For Plant Science

Title:

Tomato is a suitable material for producing recombinant miraculin protein in genetically stable manner

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ABSTRACT

Miraculin is a taste-modifying protein that turns sour tastes into sweet ones. We previously generated transgenic tomato plants that constitutively expressed miraculin. To study the stability of transgene inheritance and expression in detail, three lines of transgenic tomato that highly accumulate miraculin in the T0 generation with a single copy of the *miraculin* gene were analyzed for genomic organization, mRNA expression and miraculin accumulation up to the T5 generation, corresponding to six generations of propagation. Transgenes were stably inherited and genomic rearrangement was not detected; this was confirmed in the T5 generation in one line and the T3 generation in the other two lines. The expression of *miraculin* mRNA was stable through multiple generations and in individual plants of the same generation. The concentrations of miraculin protein ranged from 8.73 to 11.52 μg/mg total soluble protein in the transgenic tomatoes, and they were stable in each line. These results suggest that the tomato is a suitable material for producing recombinant miraculin protein.

Keywords: miraculin, recombinant protein, tomato, stability of transgenes, transgenic plants

Abbreviations: mRNA, messenger RNA; ELISA, Enzyme-linked immunosorbent assay; Real-time RT-PCR, Reverse transcription coupled with real-time polymerase chain reaction

1. Introduction

Miraculin is a taste-modifying protein that is present in the miracle fruit (*Richadella dulcifica*), a native West African shrub. Indigenous peoples often use the berries of this shrub to improve the palatability of acidic foods and beverages. Miraculin itself is not sweet, but it has the unusual property of being able to convert a sour taste into a sweet taste. The sweetness of citric acid after exposure to miraculin is estimated to be about 3,000 times that of sucrose on a per weight basis [1-4]. Because of this amazing property, interest in miraculin has been increasing.

Miraculin has great potential to be an alternative low-calorie sweetener for diabetic and dietetic purposes, but miracle fruit production is limited because it is a tropical plant. To date, several attempts have been made to produce miraculin in foreign hosts including *Escherichia coli* [5], yeast and tobacco [3]. Although miraculin was successfully expressed in these hosts, the resulting recombinant protein did not possess taste-modifying activity. Recently, however, when *Aspergillus oryzae* was used as a host for expressing miraculin, the recombinant protein did exhibit taste-modifying properties [6]. Our group has also successfully expressed recombinant miraculin protein in seed-propagated crop species such as lettuce [7] and tomato [8], as well as the vegetative-propagated crop species strawberry [9], using the same transgene construct. However, miraculin expression was barely detectable in subsequent generations of lettuce [7]. Although miraculin was stably expressed and accumulated in the vegetative progeny of transgenic strawberry plants, the level of accumulation was significantly lower than in the T0 generation of tomato and lettuce [9]. By contrast, higher miraculin accumulation was confirmed in transgenic tomatoes through the T2 generation [8]. These results

indicate that the plant species used for transformation and the mode of seedling propagation are both important factors in producing miraculin-expressing transgenic plants.

In this study, three lines of transgenic tomatoes that highly accumulate miraculin in the T0 generation with a single copy of the *miraculin* gene were selected, and the genomic organization of the transgene was investigated to study the influence of transgene integration on the endogenous genes. The expression level of *miraculin* mRNA and the concentration of miraculin protein were also measured up to the T5 generation, which corresponds to six generations of propagation.

2. Materials and Methods

2.1. Plant materials and plant growth

Three lines (56B, 2A, and 5B) of transgenic tomato (*Solanum lycopersicum* L., cv. Moneymaker) plants that highly accumulate miraculin protein in the T0 generation with a single copy of the *miraculin* gene were selected from our previous study [8]. These plants were grown in a screened greenhouse for transgenic plants and allowed to self-pollinate. Homozygous lines of each transgenic tomato were screened in the T2 generation. Seeds of line 56B were obtained through the T5 generation. Seeds of lines 2A and 5B were obtained through the T3 generation. These seeds were sown in 5 x 5 x 5 cm (height x length x width) rockwool cubes and grown in a hydroponics system in an environmentally controlled growth room at 25˚C/20˚C and 1000 ppm CO_2 concentration under a light condition as follow, 400 μmol/m^2/s photosynthetic photon flux density

(PPFD) with a light/dark cycle of 16 hr/8 hr.The seedlings were subjected to the following experiments.

