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ORIGINAL PAPER

Ubiquitin promoter–terminator cassette promotes genetically stable expression of the taste-modifying protein miraculin in transgenic lettuce

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Abstract Lettuce is a commercially important leafy vegetable that is cultivated worldwide, and it is also a target crop for plant factories. In this study, lettuce was selected as an alternative platform for recombinant miraculin production because of its fast growth, agronomic value, and wide availability. The taste-modifying protein miraculin is a glycoprotein extracted from the red berries of the West African native shrub *Richadella dulcifica*. Because of its limited natural availability, many attempts have been made to produce this protein in suitable alternative hosts. We produced transgenic lettuce with miraculin gene driven either by the ubiquitin promoter/terminator cassette from lettuce or a 35S promoter/nos terminator cassette. Miraculin gene expression and miraculin accumulation in both cassettes were compared by quantitative real-time PCR analysis, Western blotting, and enzyme-linked immunosorbent assay. The expression level of the miraculin gene and protein in transgenic lettuce was higher and more genetically stable in the ubiquitin promoter/terminator cassette than in the 35S promoter/nos terminator cassette. These results demonstrated that the ubiquitin promoter/terminator cassette is an efficient platform for the genetically stable expression of the miraculin protein in lettuce and hence this platform is of benefit for recombinant miraculin production on a commercial scale.

Keywords Miraculin · Stable expression · Transgenic lettuce · Ubiquitin promoter · 35S promoter

Abbrevations

Ubi	Ubiquitin
MIR	Miraculin
NOS	Nopaline synthase
PCR	Polymerase chain reaction

Introduction

Lettuce is a popular and easy-to-grow leafy vegetable cultivated worldwide. Lettuce is a typical crop that is commercially cultivated in plant factories, which are indoor cultivation systems with a controlled light period, light intensity, temperature, and CO_2 concentration for mass production of target plants (Hirai et al. 2010). In a plant factory, it takes approximately 3 weeks from seed sowing to harvest in lettuce under optimal growth condition, and therefore we can harvest lettuce over 20 times per year. Thus, if we can stably express a target protein of interest, lettuce will be an alternative platform for mass production of recombinant proteins in plant factories.

Many attempts have been made to produce transgenic lettuces, and a number of useful traits have been introduced into the crop (Torres et al. 1993; Curtis et al. 1994; McCabe et al. 1999; Park et al. 2005; Sun et al. 2006). A

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high degree of transgene silencing in lettuce is a major barrier to commercialize transgenic lettuce. For example, research on miraculin production in transgenic lettuce using a 35S promoter failed to obtain stable expression of the miraculin gene in successive generations (Sun et al. 2006). For this reason, it is important to develop a strategy for stably expressing transgenes in lettuce.

Genetic engineering is an important tool for inserting genes of interest into selected plant genomes. Expression efficiency depends on the stability of the inserted gene expression in successive generations of the host. Until now, a major drawback in transformation experiments has been the instability of the transgene or transgene silencing. Gene silencing occurs at transcriptional and post-transcriptional levels (Stam et al. 1997; Fagard and Vaucheret 2000). After integration of a foreign gene into a host genome, transgene instability or transgene silencing appears to occur within a few generations, illustrating the inherent defense mechanisms of plants against foreign DNA invasion and expression (Matzke et al. 1996; Kumpatla et al. 1997; Demeke et al. 1999). The promoter is a major factor influencing the level and stability of transgene expression. Curtis et al. (1994) compared several promoter-gus gene fusions in transgenic lettuce plants and found that the plastocyanin gene (petE) promoter gave higher expression than the mannopine synthase gene (MAS) (Teeri et al. 1989), hybrid 35S/Mas promoter 5′ region (Mac) (Comai et al. 1990), or cauliflower mosaic virus (CaMV) 35S promoters in first seed generation (T_1) plants. The choice both of promoter and transgene construct is important for long-term expression of transgenes in lettuce (McCabe et al. 1999). Unstable gene expression is also often related to the integration of multiple copies of the transgene in the plant genome (Muller et al. 1996), position effects (Weiler and Wakimoto 1995), and the extent of DNA methylation in the transgene loci (Srivastava et al. 1996).

Ubiquitin is a small, highly conserved protein, consisting of 76 amino acid residues, present in all eukaryotes. The ubiquitins are encoded by gene families that contain two types of structures: polyubiquitin genes and ubiquitin extension protein genes (Monia et al. 1990; Ozkaynak et al. 1987). Both types of genes are translated as polyprotein precursors and then proteolytically processed to ubiquitin monomers (Callis and Vierstra 1989). Polyubiquitin genes are constitutively expressed in all kinds of plant tissues, with increased levels in young tissues (Burke et al. 1988; Cornejo et al. 1993). Various promoters from ubiquitin genes have been tested for their potential use in driving expression of foreign genes in plant transformation systems. Ubiquitin promoters have been successfully used to transfer selected genes in many plants, including monocots and dicots (e.g., Arabidopsis, sunflower, potato; Callis et al. 1990; Binet et al. 1991; Garbarino et al. 1992; Wang et al. 2000).

Miraculin is a plant protein that can modify sour taste into sweet taste. This unique protein is extracted from the pulp of the red miracle fruit berry (*Richadella dulcifica*), a native shrub in West Africa. Miraculin itself is not sweet, but the human tongue once exposed to it perceives ordinarily sour foods, such as lemons and citrus, as sweet for up to an hour afterward. Because of its ability to modify a sour taste into a sweet one, this fruit is known as "miracle fruit." Along with miraculin, six other sweet-tasting proteins have been discovered to date; all of these have been extracted from tropical fruits and are low molecular mass compounds (~6–22 kDa; reviewed by Faus 2000). The nucleotide sequence of miraculin has been determined, and the deduced amino acid sequence suggests that a precursor of miraculin is composed of 220 amino acid residues, including 29 amino acids in a signal sequence (Masuda et al. 1995). The mature miraculin protein consists of 191 amino acids, with an N-linked oligosaccharide (Theerasilp et al. 1989). Market demands and research interest in the miracle fruit, fruit product, and recombinant miraculin have been increasing. Fresh miracle fruit, dried fruit powder, and miracle fruit pulp in tablet form are available in the world market, including Japan. Miraculin also has great potential as an alternative low-calorie sweetener. Thus, these products have been purchased by diabetics and dieters in many countries. However, the natural source of this protein is limited. Thus, several attempts have been made to produce miraculin in foreign hosts, such as *Escherichia coli* (Kurihara 1992; Matsuyama et al. 2009), yeast, transgenic tobacco (Kurihara and Nirasawa 1997), lettuce (Sun et al. 2006), tomato (Sun et al. 2007), and strawberry (Sugaya et al. 2008). Among these plant species, tomato was a suitable platform for producing recombinant miraculin in genetically stable manner (Yano et al. 2010), whereas transgene silencing frequently occurred in transgenic lettuce when the miraculin gene was driven under the 35S promoter.

To achieve stable miraculin expression in lettuce, we compared the level of miraculin accumulation between CaMV 35S promoter/nos terminator cassette and the native lettuce ubiquitin promoter/terminator cassette in transgenic lettuce. In this paper, we report that expression of the miraculin gene under the endogenous ubiquitin promoter/terminator cassette in transgenic lettuce was more efficient than that in the 35S promoter/nos terminator cassette.

