

# Functional expression of the taste-modifying protein, miraculin, in transgenic lettuce

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**Abstract** Taste-modifying proteins are a natural alternative to artificial sweeteners and flavor enhancers and have been used in some cultures for centuries. The taste-modifying protein, miraculin, has the unusual property of being able to modify a sour taste into a sweet taste. Here, we report the use of a plant expression system for the production of miraculin. A synthetic gene encoding miraculin was placed under the control of constitutive promoters and transferred to lettuce. Expression of this gene in transgenic lettuce resulted in the accumulation of significant amounts of miraculin protein in the leaves. The miraculin expressed in transgenic lettuce possessed sweetness-inducing activity. These results demonstrate that the production of miraculin in edible plants can be a good alternative strategy to enhance the availability of this protein.

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**Keywords:** Miraculin; Sweetness-inducing activity; Taste-modifying protein; Transgenic lettuce

## 1. Introduction

Bulk sweeteners and flavorsome ingredients, such as sugars, are essential foods that are important in food processing and contribute many benefits to foods. In recent years, the demand for “low-calorie” sweeteners has increased [1]. Artificial sweeteners like saccharin, aspartame, cyclamate, and acesulfame K are used worldwide as low-calorie sweeteners by patients with diseases linked to the consumption of sugar, such as diabetes, hyperlipemia, caries, and obesity; however, these substances can cause side effects, including psychological problems, mental disorders, bladder cancer, heart failure, and brain tumors [2]. Therefore, there is an intense, ongoing search for alternative sweeteners. Generally, high-molecular-weight substances do not stimulate taste cells and therefore have no taste. However, higher-molecular-mass sweet and taste-modifying proteins have been discovered that interact with the taste receptors in a potent and specific manner [3].

Sweet and taste-modifying proteins have traditionally been used by West Africans to improve flavor and suppress bitterness in food and drink. For example, they are used to improve the flavor of maize dishes, such as *agidi*, and beverages, such as palm wine or tea. In modern times, these proteins have been used in the food-processing industry as sweetening agents, flavor enhancers, and animal fodder supplements. These proteins can act at extremely low concentrations, and because of this low effective dose, they are effectively non-cariogenic and acceptable for diabetics in flavor and sweetening formulations. With the commercialization of thaumatin [4], there has been an increasing interest in these compounds. There are seven known sweet or taste-modifying proteins: thaumatin, monellin [5], mabinlin [6], pentadin [7], brazzein [8], curculin [9], and miraculin [10]. The genes for these proteins have been cloned and sequenced, and many have been expressed in foreign hosts [1,3,4,11].

*Richadella dulcifica*, a shrub native to tropical West Africa, produces red berries that have the unusual ability to modify a sour taste into a sweet taste. For example, lemons taste like oranges when they are eaten after these berries have been chewed. Owing to this unique property, the berry has been called the miracle fruit. The active ingredient in the berry, miraculin, is a taste-modifying protein that causes citric acid, ascorbic acid, and acetic acid, which are normally sour, to be perceived as sweet after the berry has been held in the mouth.

Miraculin was first isolated by Kurihara and Beidler [12]. The complete amino acid sequence of miraculin has been determined [13], and the cDNA corresponding to miraculin has been cloned and sequenced [14]. Miraculin by itself does not elicit a sweet response. Like curculin, however, it can modify a sour taste into a sweet taste. Kurihara and Beidler [12] therefore termed the protein a taste-modifying protein. The maximum sweetness after exposure to 0.4  $\mu$ M miraculin induced by 0.02 M citric acid was estimated to be around 400 000 times that of sucrose on a molar basis [10,11,15,16]. Therefore, to supply the same sweetening effect as sucrose, only minute amounts of miraculin are required. This results in an almost negligible addition to the calorie count. Therefore, miraculin could be used as a natural low-calorie sweetener by individuals suffering from diseases linked to the consumption of sugars, including obesity, diabetes, and hyperlipemia, and to control the palatability of foods.

The taste-modifying protein, miraculin, is obviously an attractive alternative to some of the more traditional sweeteners, such as sucrose. However, the commercial feasibility of miraculin is very low because the natural source of this protein is a tropical plant that is difficult to cultivate outside of its

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**Abbreviations:** BAP, 6-benzylaminopurine; CaMV, cauliflower mosaic virus; NAA, 1-naphthaleneacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate

natural environment. Depending on the market need for the particular product, extraction from the natural plant may not be able to keep up with demand. Therefore, as an alternative to the production of miraculin from its natural source, attempts have been made to produce recombinant miraculin in *Escherichia coli* [17], yeast [16], and transgenic tobacco [16]. Although miraculin has been expressed in these organisms, recombinant miraculin lacks the sweetness-inducing activity.