2.2. Genomic Southern blot analysis

Genomic DNA was isolated using the CTAB method [10]. For Southern blot analysis, 20 μg of genomic DNA was digested with *Xba*I, which cleaves only once outside the *miraculin* gene, and the fragments were separated in a 1% agarose gel run at 50 V for 3 h and then transferred to a Hybond-N+ nylon membrane (GE Healthcare UK Ltd.). A thermostable alkaline phosphatase (AP)-labeled *miraculin* gene-specific probe and an *nptII* gene-specific probe were generated using the CDP-Star AlkPhos Direct Labelling Kit, following the manufacturer's instructions (GE Healthcare UK Ltd). The membrane was hybridized with the probe overnight at 55°C. Hybridization signals were detected by chemiluminescence using CDP^{star} (Roche Diagnostics GmbH, Mannheim, Germany) followed by exposure in the LAS4000mini Image Analyzer (Fujifilm Co. Ltd., Tokyo, Japan).

2.3. Isolation of sequences flanking the transgene

To get the flanking sequence for the transgene, we used the adapter ligation PCR method that was described by Siebert et al. [11]. Genomic DNA was digested using the restriction enzyme EcoRV and ligated using the specific sequence adapter by Ligation high (TOYHOBO, Osaka, Japan). Adapter-ligated DNA was used as the template for PCR amplification. PCR was performed using LA Taq (TAKARA-BIO INC., Otsu, Japan)

with the adapter-specific primer (AP1) and *NPTII*-specific primer (npr2), and then nested PCR was performed with the adapter-specific primer (AP2) and *NPTII*-specific primer (npr1). Amplification products were sequenced and the flanking sequences of the transgene were searched against the tomato genome sequence released at 2 February 2010 using the sol genomics network web site (http://www.sgn.cornell.edu/tools/blast/). The adapter sequence was GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT; the sequence of primer AP1 was CCATCCTAATACGACTCACTATAGGGC; AP2, CTATAGGGCACGCGTGGT; npr1, GCTCATTAAACTCCAGAAACCCGCGGC; and npr2, AGGCGACTTTTCAACGCGCAATAATGG.

2.4. Quantitative real-time RT-PCR analysis

The expression level of *miraculin* mRNA in transgenic tomatoes was determined by quantitative real-time RT-PCR. Total RNA was isolated from frozen leaves using the RNeasy Plant Minimini Kit (Qiagen, Valencia, CA, USA) with RNase-free DNase (Qiagen). Reverse transcription was performed with SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), using a mixture of random hexamers as the primers. Quantitative real-time PCR was performed using the Thermal Cycler Dice Real Time System TP800 (TAKARA-BIO INC.) with SYBR Premix Ex Taq II (TAKARA-BIO INC.), following the manufacturer's instructions. Relative quantification of *miraculin* gene expression was calculated in reference to *Slubiquitin3* expression, which has been used as an internal control for tomato expression in several studies [12-13]. The primer sequence for miraculin forward (fw) was CACCCAATCCGGTTCTTGAC; miraculin

reverse (rev), GTGGTGGCGGATACTGTAAGG; ubiqitin fw, CACCAAGCCAAAGAAGATCA; and ubiquitin rev, TCAGCATTAGGGCACTCCTT.

2.5. Protein extraction, western blot analysis and ELISA

The levels of accumulated recombinant miraculin protein in transgenic tomatoes were determined using immunological measurements. The soluble protein extracted from the leaves of transgenic tomatoes was used for western blot analysis and ELISA. These methods were described in our previous work [7].