Materials and methods

Plasmid construction and transformation of lettuce

Two different cassettes were used to express the miraculin gene. Firstly, the miraculin gene was fused to CaMV 35S

promoter and terminated by a nos terminator, named 35S-MIR. Secondly, the miraculin gene was fused to the lettuce ubiquitin promoter and the lettuce ubiquitin terminator, named Ubi-MIR. The construction of the 35S MIR expression vector has been described in Sun et al. (2006). To make the Ubi-MIR expression vector, we used a pUC18-based lettuce ubiquitin promoter and terminator cassette provided by Dr. H. Fukuoka of the National Institute of Vegetables and Tea Science, Japan (unpublished data). The 1.9-kb ubiquitin promoter region was cloned as follows. The ubiquitin promoter region was amplified by PCR to introduce a *Xho*I site and was inserted into the pGEM-T easy vector. PCR primers used were forward, 5′-CTCGAGGGCGCGCCAAGCTTGCATGC GAAAC-3′; and reverse, 5′-ACATAAGGGACTGACCAC CCGGGCT-3′. The 1.9-kb ubiquitin promoter from the pGEM-T easy vector was digested with *Xho*I and *Xba*I; there was one *Xba*I site downstream in the 3′ region, −311 bp, in the promoter region. The digested 1.6-kb ubiquitin promoter fragment was cloned into the *Xho*I and *Xba*I sites in a modified pBI121 and replaced the 35S promoter. The ubiquitin terminator in pUC18 was amplified using PCR to introduce *Sac*I and *Eco*RI sites (forward, 5′-GAGCTCATTGCTACCGAGCTCTGGTTTGGTG-3′; reverse, 5′-GAATTCGGCGCGCCAGAATTCAACGCGG GCT-3′). The ubiquitin terminator fragment was cloned into 35S-MIR (Sun et al. 2006) between the *Sac*I and *Eco*RI sites, and the vector was digested with *Xba*I and *Eco*RI; the fragment contained the miraculin gene and the ubiquitin terminator fragment and was inserted into the modified pBI121 containing the ubiquitin promoter. These Ubi-MIR (Fig. 1a) and 35S-MIR (Fig. 1d) constructs were transferred into *Agrobacterium tumefaciens* GV2260 (Deblaere et al. 1985) using the method of Shen and Forde (Shen and Forde 1989). Surface-sterilized lettuce (*Lactuca sativa* cv Kaisar) seeds were germinated and grown on Murashige and Skoog (1962) medium with 2% (w/v) sucrose and 0.2% (w/v) Gelrite. Transformation of the lettuce was performed according to Sun et al. (2006).

PCR analysis

Lettuce genomic DNA was extracted from fresh fully expanded leaf tissue of putative transgenic and non-transgenic plants using the Maxwell 16 DNA purification kits according to the manufacturer's protocol (Promega, Tokyo, Japan). PCR was used to confirm the presence of the miraculin gene and neomycin phosphotransferase genes (NPTII) in the transgenic plants using miraculin-specific primers (forward, 5′-TTTTCTAGAATGAAGGAATTAA CAATGCT-3′; reverse, 5′-TTTGAGCTCTTAGAAGTAT ACGGTTTTGT-3′) and NPTII-specific primer (forward, 5′-ATGATTGAACAAGATGGATTGCACGC-3′; reverse, 5′-TCAGAAGAACTCGTCAAGAAGGCG-3′). A total of 100–200 ng genomic DNA were used as the template in a 25-μl PCR reaction mix, using an Applied BioSystems 2720 thermal cycler. The PCR conditions were as follows: 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final incubation at 72°C for 8 min. PCR products were electrophoresed on a 1.0% agarose gel and observed under UV light after staining with 0.1% ethidium bromide.

Southern blot analysis

Total genomic DNA (10 μg) from transgenic and non-transgenic plants was digested with the restriction enzyme *Xba*I, which cuts at a single site within the T-DNA. Digested DNA from each line was separated on 0.8% agarose gels at 50 V for 3 h, and DNA fragments were transferred to a nylon membrane (Hybond-N; GE Healthcare UK Ltd., Amersham, Buckinghamshire, UK) and cross linked to the membrane by the UV Hybrilinker (HL-2000 UVP, LLC, Canada). Thermostable alkaline phosphatase-labeled miraculin gene-specific probes were generated using a CDP Star AlkPhos Direct Labeling Kit, according to the manufacturer's protocol (GE Healthcare UK Ltd.). The membrane was hybridized overnight at 65°C with the probes, and the hybridization signals were detected by chemiluminescence using CDP-Star (Roche Diagnostics, Mannheim, Germany), followed by exposure in the LAS 4000 Mini Image Analyzer (Fujifilm Co. Ltd., Tokyo, Japan).

Isolation of total RNA and quantitative reverse transcription PCR (real-time PCR) analysis

The miraculin gene expression levels in transgenic lettuce plants were determined using real-time PCR analysis. Total RNA was isolated from 100 mg of expanded fresh leaf of transgenic and non-transgenic lettuce by the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) using RNase-free DNase (Qiagen, Tokyo, Japan), according to the manufacturer's protocol. The first-strand cDNA was synthesized from extracted total RNA (0.5 μg) using the SuperSript III VILO cDNA synthesis kit (Invitrogen). Real-time PCR analysis was performed by the Thermal Cycler Dice Real Time System TP800 (Takara-Bio Inc.) using SYBR Premix Ex Taq (Takara-Bio Inc., Otsu, Japan). The cycling parameters were at 95°C for 10 min to denature, followed by 40 cycles at 95°C for 30 s, 55°C for 10 s, and at 72°C for 60 s. Relative quantification of miraculin gene expression was calculated using the lettuce actin gene (DY975577) as an internal control. The experiments were repeated at least three times. Primer sequences were as follows: miraculin

