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Original Paper

Transgenic strawberry expressing the taste-modifying protein miraculin

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Abstract The gene encoding the taste-modifying protein miraculin was introduced under the control of the 35S or El2 promoter into strawberry (*Fragaria* x *ananassa*) by *Agrobacterium*-mediated transformation to produce transgenic plants. Although miraculin was detected in the leaves and fruits of the transgenic plants, the level of accumulation among the transgenic lines, which ranged from 0.5 to 2.0 $\mu g\, g^{-1}$ fresh fruit, was not significantly different and was lower than that in miracle fruits (145 $\mu g\, g^{-1}$ fresh fruit). High levels of miraculin accumulation were detected in the mature fruits. The transgenic lines were subsequently propagated via the runners for three vegetative generations, and miraculin was detected at equal levels in the leaves and fruits of the plants from each generation. In conclusion, although the level of accumulation was not high, miraculin was stably expressed and accumulated in the vegetative progeny of the transgenic strawberry plants.

Key words: Miraculin, strawberry, transgenic plants, vegetative propagation.

Miraculin is a taste-modifying protein found 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 induced by citric acid after exposure to miraculin is estimated to be about 3000 times that of sucrose on a per weight basis (Kurihara and Beidler 1969; Theerasilp and Kurihara 1988; Gibbs et al. 1996; Kurihara and Nirasawa 1997), and interest has been increasing in miraculin due to this amazing property. Miracle fruit is available fresh and in powdered or tablet form in Japan, where it is particularly popular among people with diabetes or on diets. The material currently on the market is a native form of miraculin derived from miracle fruit.

Miraculin has great potential as an alternative low-calorie sweetener for diabetic and dietetic purposes, but miracle fruit production is limited because of the plant's tropical origin. To date, several attempts have been made to produce miraculin in foreign hosts, including *Escherichia coli* (Kurihara 1992), yeast (Kurihara and Nirasawa 1997), and tobacco (Kurihara and Nirasawa 1997). 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 (Ito et al. 2007). We have also successfully expressed recombinant miraculin in seed-propagated crop species, including lettuce (Sun et al. 2006) and tomato (Sun et al. 2007); however, in subsequent generations, stable miraculin expression was observed in tomato but silencing of the transgene occurred in lettuce, although the recombinant protein was correctly folded in both cases. This suggests that the plant species used for transformation and the mode of seedling propagation are important for producing miraculin-expressing transgenic plants.

In this study, we expressed the taste-modifying protein miraculin in an important agricultural crop, strawberry (*Fragaria* x *ananassa*). As strawberry plants are propagated vegetatively for fruit production, we assessed the potential of strawberry as a platform for miraculin production and analyzed whether the expression of miraculin was stable in subsequent vegetative generations of the transgenic plants.

Materials and methods

Transformation of strawberry

In vitro cultured strawberry (*F.* x *ananassa* 'Anther')-derived shoot apex cultures were applied in this study, and *Agrobacterium tumefaciens* GV2260 (Deblaere et al. 1985) was used as the host for the binary vectors 35S-MIR and El2-MIR (Sun et al. 2006), which carried the miraculin gene (GenBank

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accession number D38598) coding region. Transformation of the explants was performed according to Park et al. (2006). The primary transgenic plants (T0) were transferred to soil and later to a greenhouse. Vegetative generations of the transgenic plants were propagated by runner formation. The T0 and vegetatively propagated plants (V1, V2, and V3) were subjected to Western blotting and enzyme-linked immunosorbent assay (ELISA). V1, V2 and V3 plants were derived from T0 plants via runners. The plants were hand-pollinated for fruit setting.

PCR analysis

Genomic DNA was isolated from the leaves (1 g) of the putative transgenic plants and non-transgenic plants using the CTAB method (Rogers and Bendich 1985). PCR was used to confirm the presence of miraculin gene in the transgenic plants using miraculin-specific primers (forward: 5′-TTTTCTA-GAATGAAGGAATTAACAATGCT-3′ and reverse: 5′-TTT-GAGCTCTTAGAAGTATACGGTTTTGT-3′). A total of 100 ng of genomic DNA was used as the template, and amplification was carried out under the following conditions: pre-heating 94°C for 9 min followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR amplifications were performed by AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA, USA). After amplification, the products were separated on 1% agarose gels at 100 V for 30 min and visualized by ethidium bromide staining.