3. Results and discussion

3.1. Molecular analysis of transgene inheritance through multiple generations

Genomic Southern blot analysis of transgenes was performed to confirm stable inheritance through multiple generations. Line 56B possessed one copy of *miraculin* and two copies of *NPT-II*, and these transgenes were inherited through the T5 generation (Figure 1). The detected band sizes were almost the same through all five generations and no extra bands were observed, indicating that there was stable inheritance of the transgenes in line 56B. The stability of the transgenes in lines 2A and 5B were observed through the T3 generation. Each line had one copy of *miraculin* and one of *NPT-II*. These lines also showed stable inheritance of the two transgenes (Figure 1). These results show that the *miraculin* and *NPT-II* genes were stably inherited through multiple generations in the transgenic tomatoes.

Genome rearrangement often occurs during seed propagation in plants, resulting in gene rearrangement, duplication, recombination and deletion. It is possible for genome rearrangements to occur in the area near a transgene. In previous studies, stabele inheritance of the transgene was shown through T5 generations in barely [14] and through T5 generations in maze [15]. In this study, inheritance of the *miraculin* gene in transgenic tomatoes was analyzed by genomic Southern blot analysis in subsequent generations. Genomic DNA extracted from ten individual plants in each generation was mixed for this analysis, and no extra bands, band shifts or band changes were observed. These results indicate that the *miraculin* gene was not involved in a genomic rearrangement and was inherited in subsequent generations of transgenic tomatoes as same as in transgenic barely [14] and transgenic maize [15].

3.2. Site of transgene integration

To study the influence of transgene integration on endogenous genes, the flanking genomic sequences of the transgenes were isolated and analyzed (Figure 2). In line 56B, the 798 bp genomic sequence at the 5' end of *miraculin* was obtained and the sequence showed significant homology to an mRNA that was recorded in GenBank (accession no. BT013114) and had been isolated from tomato fruit. However, a stop codon was found in this mRNA at 280 bp upstream from the 5' end of the transgene. Furthermore, the mRNA expression level of BT013114 was not different between fruits from line 56B and wild-type tomatoes (data not shown), suggesting that *miraculin* had integrated into a non-coding sequence in line 56B. In lines 2A and 5B, the 575 bp and 768 bp genomic sequences, respectively, at the 5' end of *miraculin* were obtained and the sequences did

not show significant homology to any known genes, suggesting that *miraculin* had also integrated into non-coding sequences in lines 2A and 5B.

3.3. The expression of *miraculin* mRNA through multiple generations

To confirm the stability of transgene expression through multiple generations and among individual plants of the same generation, *miraculin* mRNA expression level was measured using quantitative real-time RT-PCR (Figure 3). In line 56B, the expression level of *miraculin* mRNA in the T1 generation was lower than in later generations because the T1 generation had three genotypes for the *miraculin* gene: homozygous, hemizygous and nullizygous. There were no significant differences in the expression level of *miraculin* mRNA from the T2 to the T5 generation. Moreover, the expression levels of *miraculin* mRNA in eight individual plants from the T5 generation were similar to each other. In lines 2A and 5B, the expression levels of *miraculin* mRNA from the T1 to the T3 generations showed results similar to those of line 56B.

3.4. Accumulation of recombinant miraculin protein through multiple generations

To investigate the stability of miraculin protein accumulation through multiple generations in the three transgenic lines, the levels of the miraculin protein were confirmed by western blot analysis and the concentrations of the miraculin protein were measured by ELISA. In each of the three transgenic lines, miraculin protein was detected by western blot; the molecular weight of the miraculin protein was found to be 47 kDa, which was the same size as miraculin protein purified from miracle fruit (Figure 4). The

concentrations of miraculin protein in the three transgenic lines at the T1 generation were lower than in later generations, much like the expression levels of miraculin mRNA (Table 1). In line 56B, the concentrations of miraculin protein were between 9.74 to 10.94 μg/mg total soluble protein from the T2 to the T5 generations. In lines 2A and 5B, the accumulation of miraculin protein was also stable in the T2 and T3 generations (Table 1). The miraculin concentrations in these generations were 11.04 and 11.52 μg/mg total soluble protein in line 2A and 9.29 and 10.03 μg/mg total soluble protein in line 5B (Table 1).

