, *Agrobacterium*-mediated transformation at an efficiency of 31% has been used to produce recombinant butyryl cholinesterase (Corbin et al. 2016). In citrus (*Citrus unshiu* cv. Miyagawa Wase),

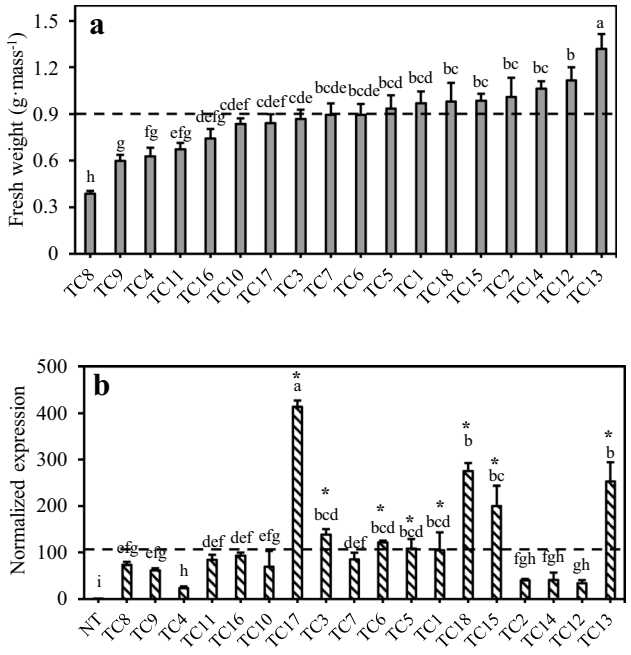

Fig. 2 The growth of transgenic callus (TC) lines and their miraculin expression level. **a** TC growth; **b** Miraculin expression level by RT-qPCR. The dashed line represents the average fresh weight and miraculin expression. NT, non-transgenic callus (control). Asterisks (*) indicate lines with good growth rate and miraculin expression. Different lowercase letters indicate statistically significant differences ($p < 0.05$; Duncan's multiple range test)

Agrobacterium-mediated transformation has been used for miraculin production at a transformation rate of approximately 22% (Bachchu et al. 2011). In some plants, *Agrobacterium*-mediated transformation which is used in this study shows high transformation efficiency and stable production of recombinant proteins over a long time because of stable insertion of the transgene in the plant genome (Chung et al. 2009). Thus, our data indicate that *Agrobacterium*-mediated transformation is suitable for the production of recombinant miraculin in carrot callus.

Cell growth and gene expression of miraculin transgenic callus

To compare the cell proliferation rate of total 18 TC lines, the fresh weight of each line was examined after 2 weeks of growth in CI medium. The fresh weight of these 18 TC lines ranged from 0.39 to 1.32 g $mass^{-1}$, with a 3.4-fold difference between the highest biomass (TC13) and lowest biomass (TC8) lines (Fig. 2a). Next, we analyzed the expression of miraculin gene in all 18 lines by RT-qPCR. The expression of the miraculin gene was the highest in TC17 and lowest in TC12, with a 12.3-fold difference in expression between these two lines (Fig. 2b). In lettuce, Jung and Kang (2018) previously reported approximately 6.5-fold higher miraculin

expression in transgenic lettuce than that in non-transformed lettuce. In the current study, miraculin expression in TC was higher than that in transgenic lettuce. These results indirectly indicate that the carrot cell cultures could be good system for the production of *miraculin* protein. No significant relationship was detected between the fresh weight of TC and the level of miraculin gene expression (Data not shown). The fresh weight of callus may change because of three reasons: (1) increase in cell number due to cell division; (2) increase in cell size; (3) cell division and thus increase in cell size. Kim et al. (2003) and Choi et al. (2003) found that gene expression increases with the increase in fresh weight of transgenic plants. In another study, the content of recombinant protein has been reported to increase as total protein increases in actively dividing cells (Polymenis and Aramayo 2015). However, in the current study, we observed no correlation between the fresh weight and gene expression level in TC lines. This suggests that the increase in fresh weight of TC lines was due to the increase in cell size, not due to cell division. Overall, eight TC lines (TC1, TC3, TC5, TC6, TC13, TC15, TC17, and TC18), with higher than average values of miraculin gene expression and fresh weight, were selected and used for the next experiment.