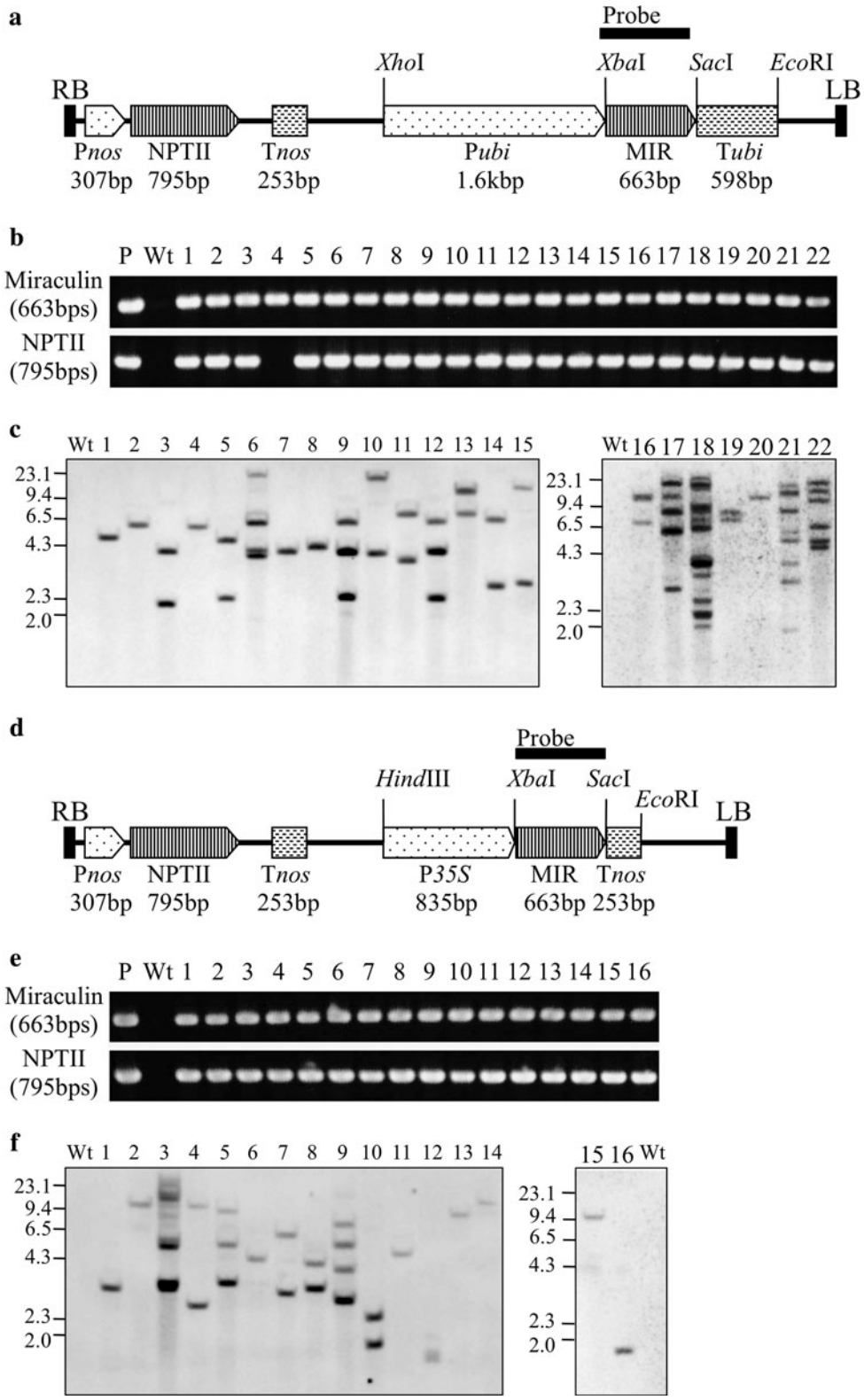

Fig. 1 Production of transgenic lettuce plants with the miraculin gene driven by the lettuce ubiquitin promoter and CaMV 35S promoter. T-DNA construct of binary vector Ubi-MIR for transformation (**a**). Detection of miraculin and NPTII genes through genomic PCR (**b**) and Southern blot analysis (**c**) in 22 putative transformants. Genomic DNA (10 μg) was digested with *Xba*I. T-DNA construct of binary vector 35S-MIR for transformation (**d**). Detection of miraculin and NPTII genes through genomic PCR (**e**) and Southern blot analysis (**f**) in 16 putative transformants. Genomic DNA (10 μg) was digested with *Xba*I. *RB* right border, *LB* left border, *Pnos* nos promoter, *Tnos* nos terminator, *MIR* miraculin, *Pubi* ubiquitin promoter, *Tubi* ubiquitin terminator, *P* positive control, *Wt* wild-type lettuce

forward, 5′-CCACCCAGAGTTGTCCAAAC-3′; miraculin reverse, 5′-TGATGTTGAGATCGGTGGAG-3′; actin forward, 5′-AGAAAATGGCCGACACTGAG-3′; actin reverse, 5′-CTAGGAAACACTGCCCTTGG-3′.

Protein extraction, Western blot analysis, and ELISA

The miraculin accumulation levels in transgenic lettuce plants were assessed immunologically. Lettuce leaf

(100 mg) was ground to a fine powder in liquid nitrogen and homogenized in two volumes of protein extraction buffer consisting of 20 mM of Tris–HCl (pH 8.0), 500 mM NaCl, and 2% polyvinylpolypyrrolidone. The extracts were centrifuged (12,000 rpm, 20 min, 4°C), and the resulting supernatants were subjected to Western blot analysis and ELISA. The protein concentrations of the extracts were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The extracted proteins (3.3 mg of fresh weight equivalents of lettuce leaf) were resolved by SDS-PAGE on 12% gels and then transferred to Hybond-P membrane (GE Healthcare UK Ltd.). After blocking with 5% skim milk, the blots were reacted with an affinity-purified anti-miraculin antibody and an anti-LHCII-type chlorophyll a/b binding protein (Lhcb2) (Agrisera AB, Sweden) as an internal control, followed by incubation with anti-rabbit immunoglobulin G, coupled to horseradish peroxidase. Immunoreactive signals were detected using an Immun-Blot Assay Kit (Nacalai Tesqu, Kyoto, Japan), according to the manufacturer's protocol. The amounts of miraculin in the transgenic lettuce plants were determined using ELISA according to the procedure of Kim et al. (2010).

Results

Development of transgenic lettuce with miraculin gene and confirmation of transgene in transgenic plants by Southern blot analysis

Putative transgenic lettuce plants with Ubi-MIR or 35S-MIR genes were generated by *Agrobacterium*-mediated genetic transformation. The transgenic plants were acclimatized in the growth room and subjected to genomic DNA polymerase chain reaction (PCR) to confirm the presence of the miraculin and NPTII genes. PCR analysis confirmed the presence of transgenes in most lines of putative transgenic lettuces with Ubi-MIR (Fig. 1b) and with 35S-MIR (Fig. 1e). In the Ubi-MIR transgenic lettuce line 4, the band of NPTII gene was not amplified. It might be only a fragment of the NPTII gene (lacking the PCR primer sites) that was integrated into lettuce genome.

Transgenic lines were subjected to genomic Southern blot analysis to confirm the copy number of the transgene. The restriction enzyme *Xba*I was chosen to produce fragments of the T-DNA in DNA extracted from selected lines. Only one *Xba*I site exists outside the miraculin gene in the binary vector (Fig. 1a, d), so that the number of obtained bands reflects the number of insertion events in the transgenic plants. Out of 22 Ubi-MIR transgenic lettuce plants, six plants (line nos. 1, 2, 4, 7, 8, 20) carried one copy, nine plants (3, 5, 10, 11, 13, 14, 15, 16, 19) carried two copies and seven plants (6, 9, 12, 17, 18, 21, 22) carried multicopies of the miraculin gene (Fig. 1c). Out of the 16 35S-MIR transgenic lettuce plants, nine (line nos. 1, 2, 6, 11, 12, 13, 14, 15, 16) carried one copy of the miraculin gene, four (4, 7, 8, 10) carried two copies and three (3, 5, 9) carried multicopies of the miraculin gene (Fig. 1f).