Genetic engineering of plants may offer a solution to this problem. The development of genetic transformation technology for plants has enabled the expression of foreign genes in different plant species, allowing the use of plants as bioreactors to produce recombinant proteins. Transgenic plants are being used to produce desired recombinant proteins, such as vaccines, antibodies, mammalian hormones, biopharmaceuticals, and food additives [18–22].

In this study, we attempted to express the taste-modifying protein, miraculin, in lettuce as a potential alternative sweetener. Lettuce is an agricultural crop that is widely grown worldwide. A synthetic gene encoding miraculin was assembled and inserted into plant expression vectors. Recombinant miraculin was expressed in transgenic lettuce and showed strong sweetness-inducing activity. To our knowledge, this is the first report on the production of the biologically active form of miraculin in a foreign host.

## 2. Materials and methods

### 2.1. Synthesis of miraculin cDNA and construction of plasmids for its expression

Total RNA was isolated from the pulp of *R. dulcifica* berries using an RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from a 1- $\mu$ g total RNA sample following the protocol of the RT-PCR High kit (Toyobo). Based on the published DNA sequence of miraculin [14] (Accession No. D38598), a pair of specific primers (forward primer: 5'-TTTTCTAGAATGAAGGAATTAACAATGCT-3', and reverse primer: 5'-TTTGAGCTCTTAGAAGTATACGGTTTTGT-3') was designed and used to amplify the coding region of miraculin. The forward primer contained the recognition sequence for *Xba*I, and the reverse primer contained the recognition sequence for *Sac*I. The amplification reaction consisted of 95 °C for 5 min, 40 cycles of amplification (95 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min), and a final extension at 72 °C for 10 min. The resulting polymerase chain reaction (PCR) fragments were purified and subcloned into the *Xba*I/*Sac*I sites of the plasmid pUC19. The sequences of this clone were confirmed by DNA sequencing. The plasmid pUC19 was digested with *Xba*I and *Sac*I to clone the miraculin-encoding 660-bp DNA fragment into the *Xba*I/*Sac*I sites of the plant transformation vectors pBI121 (Clontech) and pBE2113-GUS [23] after removing the GUS-coding region. The resulting plasmids, named 35S-MIR and E12-MIR, contained the miraculin coding region. 35S-MIR and E12-MIR were transferred to *Agrobacterium tumefaciens* GV2260 [24] using the method of Shen and Forde [25].

### 2.2. Transformation of lettuce plants

Lettuce seeds (*Lactuca sativa* cv Kaiser) were surface sterilized and germinated on Murashige and Skoog (MS) medium [26] with 2% (w/v) sucrose and 0.2% (w/v) Gelrite under a 16-h light/8-h dark photoperiod at 25 °C. The lettuce plants were transformed by infection with *A. tumefaciens* GV2260 harboring the binary vectors 35S-MIR and E12-MIR. *Agrobacterium* strains were grown in 2 ml of LB medium containing 100 mg/l kanamycin at 28 °C for 24 h and then diluted 1:40 (v/v) in liquid MS medium containing 0.1 mM acetosyringone (Sigma–Aldrich) and 0.01 mM 2-mercaptoethanol. Excised cotyledons from 5-day-old seedlings were inoculated by immersion for 5 min in the diluted bacterial suspension and transferred to a solid co-cultivation medium supplemented with 1 mg/l 6-benzylaminopurine (BAP) and 0.1 mg/l 1-naphthaleneacetic acid (NAA). After 3 days of

co-cultivation, the explants were transferred to solid MS medium containing 0.1 mg/l BAP, 0.1 mg/l NAA, 100 mg/l kanamycin, and 375 mg/l Augmentin (GlaxoSmithKline) for the selection of transformed shoots. About 4 weeks after explant inoculation, the levels of kanamycin, BAP, and NAA were reduced to 50, 0.01, and 0.05 mg/l, respectively. Approximately 2- to 3-cm-long shoots were excised and transferred to solid MS medium containing 50 mg/l kanamycin for rooting. After incubation in the conditioned medium, transformants were selected. The transgenic plants were kept at 25 °C under a 16-h light/8-h dark photoperiod with fluorescent light at an intensity of 60  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>.

### 2.3. Southern hybridization analysis

Genomic DNA was extracted from 0.5 g of fresh young leaves using the CTAB extraction method of Rogers and Bendich [27]. For Southern blot analysis, 10  $\mu$ g of genomic DNA were digested with *Xba*I, electrophoresed on a 1% agarose gel at 50 V for 4 h, and transferred to a Hybond-N+ (Amersham Biosciences) nylon membrane under alkaline conditions. The membrane was hybridized overnight at 60 °C with a 660-bp <sup>32</sup>P-labeled miraculin gene fragment amplified by PCR using the two primers described above. The membrane was given a final wash in 2 $\times$ SSC containing 0.1% sodium dodecyl sulfate (SDS) at 60 °C for 15 min. The hybridization signals were detected using BAS-5000 image analyzer (Fuji Photo Film, Japan).

