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, one that is cultivated worldwide and a target crop for a plant factory. 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 and a 35S promoter/nos terminator cassette. Miraculin gene expression and miraculin accumulation in both cassettes were compared by real-time polymerase chain reaction, Western blotting, and enzyme-linked immunosorbent assay. The expression level of 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. The results suggest the utility of the ubiquitin promoter/terminator cassette as an efficient platform for the genetically stable expression of miraculin protein in lettuce and hence 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

Introduction

Lettuce is a popular and easy-to-grow leafy vegetable cultured worldwide. Lettuce is a typical crop that is commercially cultivated in a plant factory, which is a cultivation system with a controlled light period, light intensity, temperature, and CO2 concentration for mass production of target plants (Hirai et al. 2010). In a plant factory, we can harvest lettuce over 20 times per year. Thus, if we can stably express a target protein of interests, lettuce will be an alternative platform for mass production of recombinant proteins in plant factory.

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 high degree of transgene silencing in lettuce is a major barrier to commercializing 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). Transgene silencing occurred in transgenic lettuce when the miraculin gene failed to express under the 35S promoter in the T_1 and later generations. For this reason, it is important to develop a strategy for stably expressing transgenes in target genome.

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 et al., 2000). After integration of a foreign gene into a host genome, transgene instability or transgene silencing can 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 petE promoter gave higher expression than the MAS (Teeri et al., 1989), Mac (Comai et al., 1990), or CaMV 35S promoters in first seed generation (T_1) plants. The choice of promoter and T-DNA 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), to position effects (Weiler and

Wakimoto, 1995), and to the extent of 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; Garbarino et al., 1992; Wang et al., 2000).

Miraculin is plant protein that can transform a sour taste into a 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 transform 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 were extracted from tropical fruits and are low molecular mass compounds (~6–22 kDa; reviewed by Faus, 2000). The amino acid sequence of miraculin was predicted and consists of 191 amino acids, with an N-linked oligosaccharide (Theerasilp et al., 1989). The nucleotide sequence of miraculin was 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). Market demands and research interest in the miracle fruit, fruit product, and recombinant miraculin have increased. Fresh miracle fruit, dried fruit powder, and miracle fruit pulp in tablet form are available on the world market, including in Japan. These products are being purchased by diabetics and dieters in many countries. Miraculin also has great potential as an alternative low-calorie sweetener. However, the natural source of this protein is limited. Thus, 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 occurred in transgenic lettuce when the miraculin gene was driven under the 35S promoter.

To achieve stable miraculin expression in lettuce, we compared the CaMV 35S promoter/nos terminator cassette with the ubiquitin promoter/terminator cassette from 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. One was the miraculin gene with the CaMV 35S promoter and terminated by a nos terminator, named 35S-MIR; the other was the miraculin gene with the lettuce ubiquitin promoter and terminator, named Ubi-MIR. The 35S MIR expression vector construct was described in Sun et al. (2006). To construct 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' CTCGAGGGCGCGCCAAGCTTGCATGCGAAAC-3'; and reverse, 5' ACATAAGGGACTGACCACCCGGGCT-3'. The 1.9-kb ubiquitin promoter from the pGEM-T easy vector was digested with *Xho*I and *Xba*I; there is 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' GAATTCGGCGCGCCAGAATTCAACGCGGGCT-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 to Agrobacterium tumefaciens GV2260 (Deblaere et al., 1985) using the method of Shen and Forde (Shen et al., 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 full-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' TTTTCTAGAATGAAGGAATTAACAATGCT 3'; reverse, 5' TTTGAGCTCTTAGAAGTATACGGTTTTGT 3') and NPTII-specific primer (forward, 5'-ATGATTGAACAAGATGGATTGCACGC-3';. reverse, 5'-TCAGAAGAACTCGTCAAGAAGGCG-3'). A total of 100-200 ng genomic DNA was used as the template in a 25 µl PCR reaction mix, using an Applied BioSystems 2720 thermal cycler. The PCR conditions were 94°C for 3 min; followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; followed by a final incubation of 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 fragments were transferred to a nylon membrane (Hybond-N; GE Healthcare UK Ltd., Amersham, Buckinghamshire, UK) and