The recombinant miraculin protein was purified from the transgenic tomato fruits of all generations, and the taste-modifying properties were confirmed. The recombinant miraculin produced in transgenic tomato showed similar taste-modifying activity to natural miraculin as in Sun et al. [8] while that produced in *Aspegillus oryzae* showed 1/5 of taste-modifying activity to natural miraculin [6]. Miraculin protein is stable in acidic condition, while tomato fruits exhibit acidic condition that may allow the miraculin protein to be stable in the tomato fruits. These evidences suggest an advantage of the recombinant miraculin production in tomato fruits. Effects of recombinant miraculin accumulation on the tomato fruit quality may be another matter of interests. Currently intensive metabolic profiling of the transgenic tomato fruits has been performed and significant differences between the transgenic and the non-transgenic tomato fruits have not been detected (data not shown).

3.5. Conclusion

Plants are usuful platforms for the mass production of valuable recombinant proteins

[16-17]. Research over the past 10 years has remarkably increased our ability to produce recombinant proteins in different plants. Leafy crops, cereal and legume seeds, fruits and so on are used to produce recombinant proteins. Commercially useful proteins for pharmaceutical and industrial applications have been produced in several plant systems [18]. Taste-modifying proteins have also been produced via transgenic plants [19], but only the recombinant monellin protein in transgenic tomatoes [20] and the miraculin protein in transgenic lettuce and tomatoes [7-8] have been highly produced. The concentrations of recombinant miraculin protein in transgenic tomatoes were much higher, but these proteins have not yet been commercialized and there have been no studies investigating the stability of transgene inheritance and expression through multiple generations. This study is the first to show the stability of miraculin transgene inheritance and expression, as well as the accumulation of recombinant miraculin protein, through multiple generations in transgenic tomatoes. Thus, the tomato is a promising material for the mass production of recombinant miraculin protein.

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Figure Legends

Figure 1 Map of the T-DNA region of the binary vector (A) and genomic Southern blot analysis to indicate the stability of the transgenes *miraculin* (B) and *NPTII* (C).

Figure 2 Schematic diagram of the T-DNA insertion regions. Flanking sequences obtained for each transgene were searched against the tomato genome sequence using the sol genomics network web site (http://www.sgn.cornell.edu/tools/blast/). The nearest genes were found by using BLAST in the above-mentioned site, and they are indicated with boxes and arrows (indicating transcriptional directions). In line 2A, only 600 bp of the genome sequence downstream from the 3' end of the transgene were determined.

Figure 3 The expression level of *miraculin* mRNA in transgenic tomatoes was analyzed by quantitative real-time RT-PCR. (A) The expression level of *miraculin* mRNA in all generations for each of the three transgenic lines (56B, 2A and 5B). Total RNA was isolated from leaves of 10 different plants in each line and each generation. (B) The expression level of *miraculin* mRNA in the T5 generation of line 56B. Total RNA was isolated from the leaves of 8 individual plants.

Figure 4 Accumulated recombinant miraculin protein was detected by western blot analysis. Soluble protein was extracted from the leaves of 10 different plants in each of the three transgenic lines (56B, 2A and 5B) and each generation. Twenty micrograms of soluble protein were applied per lane. W: wild type tomato, M: purified miraculin protein.