Miraculin expression and accumulation in transgenic lettuce plants with the Ubi-MIR and 35S-MIR gene in T_0 generation

The miraculin expression level was measured by real-time PCR analysis. In Ubi-MIR transgenic lettuce, miraculin gene was expressed in all transgenic lettuces except in two transgenic lines (line nos. 18 and 21) which had multicopies of miraculin gene (Fig. 2a). The level of miraculin was determined by Western blot analysis and ELISA. Miraculin was detected in all transgenic lettuces except for two lines (line nos. 18 and 21); the molecular weight of miraculin was almost the same as that of the homodimer form of purified miraculin (Fig. 2b). The level of miraculin accumulation was about 3.0–9.0 μg per mg total soluble protein (Fig. 2c).

In the 35S-MIR transgenic lettuce plants, mRNA expression of miraculin was observed in all transgenic plants having a single copy of T-DNA insertion (Fig. 3a). The accumulation of miraculin protein was likely correlated with levels of mRNA expression in these plants (Fig. 3b). However, mRNA expression of miraculin was not observed in transgenic lettuce plants having two or multiple copies of T-DNA insertion, resulting in no miraculin protein accumulation, except for line no. 7 showing relatively high expression of miraculin mRNA and protein. Subsequently, ELISA was performed to calculate the quantitative levels of miraculin protein. This analysis revealed that miraculin accumulation in the transgenic plants that produced miraculin ranged from 1.5 to 3.0 per mg total soluble protein, except for line 14 accumulating 7.0 μg per mg total soluble protein (Fig. 3c).

The real-time PCR analysis demonstrated that mRNA expression level of miraculin in plants with a single copy of T-DNA insertion was higher in the Ubi-MIR plants (relative expression level of miraculin to actin: 1.07) compared to that in the 35S-MIR plants (relative expression level of miraculin to actin: 0.18). In addition, the average of miraculin accumulation level (4.81 μg/mg total soluble protein) in Ubi-MIR transgenic lettuce with a single copy of miraculin gene was higher than that (2.72 μg/mg total soluble protein) in 35S-MIR transgenic lettuce, according to *t* test.

Miraculin gene inheritance into T_1 generation of Ubi-MIR and 35S-MIR

All transgenic lettuce plants were self-pollinated, but several lines did not set a seed. Three lines of lettuce seeds

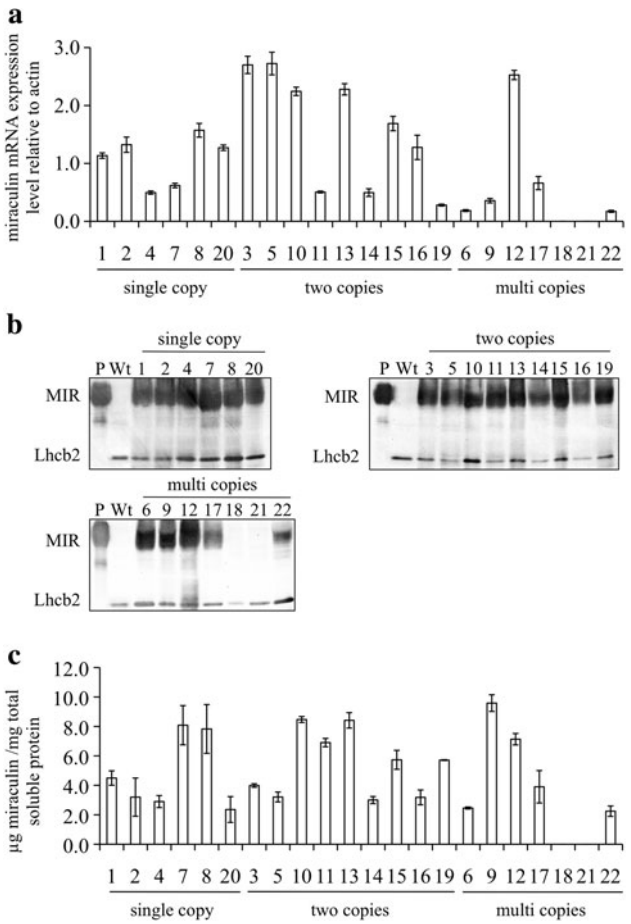

Fig. 2 Characterization of transgene transcription and translation in the T_0 generation of Ubi-MIR transgenic lettuce. Miraculin gene expression level was measured by RT-PCR (**a**). The soluble proteins from 3.3 mg fresh weight equivalents of lettuce leaf were separated by SDS-PAGE and miraculin protein accumulation was detected by Western blot analysis (**b**). Miraculin protein accumulation level was measured by ELISA (**c**). The experiment was repeated three times. *Bars* indicate standard error. *P* 350 ng purified miraculin protein, *Wt* wild-type lettuce, *MIR* miraculin, *Lhcb2* LHCII-type chlorophyll a/b binding protein

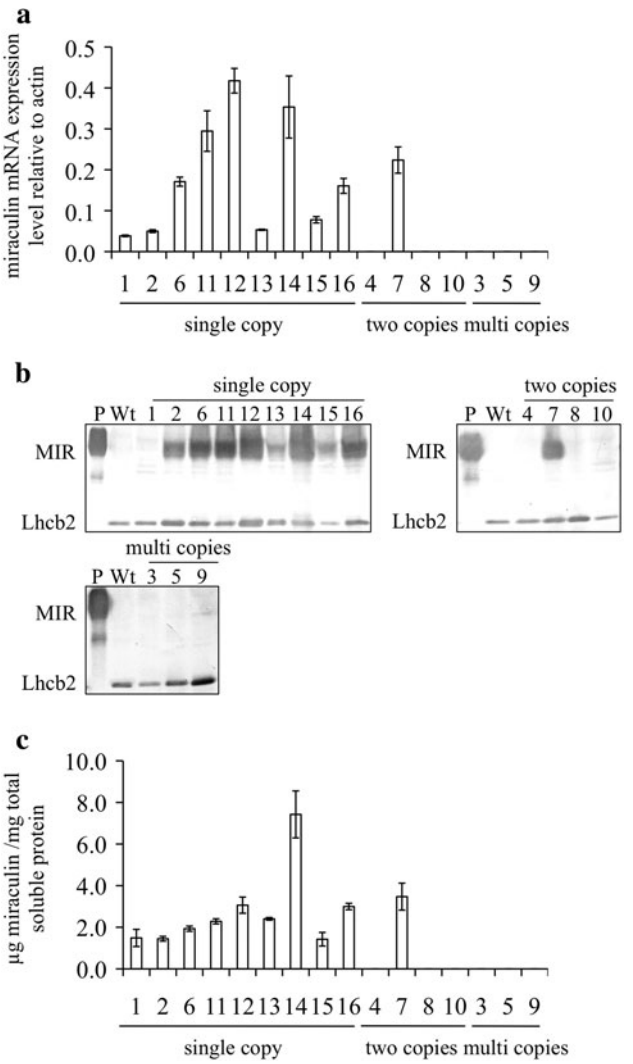

Fig. 3 Characterization of transgene transcription and translation in the T_0 generation of 35S-MIR transgenic lettuce. Miraculin gene expression level was measured by RT-PCR (**a**). The soluble proteins from 3.3 mg of fresh weight equivalents of lettuce leaf were separated by SDS-PAGE, and miraculin protein accumulation was detected by Western blot analysis (**b**). Miraculin protein accumulation level was measured by ELISA (**c**). The experiment was repeated three times. *Bars* indicate standard error. *P* 350 ng purified miraculin protein, *Wt* wild-type lettuce, *MIR* miraculin, *Lhcb2* LHCII-type chlorophyll a/b binding protein

with a single copy of transgene were sown for each construction and cultivated in a growth room. In the T_1 generation of Ubi-MIR transgenic lettuce, the inheritance, expression level of the miraculin gene and accumulation level of miraculin protein were analyzed in lines 1, 7, and 8 by PCR, real-time PCR, Western blot analysis, and ELISA, respectively (Fig. 4a–c). The results showed a good correlation between genomic PCR, real-time PCR, and ELISA. Interestingly, the levels of miraculin mRNA expression and protein accumulation in the T_1 generation were higher than those in the T_0 generation.