### 2.4. Northern hybridization analysis

Total RNA was isolated from leaf tissue of transgenic and wild-type lettuce plants using an RNeasy Plant Mini Kit (Qiagen). Fifteen micrograms of total RNA were size-fractionated on an agarose gel containing 1% formaldehyde and transferred to a Hybond-N+ (Amersham Biosciences) nylon membrane. Northern blot hybridizations were carried out under the same conditions as described for Southern blot hybridization.

### 2.5. Preparation of anti-miraculin antibody

Anti-miraculin antibody was prepared as follows: recombinant protein containing amino acids 1–191 of mature miraculin was produced in *E. coli* using pQE30 (Qiagen) and used to raise antiserum in rabbits with the assistance of the Scrum Inc. (Tokyo, Japan). Rabbit anti-miraculin antibody was purified by fractionation with ammonium sulfate (40% saturation) followed by immunoaffinity chromatography on a column of Sepharose 4 Fast Flow (Amersham Biosciences) which was coupled with the N-terminal 89-residue polypeptide of mature miraculin (residues 1–89 of miraculin) produced in *E. coli* using pGEX-4T-1 (Amersham Biosciences) following the cyanogen bromide procedure [28].

### 2.6. Purification of miraculin from miracle fruit and transgenic lettuce

Native miraculin was purified from the pulp of *R. dulcifica* according to a described method [10]. Recombinant miraculin was purified from the transgenic lettuce plant lines expressing the most miraculin (35S-MIR 29B and 16B and E12-MIR 13A and 38A). Fully expanded young leaves (100 g fresh weight) were collected and ground in liquid nitrogen to a fine powder. The powder was resuspended in 200 ml of extraction buffer, consisting of 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 2 mM EDTA, and 4% polyvinylpyrrolidone (PVP). The leaf extract was centrifuged at 15000 rpm for 20 min at 4 °C and the resultant supernatant was dialyzed against 0.02 M sodium phosphate buffer (pH 7.2). The dialyzed solution was applied to a column (2.0 $\times$ 35 cm, bed volume: 94 ml) of CM-Sepharose Fast Flow (Amersham Biosciences), which was equilibrated with 0.02 M sodium phosphate buffer (pH 7.2) at a flow rate of 25 ml/h. It was eluted with a linear gradient starting with 200 ml of the 0.02 M sodium phosphate buffer (pH 7.2) in a mixing flask and 200 ml of the same buffer solution containing 1.0 M of NaCl. The fractions containing miraculin were collected and dialyzed against 0.02 M sodium phosphate buffer (pH 7.2) containing 0.5 M NaCl. The dialyzed solution was applied to a Con A Sepharose 4B (Amersham Biosciences) column (1.6 $\times$ 10 cm, bed volume: 14 ml) equilibrated with 0.02 M sodium phosphate buffer (pH 7.2) containing 0.5 M NaCl at a flow rate of 30 ml/h. The column was washed with the starting buffer. It was then eluted with a linear gradient starting with 50 ml of the starting buffer in a mixing flask and 50 ml of the same buffer solution containing 0.15 M of methyl- $\alpha$ -D-glucoside (Tokyo

Kassei). The fractions containing miraculin were collected and concentrated. The concentrated solution was applied to a Sephacryl S-200 HR column (2.6 × 110 cm, Amersham Biosciences) equilibrated with 0.05 M phosphate buffer solution (pH 6.4) containing 0.15 M NaCl at a flow rate of 60 ml/h. The active fractions were collected and used as purified recombinant miraculin.

### 2.7. Western blot analysis and enzyme-linked immunosorbent assay (ELISA)

The expression levels of the lettuce plants expressing miraculin were determined using immunological measurements. Soluble protein was extracted from frozen leaf samples. Fully expanded young leaves were collected and ground in liquid nitrogen to a fine powder. The powder (0.2 g) was resuspended in 400 µl of extraction buffer consisting of 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 2 mM EDTA, and 4% PVPP. The leaf extract was centrifuged at 15000 rpm for 20 min at 4 °C, and the resulting supernatant was used for Western blot analysis and ELISA. The extracted proteins (25 µg per lane) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto Hybond-P membrane (Amersham Biosciences). After blocking with 3% gelatin, the blots were reacted with affinity-purified anti-miraculin antibody at room temperature for 1 h. The immunoreactive proteins were detected using a Western blotting detection kit (Bio-Rad). In addition, the miraculin concentration of the transgenic lettuce was measured using an ELISA using anti-miraculin IgG and peroxidase-conjugated anti-miraculin IgG as described [29]. Peroxidase-conjugated anti-miraculin IgG was prepared using a Peroxidase Labelling Kit-SH (Dojindo, Japan). A dilution series of purified native miraculin and a non-transgenic lettuce sample were included in the assay for reference. The protein concentration of extracts was determined using a BCA protein assay kit (Pierce).