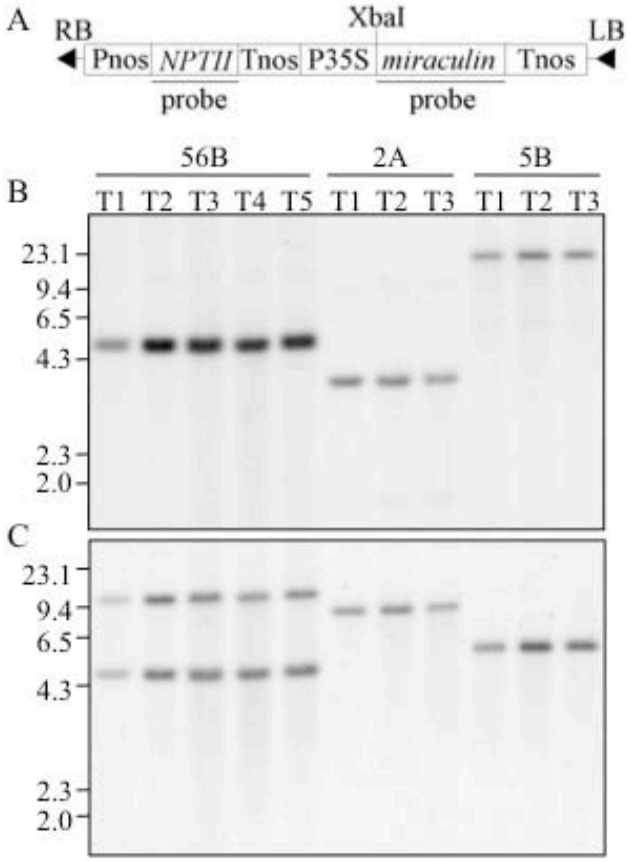
Table 1 Miraculin concentrations in all generations for each of the three lines of transgenic tomatoes.

transgenic tomato lines	generation	μg miraculin / mg protein	mg total soluble / g fresh weight	μg miraculin / g fresh weight
56B	T1	8.97 ± 0.31	11.2	100.5 ± 3.4
	T2	9.74 ± 0.21	11.1	108.1 ± 2.3
	T3	10.43 ± 0.19	8.1	84.5 ± 1.5
	T4	10.19 ± 0.37	10.0	101.9 ± 3.7
	T5	10.94 ± 0.17	10.2	111.6 ± 1.7
2A	T1	9.87 ± 0.30	11.4	112.6 ± 3.4
	T2	11.04 ± 0.99	10.6	117.1 ± 10.5
	T3	11.52 ± 1.03	11.0	126.7 ± 11.3
5B	T1	8.73 ± 0.30	10.1	88.2 ± 3.0
	T2	9.29 ± 0.31	9.8	91.0 ± 3.1
	T3	10.03 ± 0.76	10.5	105.3 ± 8.0

The concentrations of miraculin protein in extracts obtained from transgenic tomatoes were measured by ELISA. The soluble proteins were extracted from the leaves of 10 different plants in each line and each generation. The data are presented as means ± standard error (n=3).

368

Fig. 1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XbaI
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23.1
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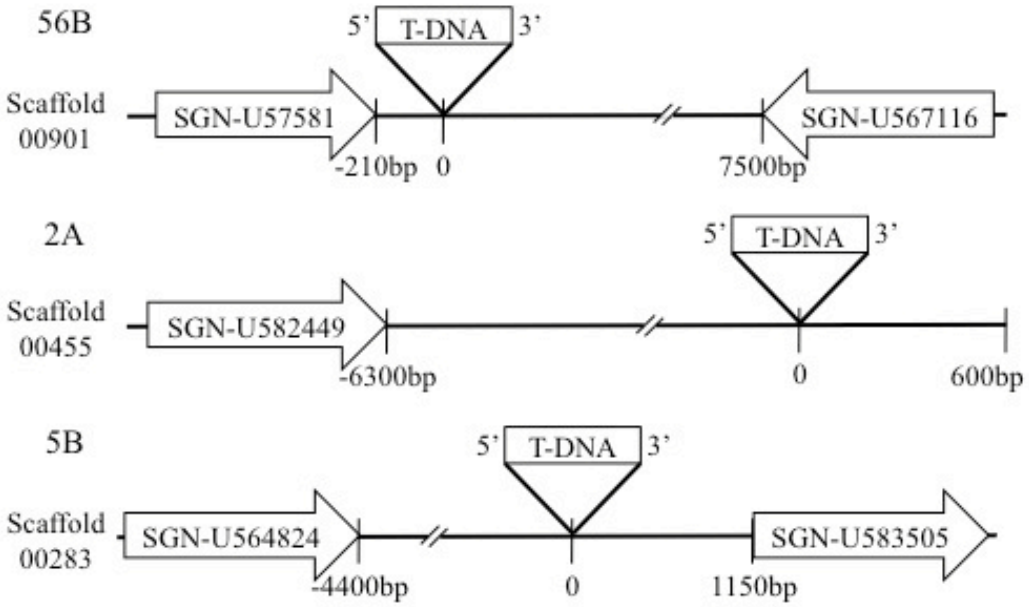
2.3
2.0