Analysis of miraculin protein in TC lines

To examine miraculin protein production in TC lines, we performed SDS-PAGE and western blot analysis. SDS-PAGE revealed a 28 kDa band on the gel (Data not shown). Western blot analysis of the TSP revealed two protein bands at 28 and 45 kDa (Fig. 3). According to Jin et al. (2013), the 28 kDa band represents the miraculin monomer, while the 45 kDa band represents the miraculin dimer. In nature, miraculin forms a disulfide bond and exists as a 45 kDa dimer (Bachchu et al. 2011). Therefore, in this study, recombinant miraculin is expected to form a dimer (45 kDa) with a disulfide bond during normal post-translational modification in TC. When DTT was used for western blot analysis, the dimer was converted into the monomer (28 kDa). In this experiment, the 45 kDa band probably represented the dimeric from of miraculin, which was not completely cleaved by DTT. This indirectly shows that the recombinant miraculin was correctly biosynthesized in TC lines. Next, we performed ELISA to determine the yield of miraculin in TC13, TC15, TC17, and TC18 lines. The production of miraculin protein was the highest in TC17 (5.9 ng μl^{-1}) (Table 2), which accounted for 0.24% of the TSP.

Previously, the yield of miraculin protein in transgenic tomato, lettuce, and strawberry plants was reported to account for 0.55–1.15% of the TSP (Sun et al. 2006, 2007; Yano et al. 2010). The yield of recombinant miraculin protein in TC was lower than that in transgenic plants, however these transgenic cell culture system could be safer and more efficient than intact plant system for mass production of recombinant miraculin.

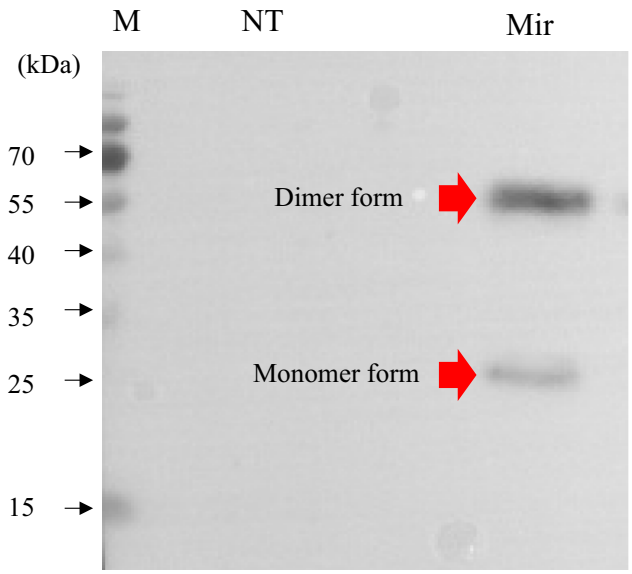

Fig. 3 Western blot analysis of transgenic callus TC17 (Antibody: ANTI-FLAG M2-Peroxidase). Lane *M*: maker: *NT*: Non-transgenic callus, Lane *Mir*: miraculin transgenic callus TC17

Table 2 Concentration of miraculin in transgenic callus (TC) lines

Cell line	Total soluble protein (TSP) (μg/μl)	Purified miraculin protein (ng/μl)	Purity[z] (% TSP)
TC13	2.046 c[y]	1.9 b	0.09 b
TC15	2.376 b	1.0 b	0.04 bc
TC17	2.489 a	5.9 a	0.24 a
TC18	2.366 b	0.1 c	0.01 c