### 2.8. Analysis of dimerization and N-glycosylation of recombinant miraculin expressed in transgenic lettuce

Dimerization of the recombinant miraculin expressed in transgenic lettuce was confirmed using non-reducing and reducing SDS-PAGE. Extracts (25 µg per lane) from transgenic lettuce plants were separated by SDS-PAGE under non-reducing and reducing conditions and analyzed by Western blotting as described above. The N-glycosylation of recombinant miraculin was analyzed using Western blot after treatment with N-glycosidase A (Roche). The total soluble proteins (50 µg) extracted from transgenic lettuce were prepared in 0.01 M sodium acetate buffer (pH 5.1) and boiled for 5 min, and then 4 mU N-glycosidase A (Roche) were added. Enzyme digests were conducted at 37 °C for 24 h, and aliquots of protein were subjected to SDS-PAGE in the presence of dithiothreitol and then to Western blot analysis. Purified native miraculin (0.5 µg) was treated with 2 mU N-glycosidase A under the same conditions and included in the analysis for reference.

### 2.9. Measurements of taste-modifying activity

The taste-modifying activity of miraculin was assayed using six subjects, as described previously [15]. Prior to evaluation, subjects tasted repeatedly a series of standard sucrose solutions (0.1–1.0 M). Then, they either chewed one or two grams of lettuce leaf for 3 min or held 5 ml of 0.4 µM purified miraculin solution in the mouth for 3 min and spat out. The mouth was rinsed with water and then 0.02 M citric acid was tasted. The sweetness induced by 0.02 M citric acid was evaluated by comparing its sweetness to that of a series of standard sucrose solutions. The subjects were asked to choose which of ten sucrose solutions best approximated the intensity of sweetness induced by the acid. At least 3 h was interposed between each test.

## 3. Results

### 3.1. Transforming lettuce plants with the miraculin gene

Miraculin accumulates specifically in the pulp of the miracle fruit [14]. To determine if miraculin could be produced in other plant tissues, we constructed chimeric genes using the *miraculin* gene and two different promoters (Fig. 1A). Lettuce plants were transformed by infection with *A. tumefaciens* strain

GV2260 [24] harbouring the binary vectors 35S-MIR and E12-MIR. The presence of the *miraculin* gene in these plants was confirmed by Southern blot analysis (Fig. 1B). Genomic DNA from 40 transformed plants was digested with *Xba*I endonuclease, which cuts the T-DNA in plasmids 35S-MIR and E12-MIR only once outside the *miraculin* gene, so the number of bands, in most cases, should be equal to the number of transgenes. The hybridization of restricted genomic DNA from the selected transformed lines with radiolabeled probe for the *miraculin* gene revealed that the clones represented independent transformation events, and the number of transgene copies varied from one to several in different transformed lines. Thirty-six independent transgenic lettuce plants were obtained (Fig. 1B).

### 3.2. Accumulation of miraculin mRNA in transgenic lettuce

To determine whether transgenic lettuce plants expressed the miraculin mRNA, Northern blot analysis was carried out using total RNA isolated from 19 randomly selected transformed lines. Northern blot analysis showed the presence of miraculin transcripts in 13 transformed lines, whereas no transcripts were detected in untransformed plants. The transformants showed variation in expression. We found no correlation between the number of copies of the *miraculin* gene in a lettuce genome and its level of expression, although the level of *miraculin* gene expression was higher in single-copy transformants than in multi-copy transformants. These results indicate that miraculin mRNA was successfully expressed in lettuce leaf tissue (Fig. 2A).

### 3.3. Expression of the miraculin protein in transgenic lettuce

To assay for the production of miraculin, lettuce leaf protein was isolated and analyzed using Western blotting (Fig. 2B). As expected, miraculin was not detected in leaves harvested from untransformed control lettuce. In contrast, miraculin was detected in three transgenic lettuce plants bearing an E12-miraculin gene and in five transgenic lettuce plants bearing a 35S-miraculin gene (Fig. 2B). The expression levels in the different plant lines varied markedly. Unexpectedly, a comparison of the expression level in E12-35S-Ω and 35S promoter plants showed that the expression was not higher with the E12-35S-Ω promoter. The miraculin protein in lettuce plants was very similar in size to the native miraculin extracted from the pulp of *R. dulcifica* berries.

### 3.4. Analysis of dimerization and N-glycosylation of recombinant miraculin expressed in transgenic lettuce

Miraculin exists naturally as a disulfide-linked dimer and is a basic glycoprotein. The molecular weight of the miraculin dimer was 43 kDa on SDS-PAGE [17]. Since the molecular weight of the recombinant miraculin expressed in lettuce was about 45 kDa on non-reduced SDS-PAGE (Fig. 2B), it is possible that the recombinant miraculin forms a disulfide-linked dimer and is glycosylated.