369

Fig. 2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5'
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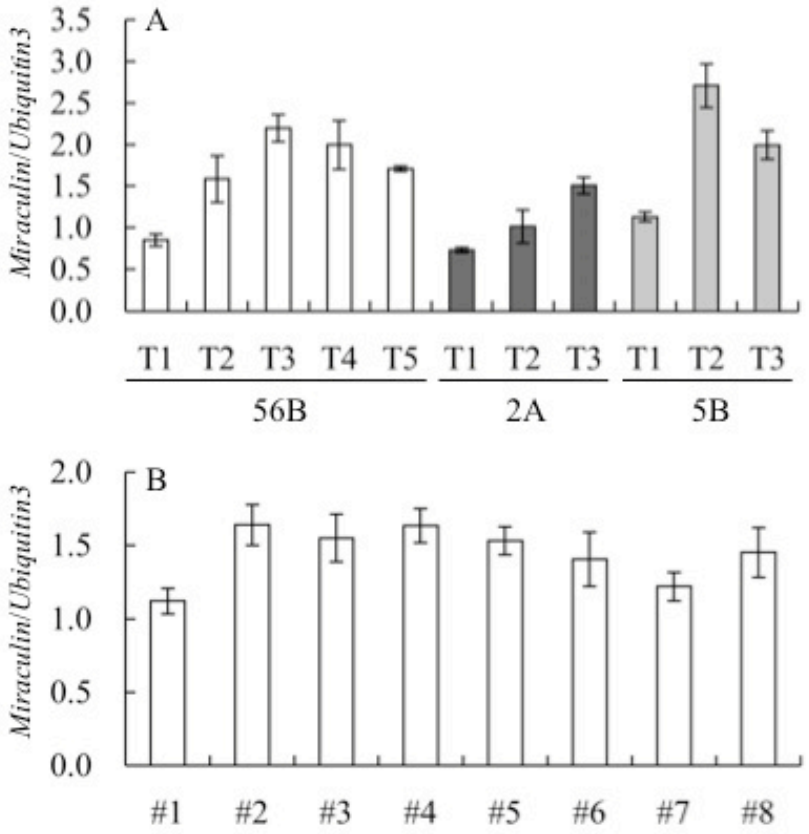
Fig. 3


A
Miraculin/Ubiquitin3
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Miraculin/Ubiquitin3
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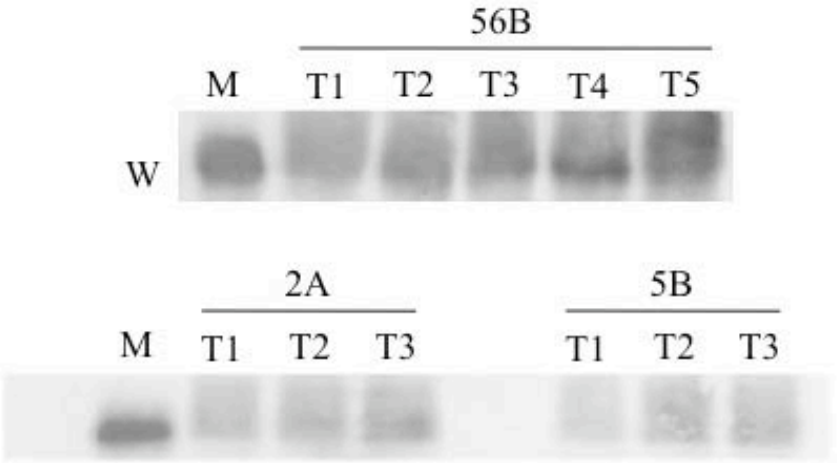
372

373

Fig. 4



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