cross linked to the membrane by UV using a 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℃ 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. Total RNA was isolated from 100 mg 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 was performed with 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 95ºC for 10 min to denature, 40 cycles of 95ºC for 30 s, 55ºC for 10 s, and 72ºC 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 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 level 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 Tris-HCl (pH 8.0), 500 mM NaCl, and 2% polyvinylpolypyrrolidone. The extracts were centrifuged (12000 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 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 plants obtained 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 all 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 a miss-integration of NPTII gene into lettuce genome.

Transgenic lines were subjected to genomic Southern 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. The Ubi-MIR transgenic lettuces of 6 of the plants (lines no. 1, 2, 4, 7, 8, 20) carried one copy of the miraculin gene, 9 of plant (3, 5, 10, 11, 13, 14, 15, 16, 19) carried two copies of the miraculin gene and 7 of plant (6, 9, 12, 17, 18, 21, 22) carried multi copies of the

miraculin gene (Fig. 1C). The 35S-MIR transgenic lettuces of 9 of the plants (lines no. 1, 2, 6, 11, 12, 13, 14, 15, 16) carried one copy of the miraculin gene, 4 of plant (4, 7, 8, 10) carried two copies of the miraculin gene and 3 of plant (3, 5, 9) carried multi copies 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 with real time PCR. In Ubi-MIR transgenic lettuce, miraculin gene was expressed in all transgenic lettuces except two transgenic lines (line no. 18 and 21) which had multi copies of miraculin gene (Fig. 2A). Miraculin accumulation and accumulation level was measured with Western blot analysis and ELISA, respectively. The band of miraculin was detected in all transgenic lettuces expected two lines (Line no. 18 and 21), and miraculin band of transgenic lettuce size was almost same as homodimer form of purified miraculin (Fig. 2B). The accumulation level of miraculin was about 3.0-9.0 μg per mg total soluble protein (Fig. 2C).

In 35S-MIR transgenic lettuce, miraculin gene was expressed in all transgenic lettuce had single copy of miraculin gene and only one transgenic lettuce (line no. 7) had two copies of miraculin gene, while miraculin gene expression was not detected in other transgenic lettuce had two copies and multi copies of miraculin gene (Fig. 3A). The transgenic lettuce in which the miraculin gene expression was detected by RT-PCR were detected the accumulation of miraculin protein using Western blot analysis in the T_0 generation (Fig. 3B). Miraculin accumulation level of miraculin detected transgenic lettuce was 1.5-3.0 μg per mg total soluble protein except line 14 accumulating 7.0 μg miraculin per mg total soluble protein (Fig. 3C).

The average of miraculin expression level ( ) in Ubi-MIR transgenic lettuce with a single copy of miraculin gene was higher than that ( ) in 35S-MIR transgenic lettuce and the average of miraculin accumulation level ( ) in Ubi-MIR transgenic lettuce with a single copy of miraculin gene was higher than that ( ) in 35S-MIR transgenic lettuce, according to ????? test.

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

All transgenic lettuce line was self-pollinated, but several lines did not set a seed. Three lines of lettuce seeds with a single copy of transgene was sown for each construction and cultivated in growth room. In 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 using genomic DNA PCR, real-time PCR, Western blot analysis and ELISA, respectively (Fig. 4A, B, C). The results showed a good correlation between genomic PCR, real-time PCR and ELISA. The miraculin expression and accumulation level in T_1 generation was higher than in the T_0 generation.

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

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

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

In all T_2 generation of Ubi-MIR transgenic lettuce, miraculin gene and miraculin protein was detected with genomic PCR and Western blot analysis, respectively (Fig. 6A, B). Expression level of miraculin gene measured using RT-PCR and concentration of miraculin protein measured using ELISA was not significant difference among individual plant in each transgenic line. Miraculin concentration in line No. 1 was highest (about 14 μg miraculin per mg total soluble protein) and line No. 8 was lowest (about 8 μg miraculin per mg total soluble protein) in each transgenic lettuce.