Southern blotting

Genomic DNA was isolated from the unfolding leaves (80 mg) of transgenic and non-transgenic plants in a greenhouse using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). For Southern blot analysis, 15 μg of genomic DNA was digested with *Xba*I, which cuts a single site within the T-DNA, separated on 1% agarose gels at 50 V for 3 h, and transferred to Hybond-N^+ nylon membranes (GE Healthcare UK Ltd., Buckinghamshire, UK). Thermostable alkaline phosphatase (AP)-labeled *miraculin*- and *nptII*-gene specific probes were generated using a CDP-Star AlkPhos Direct Labeling Kit according to the manufacturer's instructions (GE Healthcare UK Ltd.). The membrane was hybridized overnight at 55°C with the probes, and the hybridization signals were detected by chemiluminescence using CDP-Star (Roche Diagnostics, Mannheim, Germany) and an LAS4000mini Image Analyzer (Fujifilm Co. Ltd., Tokyo, Japan).

Western blotting and ELISA

Western blot analysis and ELISA were used to monitor the accumulation of miraculin in the transgenic plants. The leaves and fruits of the plants were frozen in liquid nitrogen and ground to a fine powder (0.1 g), which was then resuspended and vortexed in 400 μl of extraction buffer containing 50 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 1 mM PSMF, and 1.5% (w/v) polyvinylpolypyrrolidone (PVPP). The extracts were then centrifuged at 15,000 rpm for 20 min at 4°C. The resulting supernatants were collected and the soluble protein concentration was measured using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). The extracted protein was subjected to Western blotting and ELISA according to Sun et al. (2006).

Results

Transgenic plant production

Transgenic plants were regenerated from explants inoculated with *A. tumefaciens* harboring 35S-MIR or El2-MIR (Figure 1A), and the morphologically normal plants were selected. Subsequently, the ploidy levels were determined by flow cytometry (data not shown), and plants with the same peak pattern as the non-transgenic plants were selected as candidate transgenic plants. The presence of the transgene was verified by genomic PCR for *miraculin* gene (data not shown). Two lines each for the 35S promoter construct (35S-5 and 35S-6) and El2 promoter construct (El2-2 and El2-4) were selected as transgenic plants. Finally, integration of the transgene was confirmed by genomic Southern blotting. Line 35S-5 contained more than two copies of the transgene, whereas line 35S-6 contained a single copy of the transgene (Figure 1B), indicating that they

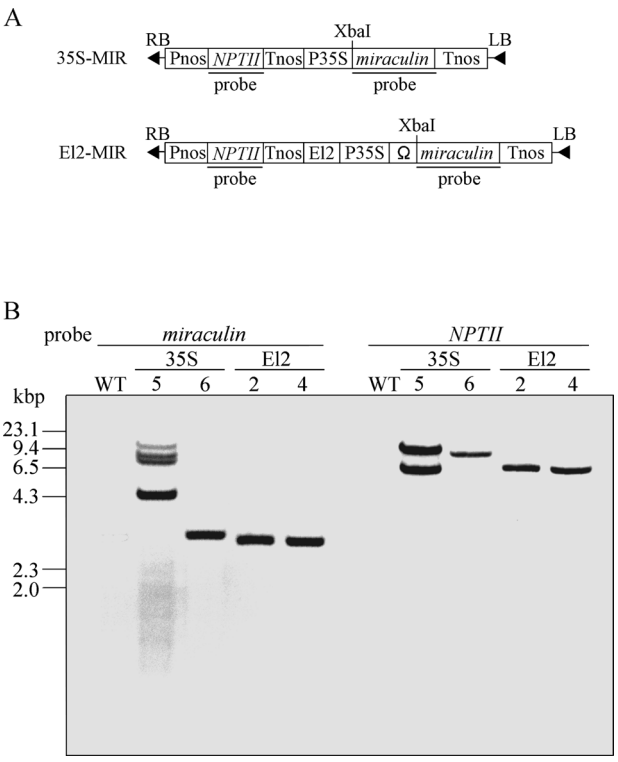

Figure 1. Map of the T-DNA region in the binary vectors 35S-MIR and El2-MIR (A) and Southern hybridization analysis of the strawberry transformants (B). RB, right border of the T-DNA; LB, left border of the T-DNA; Pnos, nopaline synthase gene promoter; *nptII*, neomycin phosphotransferase gene; Tnos, nopaline synthase gene terminator; P35S, CaMV 35S promoter; El2-P35S-Ω, the promoter cassette containing a translational enhancer (Mitsuhara et al. 1996); *miraculin*, *miraculin* gene. Genomic DNA from four transgenic lines (35S-5, 35S-6, El2-2, and El2-4) and a wild-type strawberry plant (WT) was used for Southern blot analysis. The *nptII* and *miraculin* coding sequences were used as probes.