To confirm the dimerization of the miraculin subunits expressed in transgenic lettuce, soluble protein extracts from the transgenic lettuce plants were analyzed by Western blotting, and the results are shown in Fig. 3A. The molecular weights of the miraculin under non-reducing and reducing conditions were about 45 and 28 kDa, respectively. These results suggest that the miraculin expressed in transgenic lettuce is a

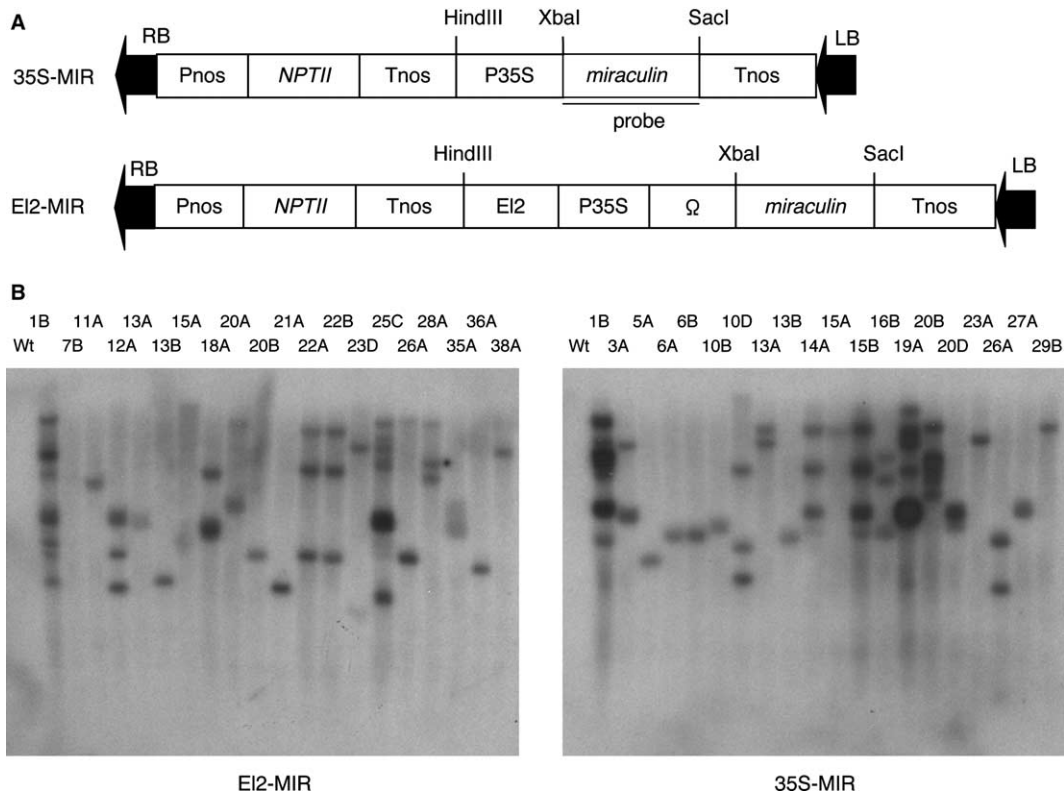


Fig. 1. Map of the *T*-DNA region of binary vectors 35S-MIR and EI2-MIR (A) and Southern hybridization analysis of lettuce transformants (B). RB, right border of *T*-DNA; LB, left border of *T*-DNA; Pnos, nopaline synthase gene promoter; *NPTII*, neomycin phosphotransferase gene; Tnos, nopaline synthase gene terminator; P35S, CaMV 35S promoter; EI2-35S-Ω, promoter cassette containing a translational enhancer; *miraculin*, miraculin gene. The genomic DNA from 40 transgenic lettuce plants (1B–29B) and a wild-type lettuce plant (Wt) were used for Southern blot analysis.