In the T_1 generation of 35S-MIR transgenic lettuce, the results of genomic PCR showed the segregation of the miraculin gene (Fig. 5a). Among these transgenic lettuces with the miraculine gene, miraculin protein was not detected by Western blot analysis, suggesting that the miraculin gene was silenced in the T_1 generation.

Miraculin gene inheritance into the T_2 generation of Ubi-MIR and 35S-MIR

Seeds of the T_2 generation were harvested from self-pollinated T_1 generation of transgenic lettuce. The seeds of transgenic lettuce line were sown on soil and cultivated in a growth room. The homozygous lines for the miraculin gene were selected by genomic real-time PCR (data not shown).

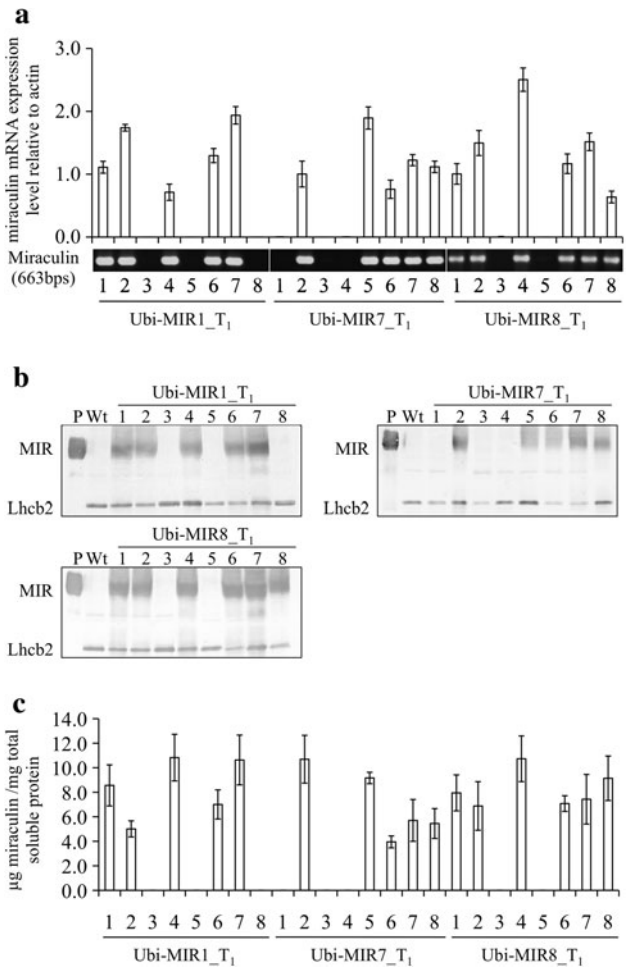

Fig. 4 Characterization of transgene transcription and translation in the T_1 generation of Ubi-MIR transgenic lettuce lines 1, 7, and 8. The presence of transgene was confirmed by genomic PCR, and miraculin gene expression level was measured by RT-PCR (**a**). The soluble proteins from 3.3 mg fresh weight equivalents of lettuce leaf were separated by SDS-PAGE, and miraculin protein accumulation was detected by Western blot analysis (**b**). Miraculin protein accumulation level was measured by ELISA (**c**). The experiment was repeated three times. *Bars* indicate standard error. *P* 350 ng of purified miraculin protein, *Wt* wild-type lettuce, *MIR* miraculin, *Lhcb2* LHCII-type chlorophyll a/b binding protein

In all T_2 generations of Ubi-MIR transgenic lettuce, the miraculin gene and miraculin protein were detected with genomic PCR and Western blot analysis, respectively (Fig. 6a, b). Both the expression level of miraculin gene measured by RT-PCR and concentration of miraculin protein measured by ELISA were not significantly different among individual plants in each transgenic line. Miraculin concentration in line no. 1 was the highest (about 14 μg miraculin per mg total soluble protein) and in line no. 8 was the lowest (about 8 μg miraculin per mg total soluble protein) in transgenic lettuce plants.

In the T_2 generation of 35S-MIR transgenic lettuce, miraculin gene insertion was detected by genomic PCR in all transgenic lettuce plants, but miraculin protein was not detected in these lines (Fig. 5b). In addition, miraculin mRNA expression was not detected in all transgenic lettuce lines with 35S-MIR in the T_1 and T_2 generations (data not shown).

Discussion

Genetic engineering to produce transgenic plants has emerged as a promising technology for the production of recombinant biopharmaceutical proteins and vaccines. They provide many advantages and their potential for use as bioreactors for the production of therapeutic molecules is an active area of research (Lindbo 2007). A wide variety of complex and valuable foreign proteins can be expressed efficiently in transgenic plants (Arntzen et al. 2005). The productivity of recombinant proteins in transgenic plants is economically compared with transgenic animals or the mammalian cell culture systems. The use of plant expression systems for recombinant protein production is considered to be at least as economical as traditional industrial facilities (fermentation processes, bioreactor systems; Obregon et al. 2006). For low-cost and commercially applicable plant expression systems, the stability of the transgene expression in the target plant species is a key. Thus, it is important to develop stable transgene-expressing lines. The promoter and terminator are key factors that influence the stability of the transgene expression in the host genome, although the interaction between promoters and plant species is variable.

The present study describes the production of the commercially important protein, miraculin, in a plant expression system. The demand and research interest for this protein for dieters and diabetics are increasing. A limited natural availability is a major barrier to the commercialization of this protein (Witty 1998). Lettuce is a widely consumed leafy vegetable that is grown worldwide and commercially cultivated in plant factories. For this reason, lettuce was chosen as a simple and readily available platform for the commercial production of recombinant miraculin.