[z]Miraculin content/TSP content) × 100

[y]Different lowercase letters within each column indicate significant differences ($p \leq 0.05$; Duncan's multiple range test)

Cell division and DNA content analysis of TC lines

Flow cytometric analysis was performed to analyze the cell cycle and DNA content of the TC lines. The ratio of step of cell cycle was determined by counting the number of cells in each phase. The proportion of S phase was the highest in TC13 (36.8%), while that of the G2/M phase was the highest in TC3 (33.1%) (Fig. 4a). The high ratio of S and G2/M phase means that cell division is active. Thus, TC lines with a high ratio of S and G2/M phase showed active cell division. The DNA content was the highest in TC9 (1.26 pg $2C^{-1}$) and lowest in TC12 (0.95 pg $2C^{-1}$), thus showing approximately 1.3-fold difference (Fig. 4b). The DNA content of each TC line was different, probably because the

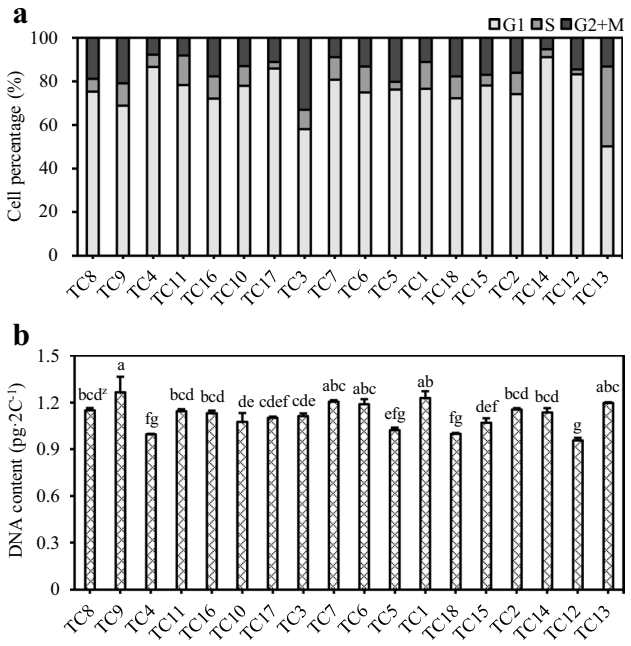

Fig. 4 Cell cycle (**a**) and DNA content (**b**) of transgenic callus lines (TC: Transgenic callus). [z]Different letters indicate significantly difference at $p < 0.05$ according to Duncan's multiple range test ($n = 5$)

DNA content gradually increased from the 2C to 4C level during the S phase (Pfosser et al. 2007). This suggests that TC lines with high DNA content contained a large number of dividing cells. Thus, measurement of the DNA content by flow cytometry enabled the identification of TC lines with active cell division.

Correlation between cell division and miraculin gene expression

We examined the correlation between DNA content, cell division cycle, and miraculin gene expression. Miraculin gene expression and DNA content was not significantly correlated (Data not shown), but the correlation between cell division cycle and miraculin expression differed according to each phase of cell cycle. The S and G2/M phases showed a positive correlation with miraculin expression (Fig. 5b, c), whereas the G1 phase showed a negative correlation (Fig. 5a). Egelkrout et al. (2012) reported that increasing the content of plant endogenous proteins also increases the yield of recombinant protein. Proteins are one of the most abundant macromolecules in proliferating cells (Polymenis and Aramayo 2015). Therefore, it is estimated that the yield of recombinant protein will increase with cell division because cells with high cell proliferation rates have high protein content. Since the cell division begins at the S and G2/M phases of the cell cycle, a high proportion of cells in the S and G2/M phase implies that cell proliferation is