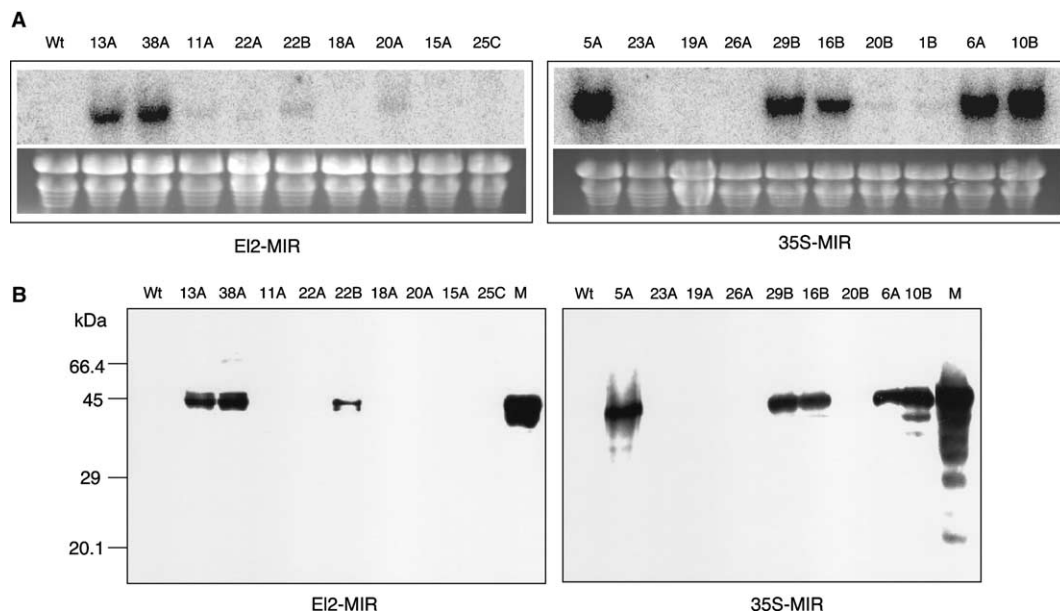


Fig. 2. Expression of miraculin RNA and protein in  $T_0$  transgenic lettuce plants. (A) Accumulation of miraculin RNA in transgenic lettuce plants. Lanes: Wt, untransformed lettuce plant; 13A–10B, transgenic lettuce plants. Bottom: ribosomal RNA after ethidium bromide staining is shown for equal loading. (B) Detection of miraculin protein in transgenic lettuce plants. Lanes: Wt, untransformed lettuce plant; M, extract from miracle fruit as positive control; 13A–25C, transgenic lettuce plants derived from EI2-MIR construct; 5A–10B, transgenic lettuce plants derived from 35S-MIR construct. The size of the protein standard is shown in kDa on the left.

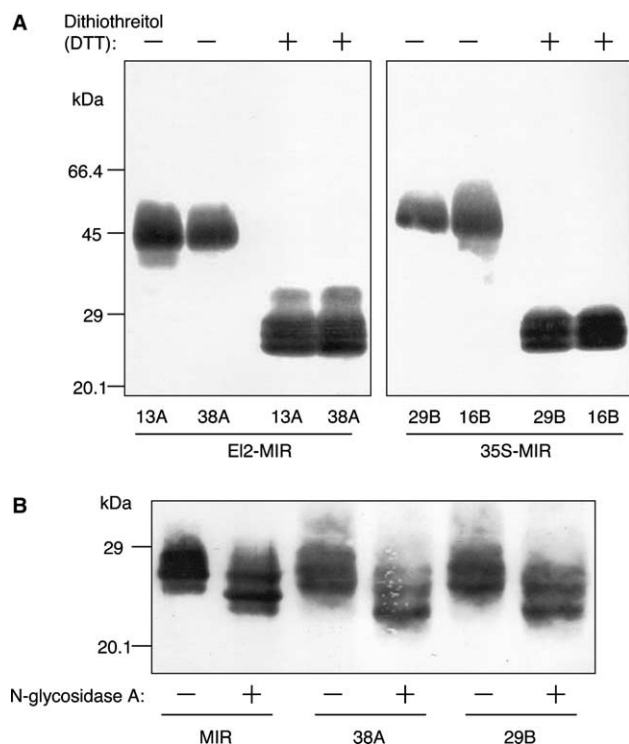


Fig. 3. Analysis of the post-translational modification of recombinant miraculin expressed in transgenic lettuce. (A) Analysis of the dimerization of recombinant miraculin expressed in transgenic lettuce. SDS-PAGE was performed under non-reducing and reducing conditions. Lanes: 13A and 38A, transgenic lettuce lines derived from E12-MIR construct; 29B and 16B, transgenic lettuce lines derived from 35S-MIR construct. (B) Analysis of the *N*-glycosylation of recombinant miraculin in lettuce. SDS-PAGE was performed under reducing conditions. Lanes: MIR, purified miraculin from miracle fruit; 38A, transgenic lettuce line derived from E12-MIR construct; 29B, transgenic lettuce line derived from 35S-MIR construct. The size of the protein standard is shown in kDa on the left.

dimer and that the dimerization results from the formation of interchain disulfide linkages between the miraculin subunits.

Since miraculin contains two *N*-glycosylation sites [13], we tested whether the miraculin in lettuce is glycosylated. Soluble protein extracts from the transgenic lettuce plants were treated with peptide *N*-glycosidase A (PNGase A, Roche), a glycoamidase that liberates *N*-linked oligosaccharides from glycopeptides and glycoproteins [30]. As shown in Fig. 3B, after *N*-glycosidase A treatment, smaller bands with a molecular mass of approximately 23 kDa were detected in lettuce, as in native miraculin. This indicates that the recombinant miraculin in transgenic lettuce is an *N*-glycosylated protein.