The stability of miraculin gene expression in lettuce was compared between the lettuce ubiquitin promoter/terminator cassette and the 35S promoter/nos terminator cassette. Transgenic lettuce expressing biologically active miraculin was firstly reported by Sun et al. (2006), but stable transgene expression was not achieved. Their results showed that transgene silencing occurred when the 35S promoter was used to drive miraculin expression. All transgenic lettuce lines with the miraculin gene under the control of the 35S and EL2-35S-Ω promoter (containing 419 bp of 5′-upstream sequence of 35S promoter and 71 bp

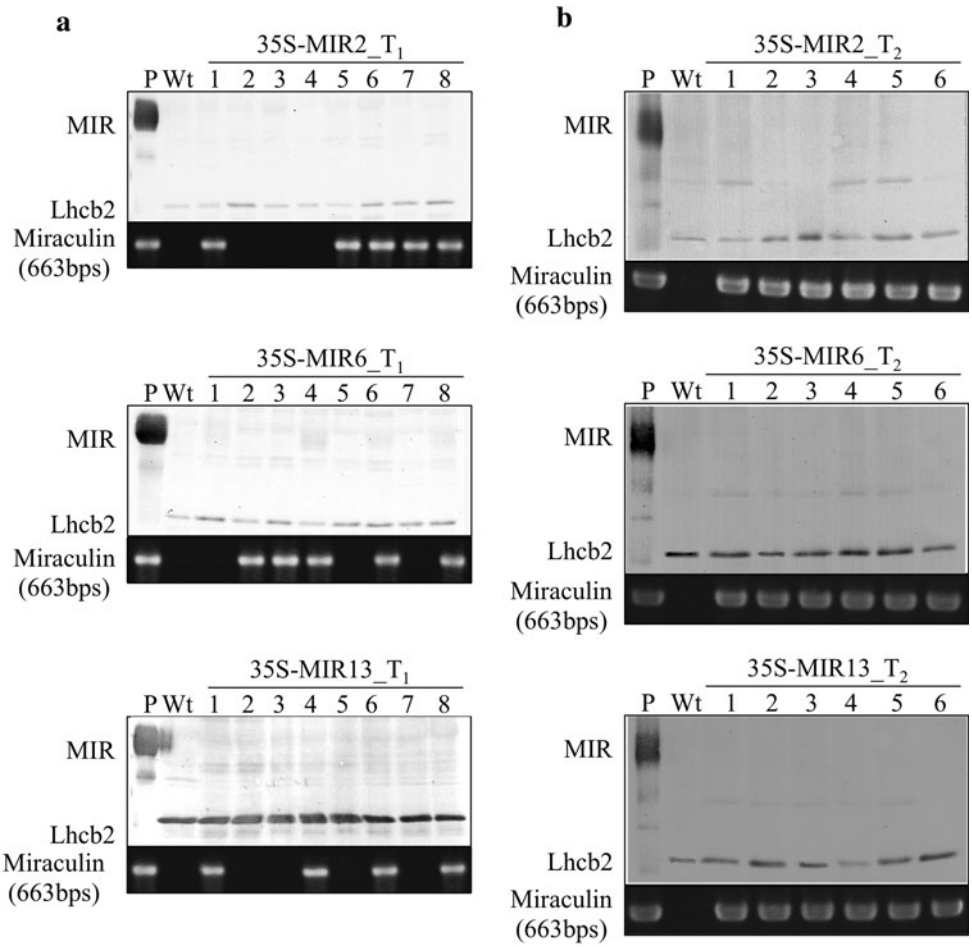

Fig. 5 Detection of miraculin protein in the T_1 (**a**) and T_2 (**b**) generations of 35S-MIR transgenic lettuce lines 2, 6, and 13. The presence of transgene was confirmed by genomic PCR. The soluble proteins from 3.3 mg fresh weight equivalents of lettuce leaf were separated by SDS-PAGE, and miraculin protein accumulation was detected by Western blot analysis. *P* 350 ng purified miraculin protein, *Wt* wild-type lettuce, *MIR* miraculin, *Lhcb2* LHCII-type chlorophyll a/b binding protein

of 5′-untranslated sequence of tobacco mosaic virus) showed lower expression in the T_1 generation, and few transgenic plants expressed the miraculin gene in the T_1 generation (Sun et al. 2006). These results prompted us to use a different promoter for stable miraculin gene expression in lettuce. The 35S promoter has been widely and successfully used for transformation studies in many crops. In tomatoes, stable expression of the miraculin gene was successfully achieved and stably inherited using the 35S promoter (Sun et al. 2007; Yano et al. 2010). This problem with using the 35S promoter in lettuce transformation emphasizes that expression instability is a species-specific issue. Indeed, the rates and causes of instability vary widely across species, environments, and transformation systems (Meza et al. 2001; Kohli et al. 2003). Similar situations have been reported with genetic transformation in lettuce using the 35S promoter (Curtis et al. 1994; McCabe et al. 1999).

In this study, transgenic lettuce plants using the 35S promoter showed almost complete silencing of the miraculin gene expression in the T_2 generation. These results have been supported by real-time PCR, ELISA, and Western blot analyses. The T_1 progenies from these lines had no detectable level of miraculin gene expression (only one line expressed) by real-time PCR analysis. Transgene silencing is frequently observed in transformation systems, although the mechanisms are not fully understood. This result suggests that the viral 35S promoter was more vulnerable than the ubiquitin promoter to gene inactivation following integration into the lettuce genome. Many reports have shown that the methylation of promoters directing transgene expression in transgenic plants is related to loss of transgene expression. Transgenic petunia plants carrying a 35S promoter-driven maize A1 gene failed to exhibit the expected red flower color because of the hypermethylated state of the promoter (Meyer et al. 1992). Transcriptional gene silencing is often associated with the hypermethylation of cytosine residues in promoter regions (Finnegan et al. 2001). A comparison of DNA methylation between 35S promoter and ubiquitin promoter will provide a clue to understand our observation in transgenic lettuce plants.

The present study indicates that the use of the endogenous lettuce ubiquitin promoter to drive the miraculin gene in a transformation system could overcome the transgene silencing problem. We found that all single-copy