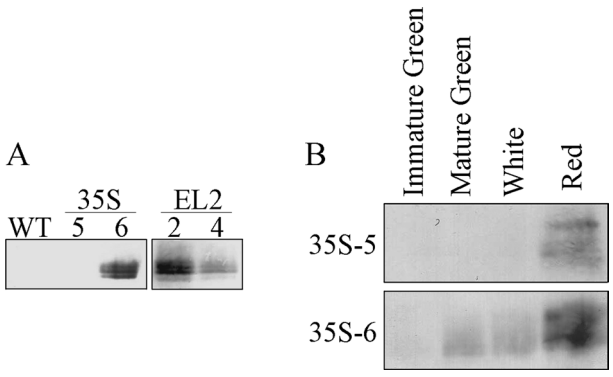

Figure 2. Analysis of miraculin accumulation by Western blotting in transgenic strawberry. (A) Miraculin accumulation in the leaves of four transgenic lines (35S-5, 35S-6, El2-2, and El2-4) and a wild-type strawberry plant (WT). (B) Miraculin accumulation in the fruits of two transgenic lines (35S-5 and 35S-6) at four developmental stages: immature green, mature green, white, and red.

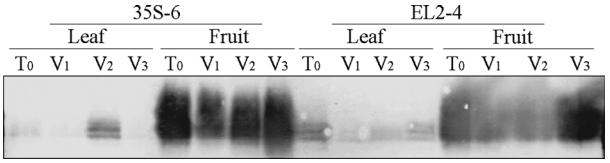

Figure 3. Analysis of miraculin accumulation by Western blotting in the transgenic lines 35S-6 and El2-4. Miraculin accumulation in the leaves and fruits at the primary transgenic (T0) and vegetative (V1–V3) generations was detected.

were independent lines. In contrast, lines El2-2 and El2-4 showed the same banding pattern using the *miraculin* and *nptII* probes, indicating that they might be clones. All four T0 lines were subjected to the following experiments.

Accumulation of miraculin protein in the T0 plants

Four transgenic lines were grown in a netted greenhouse. The leaves were analyzed by Western blotting to detect miraculin expression. Lines 35S-6, El2-2, and El2-4 showed miraculin accumulation in the leaves (Figure 2A), while miraculin accumulation in the developing fruits was detected in lines 35S-5 and 35S-6. Total proteins extracted from 0.2 g of fresh fruit were subjected to Western blotting. Accumulation of miraculin increased with fruit maturation and ripening (Figure 2B), even for those lines in which expression was driven by the constitutive 35S promoter. The detected band size was ~47 kDa corresponding to the dimeric form of miraculin, indicating that the transgenic plants expressed miraculin as dimers, as in miracle fruit (Igeta et al. 1991).

Accumulation of miraculin protein in vegetatively propagated plants

Total protein extracted from 0.2 g of fresh fruit was subjected to Western blotting. Although miraculin accumulation in young leaves and mature red fruits occurred in the vegetative generation of lines 35S-6 and El2-4 (Figure 3), a significantly higher level of accumulation was observed in the red fruit than in the leaves in both transgenic lines. Miraculin accumulation in the leaves and fruits of each primary transgenic line was nearly equal to that in the second, third, and fourth vegetative generations. Major bands were detected at ~47 kDa, corresponding to the dimeric form of miraculin, indicating the stable expression of miraculin in the vegetative progeny.

Table 1 Concentration of miraculin in transgenic strawberry plants and miracle fruit

Line	μg miraculin g^{-1} fresh weight*
35S-5	0.5
35S-6	2.0
El2-2	1.8
El2-4	1.5
Miracle fruit	145.0

* The miraculin concentration in a strawberry fruit protein extract was determined by ELISA.

Amount of miraculin in the transgenic plants

Miraculin accumulation in mature red fruits and miracle fruit was measured by ELISA (Table 1). The level of accumulation, which ranged from 0.5 to 2.0 μg g^{-1} fresh fruit, was not significantly different among the transgenic lines, and was significantly lower than that in miracle fruit (145 μg g^{-1} fresh fruit).