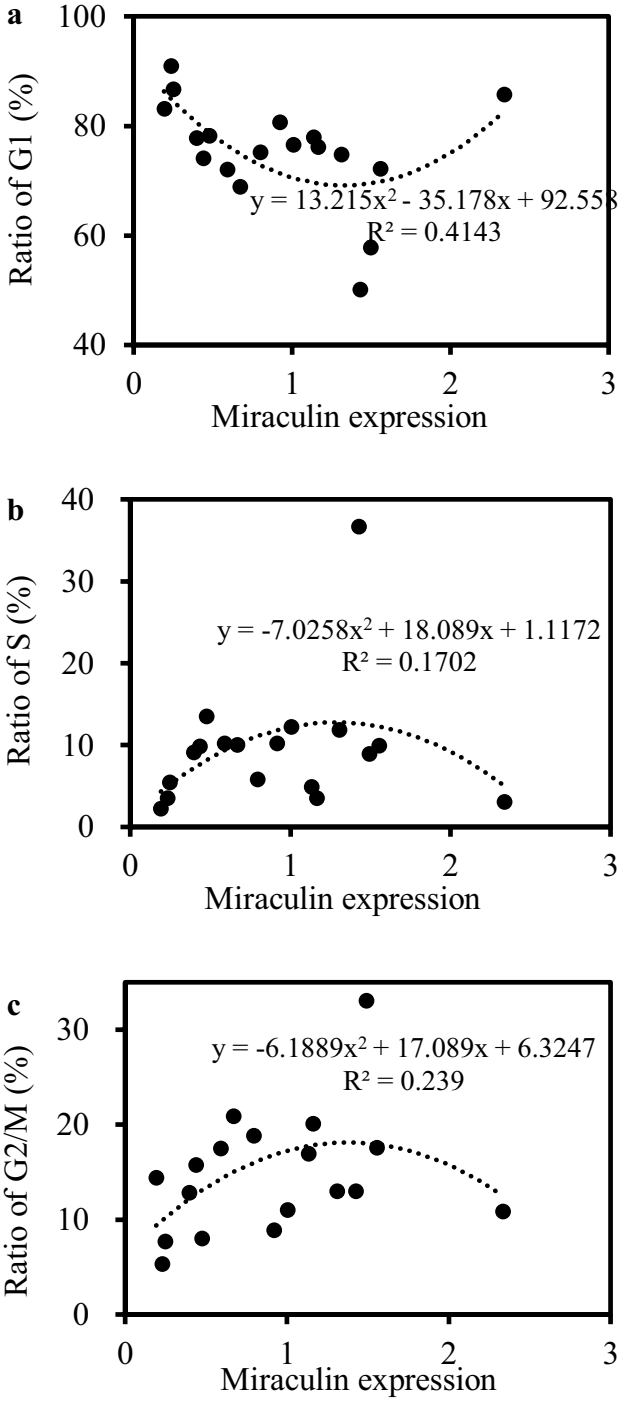

Fig. 5 Correlation analysis of miraculin expression and the phase of cell cycle. Ratio of G1 phase (**a**), S phase (**b**), and G2/M phase (**c**) in the cell cycle

active. Therefore, with the increase in cell proliferation rates of TC lines, the amount of endogenous protein and recombinant miraculin protein also increased. These results suggest that miraculin expression may be high in TC lines with high cell division; this was proven by the correlation of miraculin

expression with cell division. Because miraculin expression increased with the increase in cell division of TC lines, it is thought that DNA content was positively correlated with miraculin expression.

Conclusion

Overall, in this study, we expressed the *miraculin* gene in carrot callus via *Agrobacterium*-mediated transformation, and selected four lines (TC3, TC13, TC17, and TC18) based on miraculin expression and cell cycle analyses. Our results showed that the more active the cell division, the higher the expression of miraculin. These results lay the foundation for future use of recombinant miraculin in the food and beverage industry.

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Author contributions YJP and JEH contributed to data acquisition and wrote the manuscript. TTH and HL participated in interpreted data and revising for intellectual content. SYP made substantial contributions to the conception and design of the study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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