### 3.5. Quantification of the recombinant miraculin in transgenic lettuce

The miraculin content in crude extracts of transgenic lettuce plants was measured using an ELISA with affinity-purified anti-miraculin IgG and peroxidase-conjugated anti-miraculin IgG. The results for the two highest-producing transgenic lettuce plants for each line derived from the two constructs are shown in Table 1. The concentrations of miraculin from the two E12-MIR construct plants and two 35S-MIR construct plants were 33.7, 42.3, 43.5, and 39.8  $\mu\text{g}$  per gram fresh weight, respectively. This indicates that the recombinant miraculin

Table 1  
Concentration of miraculin in transgenic lettuce plants

	Transformants			
	E12-MIR 13A	38A	35S-MIR 29B	16B
$\mu\text{g}$ miraculin/g fresh weight <sup>a</sup>	33.7	42.3	43.5	39.8
mg total soluble protein/g fresh weight	4.7	4.5	4.3	5.1
$\mu\text{g}$ miraculin/mg total soluble protein	7.2	9.4	10.1	7.8

The miraculin concentration was determined for a protein extract obtained from transgenic lettuce.

<sup>a</sup>The miraculin content was measured using an enzyme-linked immunosorbent assay, as described in Section 2. The protein concentration was determined as described in Section 2.

constituted approximately 1% of the total soluble protein extracted from the transgenic lettuce.

### 3.6. Purification of native and recombinant miraculin

To test whether the purified miraculin retains its intrinsic sweetness-inducing activity, native and recombinant miraculin were purified. Native miraculin was purified from the pulp of *R. dulcifica* according to a described method [10], using miracle fruit pulp free from skin and seeds. Approximately 4.3 mg of pure native miraculin were obtained from 15 g of fresh miracle fruit. The purified miraculin gave a single band with a molecular weight of 45 kDa on non-reducing SDS-PAGE (Fig. 4). The recombinant miraculin was purified from the highest miraculin-expressing transgenic lettuce plant lines, as described in Section 2. The fractions generated during a representative purification step were subject to Western blot analyses using anti-miraculin antibody. The fractions were also analyzed using SDS-PAGE, and the results are shown in Fig. 4. The purified

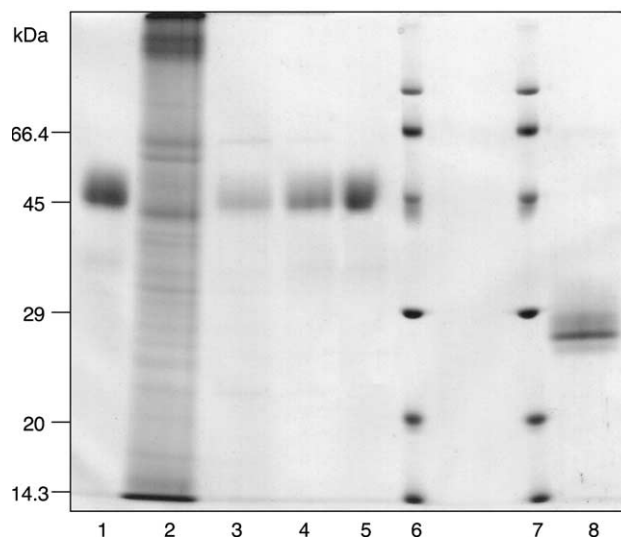


Fig. 4. Non-reducing (lanes 1–5) and reducing SDS-PAGE (lane 8) of products from the multi-step purification for the isolation of miraculin from transgenic lettuce. Lanes: 1, purified native miraculin from miracle fruit; 2, crude extract from transgenic lettuce; 3, miraculin peak from cation-exchange chromatography; 4, miraculin peak from affinity chromatography; 5, purified miraculin (dimer) from gel filtration chromatography; 6 and 7, standard marker proteins; 8, purified miraculin (monomer). The size of the protein standard is shown in kDa on the left.

Table 2  
Sweetness intensity induced by native and recombinant miraculin

Source material	Concentration of miraculin in taste sample ( $\mu\text{g}$ )	Induced sweetness <sup>a</sup> (SEV) (M)
Miracle fruit (one fresh fruit)	120.0	$0.31 \pm 0.01$
Lettuce leaf tissue (1 g fresh weight)	43.5	$0.19 \pm 0.01$
Lettuce leaf tissue (2 g fresh weight)	87.0	$0.30 \pm 0.01$
Purified native miraculin	90.0	$0.32 \pm 0.02$
Purified recombinant miraculin	90.0	$0.31 \pm 0.01$

The miraculin concentration of the source material was measured using an enzyme-linked immunosorbent assay, as described in the materials and methods.