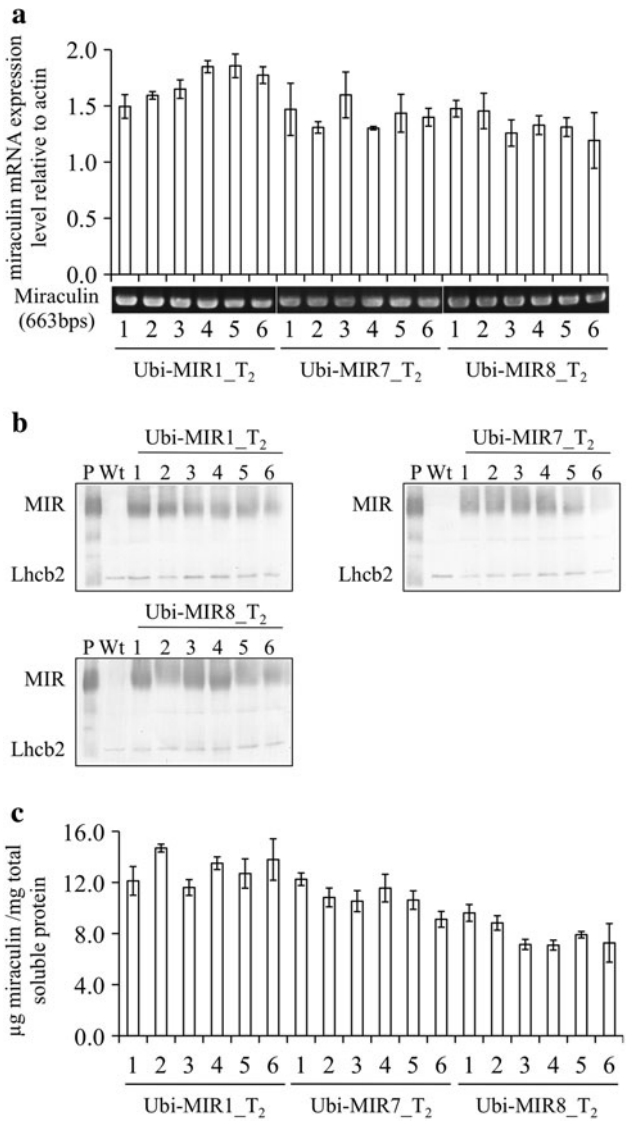

Fig. 6 Characterization of transgene transcription and translation in the T_2 generation of Ubi-MIR transgenic lettuce lines 1, 7, and 8. The presence of transgene was confirmed by genomic PCR, and miraculin gene expression level was measured by RT-PCR (**a**). The soluble protein from 3.3 mg fresh weight equivalents of lettuce leaf were separated by SDS-PAGE, and miraculin protein accumulation was detected by Western blot analysis (**b**). Miraculin protein accumulation level was measured by ELISA (**c**). The experiment was repeated three times. *Bars* indicate standard error. *P* 350 ng purified miraculin protein, *Wt* wild-type lettuce, *MIR* miraculin, *Lhcb2* LHCII-type chlorophyll a/b binding protein

transgenic lines using the ubiquitin promoter expressed the miraculin gene in the T_0 generation at a high level compared with using the 35S promoter. The expression of miraculin was also clear and stable in the T_1 and T_2 generations, as revealed by real-time PCR, Western blotting, and ELISA. The transgenic lines showed stable expression and inheritance of the miraculin gene for up to three generations. Moreover, these results showed that the transgene under the control of the CaMV 35S promoter was likely silenced in the T_1 and subsequent generations, whereas the ubiquitin promoter-driven miraculin gene was stably expressed in the T_1 and T_2 generations. The effectiveness of the maize ubiquitin promoter was reported by Chen et al. (1998, 1999). Chen et al. (1998) reported that the 35S-derived gene was silenced in the T_1 generation of transgenic maize and maize ubiquitin promoter-derived gene was expressed in the T_1 generation of transgenic maize.

Previously, we have shown that tomato is a suitable platform for producing recombinant miraculin in a genetically stable manner (Yano et al. 2010). We have also developed a cultivation system for tomato in a plant factory (Hirai et al. 2010) and bred a suitable tomato line for a plant factory (Kato et al. 2010). Although there are some studies on the production of tomato in plant factories, there are no prospects for commercial production of tomato fruits in plant factories because of its high cost. On the other hand, lettuce is the only vegetable crop that is commercially cultivated in plant factories. In this study, we have demonstrated the genetically stable expression of miraculin in transgenic lettuce, suggesting that lettuce would be a practical platform for mass production of recombinant miraculin.

In conclusion, the miraculin gene was successfully expressed under the control of a ubiquitin promoter, fused to a ubiquitin terminator, in T_0, T_1, and T_2 transgenic lettuce. This study also showed that the level of miraculin accumulation in transgenic lettuce was equivalent to that in the native miracle fruit, as well as that in transgenic tomatoes that stably accumulates miraculin. It was demonstrated that miraculin gene expression failed using 35S promoter/nos terminator cassette transgenic lettuce in the T_1 and T_2 generations. We conclude that the endogenous lettuce ubiquitin promoter, fused to a ubiquitin terminator, is a suitable driver for stable foreign gene expression in lettuce and overcomes the gene silencing problem.

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References

Arntzen C, Plotkin S, Dodet B (2005) Plant-derived vaccines and antibodies: potential and limitations. Vaccine 23:1753–1756. doi:10.1016/j.vaccine.2005.01.090

Binet MN, Weil JH, Tessier LH (1991) Structure and expression of sunflower ubiquitin genes. Plant Mol Biol 17:395–407. doi:10.1007/BF00040634

Burke TJ, Callis J, Viestra RD (1988) Characterization of a polyubiquitin gene from *Arabidopsis thaliana*. Mol Gen Genet 213:435–443. doi:10.1007/BF00339613

Callis J, Vierstra RD (1989) Ubiquitin and ubiquitin genes in higher plants. Oxford Surv Plant Mol Biol 6:1–30

Callis J, Raasch JA, Vierstra RD (1990) Ubiquitin extension proteins of *Arabidopsis thaliana*. J Biol Chem 265:12486–12493

Chen WP, Gu X, Liang GH, Muthukrishnan S, Chen PD, Liu DJ, Gill BS (1998) Introduction and constitutive expression of a rice chitinase gene in bread wheat using biolistic bombardment and the bar gene as a selectable marker. Theor Appl Genet 97:1296–1306. doi:10.1007/s001220051022

Chen WP, Chen PD, Liu DJ, Kynast R, Friebe B, Velazhahan R, Muthukrishnan S, Gill BS (1999) Development of wheat scab symptoms is delayed in transgenic wheat plants that constitutively express a rice thaumatin-like protein gene. Theor Appl Genet 99:755–760. doi:10.1007/s001220051294

Comai L, Moran P, Maslyar D (1990) Novel and useful properties of a chimeric plant promoter combining CaMV 35S and MAS elements. Plant Mol Biol 15:373–381. doi:10.1007/BF00019155

Cornejo MJ, Luth D, Blankenship KM, Anderson OD, Blechl AE (1993) Activity of a maize ubiquitin promoter in transgenic rice. Plant Mol Biol 23:567–581. doi:10.1007/BF00019304

Curtis IS, Power JB, McCabe M, de Laat A, Davey MR (1994) Promoter–GUS fusions in lettuce. In: Abstracts 4th Int Congr Plant Mol Biol (Abstract no. 1682). The International Society for Plant Molecular Biology, Amsterdam

Deblaere R, Bytebier B, Degreve H, Schell J, van Montagu M, Leemans J (1985) Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene-transfer to plants. Nucleic Acids Res 13:4777–4788. doi:10.1093/nar/13.13.4777

Demeke T, Hucl P, Baga M, Caswell K, Leung N, Chibbar RN (1999) Transgene inheritance and silencing in hexaploid spring wheat. Theor Appl Genet 99:947–953. doi:10.1007/s001220051401

Fagard M, Vaucheret H (2000) (Trans)gene silencing in plants: how many mechanisms? Annu Rev Plant Physiol Plant Mol Biol 51:167–194. doi:10.1146/annurev.arplant.51.1.167

Faus I (2000) Recent developments in the characterization and biotechnological production of sweet-tasting proteins. Appl Microbiol Biotechnol 53:145–151. doi:10.1007/s002530050001

Finnegan EJ, Wang M, Waterhouse P (2001) Gene silencing: fleshing out the bones. Curr Biol 11:R99–R102. doi:10.1016/S0960-9822(01)00039-2

Garbarino JE, Rockhold DR, Belknap WR (1992) Expression of stress responsive ubiquitin genes in potato tubers. Plant Mol Biol 20:235–244. doi:10.1007/BF00014491

Hirai T, Fukukawa G, Kakuta H, Fukuda N, Ezura H (2010) Mass production of recombinant miraculin using transgenic tomatoes in a closed cultivation system. J Agric Food Chem 58:6096–6101. doi:10.1021/jf100414v