Discussion

Previously, recombinant miraculin was expressed in the seed-propagated crop species lettuce (Sun et al. 2006) and tomato (Sun et al. 2007). Miraculin expression was completely suppressed at the T1 generation in lettuce, whereas it was stable up to the T3 generation in tomato. Although the same construct (35S promoter, miraculin gene, and nos terminator) was used in both transformations, miraculin expression was suppressed in the subsequent generations in lettuce and stably expressed in the subsequent generations in tomato, suggesting that genetic background and mode of propagation are important for the expression of miraculin in transgenic plants. To analyze the expression of miraculin in a vegetatively propagated crop species, we introduced the same miraculin construct into strawberry. The transgenic strawberry plants successfully expressed miraculin, and the expression level was constant even after three vegetative generations. Strawberry has been widely used in transgenic research; genes conferring insect resistance, fungus, bacteria, and virus resistance, stress resistance, herbicide resistance,

and fruit quality improvement have all been introduced into strawberry plants (Qin et al. 2008). Although variability in transgene expression in strawberry was described in a recent review (Qin et al. 2008), transgene inactivation or silencing was not mentioned. This indicates that strawberry may be an ideal platform for the production of recombinant miraculin.

Miraculin accumulation in the transgenic strawberry plants ranged from 0.5 to 2.0 $\mu g\,g^{-1}$ fresh fruit, which is comparable to that reported for *A. oryzae* (Ito et al. 2007). Strawberry fruit production per plant ranges from 200 to 500 g depending on the cultivar and cultivation method, and 7000–10,000 nursery plants are planted per 1000 m^2 for commercial production (Ishihara et al. 1996; Ezura et al. 1998). Consequently, strawberry fruit production may be as high as 5000 kg/1000 m^2. Therefore, when transgenic strawberry plants expressing miraculin are cultivated in an area of 1000 m^2, about 10 g of miraculin will be produced. Based on our estimate of miraculin production, if we were to grow miracle fruit trees in an area of 1000 m^2, we could expect to harvest 500 kg of miracle fruits per year. Since miracle fruits contain 150 μg of miraculin per gram fresh weight, the total amount of miraculin produced would be 75 g/1000 m^2, which is 7.5 times higher than that produced using transgenic strawberry. Therefore, increasing the level of miraculin accumulation in the fruits is necessary for the practical application of transgenic strawberry to the production of recombinant miraculin.

In tomato, we were able to produce miraculin at a concentration of more than 100 $\mu g\,g^{-1}$ fresh fruit (Sun et al. 2007). This level of accumulation is 50 times higher than that in strawberry. The reduced accumulation of miraculin in strawberry fruits can be overcome in two possible ways. In most previous studies of transgenic strawberry, the 35S promoter and nos terminator were used for transgene expression (Qin et al. 2008). In contrast, we used the 35S and El2 promoters in this study (Mitsuhara et al. 1996). Transgenic strawberry plants in which the *GUS* gene was driven by the 35S promoter showed variable levels of GUS activity depending on the line (Cordero de Mesa et al. 2004). We produced four independent lines using two promoters (35S and El2), which suggests that the production of additional transgenic lines will eventually lead to a line with high levels of miraculin expression. The second possibility is to engineer the promoter and terminator regions. Several studies have reported that promoter- and terminator-derived plant genes allow for increased transgene expression, resulting in greater gene product accumulation (Outchkourov et al. 2003). Studies involving these types of approaches are currently under way.

In this study, we produced four lines of transgenic strawberry expressing miraculin. No miraculin accumulation was detected in the leaves of line 35S-5, and little accumulation was detected in the red fruit (Figure 2). As shown by genomic Southern analysis, this line contained more than two copies of the transgene while the other three lines each contained only a single copy (Figure 1B). In our previous study on transgenic lettuce (Sun et al. 2006), those lines containing multiple copies of the transgene exhibited reduced or no accumulation of miraculin. The mechanism is unclear, but a similar mechanism is speculated to suppress the accumulation of miraculin in transgenic strawberry.

In conclusion, although the level of accumulation was not high enough for commercial production, miraculin was stably expressed and accumulated in the vegetative progeny of transgenic strawberry, suggesting that strawberry may be a viable platform for recombinant miraculin production.

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