<sup>a</sup>The sucrose equivalence value (SEV) corresponds to the sweetness intensity induced by 0.02 M citric acid evaluated by comparing its sweetness to that of a series of standard sucrose solutions (0.1–1.0 M). Data represent the mean of six subjects  $\pm$  S.E.

miraculin gave multiple broadbands on SDS–PAGE, and the molecular weights of the purified miraculin under non-reducing and reducing conditions were about 45 and 28 kDa, respectively. SDS–PAGE likely resulted in multiple broadbands because miraculin is a glycoprotein. Approximately 1 mg of purified recombinant miraculin was obtained from 100 g of fresh lettuce leaf tissue. This purification protocol was repeated multiple times to obtain a sufficient amount of purified miraculin to test its taste-modifying activity.

### 3.7. Measurements of the taste-modifying activity

As potential applications for recombinant miraculin expressed in lettuce may require the use of either a purified protein or a less purified material derived from lettuce leaf tissue, it is useful to estimate the taste-modifying activity of miraculin both within a lettuce leaf fraction and as a purified protein. To determine whether the miraculin protein within lettuce leaf tissue possesses taste-modifying activity, lettuce leaf tissue was used for sensory evaluation. The sweetness induced by 0.02 M citric acid after chewing 1 or 2 g of transgenic lettuce leaf was equivalent to that of about 0.2 and 0.3 M sucrose solution (Table 2), respectively. The sweetness induced by 2 g of transgenic lettuce was almost same as that of the sweetness induced by one miracle fruit, and the sweetening effect did not increase when more transgenic lettuce was placed in the mouth. No sweetness was induced under the conditions of this analysis with non-transgenic lettuce leaf tissue. The evaluation of the taste-modifying activity of purified miraculin solutions from miracle fruit and transgenic lettuce leaf tissues is summarized in Table 2. The sweetness induced by 0.02 M citric acid after 0.4  $\mu\text{M}$  miraculin was held in the mouth was equivalent to the sweetness of about 0.3 M sucrose solution. This value is equal to that of the maximum sweetness induced by miraculin [16]. These results clearly demonstrate that the miraculin expressed in lettuce possesses strong sweetness-inducing activity.

## 4. Discussion

Plants are increasingly being used as bioreactors for the commercial production of a number of valuable proteins [21,22]. The expression of transgenic proteins in edible plants has the particular advantage of producing materials that can be consumed directly, a characteristic that has been exploited to develop oral vaccines [31].

Here, we report the use of a lettuce expression system for the production of the taste-modifying protein miraculin as a potential alternative sweetener. In the present study, recombinant miraculin produced in transgenic lettuce plants formed disulfide-linked dimer, was *N*-glycosylated and showed strong

sweetness-inducing activity. This suggests that using 35S or EL2-35S- $\Omega$  promoters, it is possible to produce miraculin in transgenic plants. In addition, these results imply that the cleavable N-terminal signal peptide of the precursor of miraculin [14] may regulate folding of the precursor miraculin, induce translocation, and play a role in the secretion of the protein. The expression levels of miraculin in transgenic lettuce plants were estimated to be up to 1% of the total soluble protein extracted from 1 g of fresh tissue, which corresponds to approximately 40  $\mu\text{g}$  of miraculin per gram of lettuce. The sweetness after exposure to 2 g of transgenic lettuce leaf tissue induced by 0.02 M citric acid was equivalent to that of the sweetness of 0.3 M sucrose. On a molar basis, the sweetness of miraculin is 300000 times that of sucrose. This suggests that the recombinant miraculin expressed in transgenic lettuce is biologically active and that the expression level is sufficient for modifying taste. Expression of the miraculin protein was detected in  $T_1$  and  $T_2$  generation transgenic lettuce plants. However, the expression level of miraculin in the  $T_1$  and  $T_2$  progenies was at least 10 times lower than that of  $T_0$  plants (data not shown). Genetic engineering relies on stable integration, the desired level of expression, and predictable inheritance of the introduced transgenes, although many recent studies have shown that transgene instability frequently occurs in transgenic plants [32,33]. Although the mechanisms of this instability, e.g., gene silencing or loss, are not fully understood, it is a major obstacle in the transformation of plants. One of the keys to future success will undoubtedly lie in finding a way to maintain the level of expression of the recombinant protein in plants over many generations without silencing. Clearly, there will be a drive to attain higher levels of expression, and there is much more room for improvement over established systems. We are also testing new strategies to increase miraculin production in transgenic plants. In conclusion, the production of recombinant miraculin in transgenic plants will open up new ways to study taste-modifying proteins and the commercial application of miraculin as a low-calorie sweetener.

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