Kato K, Yoshida R, Kikuzaki A, Hirai T, Kuroda H, Hiwasa-Tanase K, Takane K, Ezura H, Mizoguchi T (2010) Molecular breeding of tomato lines for mass production of miraculin in a plant factory. J Agric Food Chem 58:9505–9510. doi:10.1021/jf101874b

Kim YW, Kato K, Hirai T, Hiwasa-Tanase K, Ezura H (2010) Spatial and development profiling of miraculin accumulation in transgenic tomato fruits expressing the miraculin gene constitutively. J Agric Food Chem 58:282–286. doi:10.1021/jf9030663

Kohli A, Twyman RM, Abranches R, Wegel E, Stoger E, Christou P (2003) Transgene integration, organization and interaction in plants. Plant Mol Biol 52:247–258. doi:10.1023/A:1023941407376

Kumpatla SP, Teng W, Buchholz WG, Hall TC (1997) Epigenetic transcriptional silencing and 5-azacytidine-mediated reactivation of a complex transgene in rice. Plant Physiol 115:361–373. doi:10.1104/pp115.2.361

Kurihara Y (1992) Characteristics of antisweet substances, sweet proteins, and sweetness-inducing proteins. Crit Rev Food Sci Nutr 32:231–252. doi:10.1016/0924-2244(94)90069-8

Kurihara Y, Nirasawa S (1997) Structures and activities of sweetness-inducing substances (miraculin, curculin, strongin) and the heat-stable sweet protein, mabinlin. FFI J Japan 174:67–74

Lindbo JA (2007) High-efficiency protein expression in plants from agroinfection-compatible tobacco mosaic virus expression vectors. BMC Biotech 7:52. doi:10.1186/1472-6750-7-52

Masuda Y, Nirasawa S, Nakaya K, Kurihara Y (1995) Cloning and sequencing of a cDNA encoding a taste-modifying protein, miraculin. Gene 161:175–177. doi:10.1016/0378-1119(95)00198-F

Matsuyama T, Satoh M, Nakata R, Aoyama T, Inoue H (2009) Functional expression of miraculin, a taste-modifying protein in *Escherichia coli*. J Biochem 145:445–450. doi:10.1093/jb/mvn184

Matzke MA, Matzke JM, Eggleston WB (1996) Paramutation and transgene silencing: a common response to invasive DNA? Trends Plant Sci 1:382–388. doi:10.1016/S1360-1385(96)80313-X

McCabe MS, Schepers F, van der Arend A, Mohapatra U, de Laat AMM, Power JB, Davey MR (1999) Increased stable inheritance of herbicide resistance in transgenic lettuce carrying a petE promoter-bar gene compared with a CaMV 35S-bar gene. Theor Appl Genet 99:587–592. doi:10.1007/s001220051272

Meyer P, Linn F, Heidmann I, Meyer zAH, Niedenhof I, Saedler H (1992) Endogenous and environmental factors influence 35S promoter methylation of a maize A1 gene construct in transgenic petunia and its colour phenotype. Mol Gen Genet 231:345–352. doi:10.1007/BF00292701

Meza TJ, Kamfjord D, Håkelien AM, Evans I, Godager LH, Mandal A, Jakobsen KS, Aalen RB (2001) The frequency of silencing in *Arabidopsis thaliana* varies highly between progeny of siblings and can be influenced by environmental factors. Transgenic Res 10:53–67. doi:10.1023/A:1008903026579

Monia BP, Ecker DJ, Crooke ST (1990) New perspectives on the structure and function of ubiquitin. Biotechnology 8:209–215. doi:10.1038/nbt0390-209

Muller E, Lorz H, Lutticke S (1996) Variability of transgene expression in clonal cell lines of wheat. Plant Sci 114:71–82. doi:10.1016/0168-9452(95)04312-8

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Obregon P, Chargelegue D, Drake PMW, Prada A, Nuttall J, Frigerio L, Ma JK-C (2006) HIV-1 p24–immunoglobulin fusion molecule: a new strategy for plant-based protein production. Plant Biotech J 4:195–207. doi:10.1111/j.1467-7652.2005.00171.x

Ozkaynak E, Finley D, Solomon MJ, Varhavsky A (1987) The yeast ubiquitin genes: a family of natural gene fusions. EMBO J 6:1429–1439

Park BJ, Liu Z, Kanno A, Kameya T (2005) Increased tolerance to salt- and water-deficit stress in transgenic lettuce (*Lactuca sativa* L.) by constitutive expression of LEA. Plant Growth Reg 45:165–171. doi:10.1007/s10725-004-7924-y

Shen WJ, Forde BG (1989) Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. Nucleic Acids Res 17:8385

Srivastava V, Vasil V, Vasil IK (1996) Molecular characterization of the fate of transgenes in transformed wheat (*Triticum aestivum* L.). Theor Appl Genet 92:1031–1037. doi:10.1007/BF00224045

Stam M, Mol JNM, Kooter JM (1997) The silence of genes in transgenic plants. Ann Bot 79:3–12. doi:10.1006/anbo.1996.0295

Sugaya T, Yano M, Sun HJ, Hira T, Ezura H (2008) Transgenic strawberry expressing a taste-modifying protein, miraculin. Plant Biotech 25:329–333

Sun HJ, Cui ML, Ma B, Ezura H (2006) Functional expression of the taste-modifying protein, miraculin, in transgenic lettuce. FEBS Lett 580:620–626. doi:10.1016/j.febslet.2005.12.080

Sun HJ, Kataoka H, Yano M, Ezura H (2007) Genetically stable expression of functional miraculin, a new type of alternative sweetener, in transgenic tomato plants. Plant Biotech J 5:768–777. doi:10.1111/j.1467-7652.2007.00283.x

Teeri TH, Lehvaslaiho H, Frank M, Uotila J, Heino P, Palva ET, Montagu VM, Herrera-Estrella L (1989) Gene fusions to lac Z reveal new expression patterns of chimeric genes in transgenic plants. EMBO J 8:343–350

Theerasilp S, Hitotsuya H, Nakajo S, Nakaya K, Nakamura Y, Kurihara Y (1989) Complete amino acid sequence and structure characterization of the taste-modifying protein, miraculin. J Biol Chem 264:6655–6659

Torres AC, Cantliffe DJ, Laughner B, Bienie M, Nagata R, Ashraf M, Ferl RJ (1993) Stable transformation of lettuce cultivar South Bay from cotyledon explants. Plant Cell Tissue Org Cult 34:279–285. doi:10.1007/BF00029717

Wang J, Jiang J, Oard JH (2000) Structure, expression and promoter activity of two poly ubiquitin genes from rice (*Oryza sativa* L.). Plant Sci 156:201–211. doi:10.1016/S0168-9452(00)00255-7

Weiler KS, Wakimoto BT (1995) Heterochromatin and gene expression in Drosophila. Annu Rev Genet 29:577–605. doi:10.1146/annurev.ge.29.120195.003045

Witty M (1998) New technologies for taste modifying proteins. Trends Food Sci Technol 9:275–280. doi:10.1016/S0924-2244(98)00048-X

Yano M, Hirai T, Kato K, Hiwasa-Tanase K, Fukuda N, Ezura H (2010) Tomato is a suitable material for producing recombinant miraculin protein in genetically stable manner. Plant Sci 178:469–473. doi:10.1016/j.plantsci.2010.02.016