In T_2 generation of 35S-MIR transgenic lettuce, miraculin gene was detected with genomic PCR among all individual plant in each transgenic lettuce but miraculin protein was not detected in all transgenic lettuce lines (Fig. 5B). In addition, miraculin gene expression was not detected in all transgenic lettuce lines with 35S-MIR in T_1 and T_2 generations (data not shown).

Discussion

Transgenic plants have emerged as a promising technology for the production of recombinant biopharmaceutical proteins and vaccines. They offer many advantages, and their potential for used 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). Production of recombinant proteins in transgenic plants is economical compared with transgenic animals or the mammalian cell culture systems. The use of plant expression systems for recombinant protein production should 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 for and research interest in 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, one that is grown worldwide and commercially cultivated in a plant factory. 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 first 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 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. 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 complete silencing of miraculin gene expression in the T_2 generation. These results were supported by real-time PCR, ELISA, and Western blot analyses. The T_1 progenies from these lines had no significant level of miraculin gene expression (only one line expressed). We suggest that gene silencing occurred during seed formation in T_0 plants. 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 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). According to Fukuoka (personal communication), 35S-promoter was subjected to DNA methylation and resulted in the transgene silencing, while ubiquitin promoter was not subjected to DNA methylation in transgenic lettuce.

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-insert primary transgenic lines using the ubiquitin promoter expressed the miraculin gene in the T_0 generation at a high level compared with using 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 show that the transgene under the control of the CaMV 35S promoter was 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 generation. The effectiveness of the maize ubiquitin promoter was reported by Chen et al. (1998, 1999). Chen et al. (1998) reported 35S derived gene was silenced in T_1 genaretion of transgenic maize and maize ubiquitin promoter derived gene was expressed in T_1 generation of transgenic maize.

In conclusion, the miraculin gene was successfully expressed with a ubiquitin promoter, linked with a ubiquitin terminator, in T_0, T_1 and T_2 transgenic lettuce and showed an expression level similar to that of native miraculin and miraculin stably expressed in tomatoes, and miraculin gene expression was failed using 35S promoter/nos terminator cassette transgenic lettuce in T_1 and T_2 generation. We conclude that the endogenous lettuce ubiquitin promoter, linked with a ubiquitin terminator, is a suitable driver for stable foreign gene expression in lettuce and overcomes the gene silencing problem.

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Figure captions

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.

Fig. 2 Characterization of transgene transcription and translation in T_0 generation of Ubi-MIR transgenic lettuce. 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, 350ng 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 T_0 generation of 35S-MIR transgenic lettuce. 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, 350ng purified miraculin protein; Wt, wild type lettuce; MIR, miraculin; Lhcb2, LHCII type chlorophyll a/b binding protein.

Fig. 4 Characterization of transgene transcription and translation in T_1 generation of Ubi-MIR

transgenic lettuce lines 1, 7, and 8. 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, 350ng purified miraculin protein; Wt, wild type lettuce; MIR, miraculin; Lhcb2, LHCII type chlorophyll a/b binding protein.

Fig. 5 Detection of miraculin protein in T_1 (A) and T_2 (B) generations of 35S-MIR transgenic lettuce lines 2, 6, and 13. Presence of transgene was confirmed by genomic PCR. 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. P, 350ng purified miraculin protein; Wt, wild type lettuce; MIR, miraculin; Lhcb2, LHCII type chlorophyll a/b binding protein.

Fig. 6 Characterization of transgene transcription and translation in T_2 generation of Ubi-MIR transgenic lettuce lines 1, 7, and 8. 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, 350ng purified miraculin protein; Wt, wild type lettuce; MIR, miraculin; Lhcb2, LHCII type chlorophyll a/b binding protein.

Figure 1

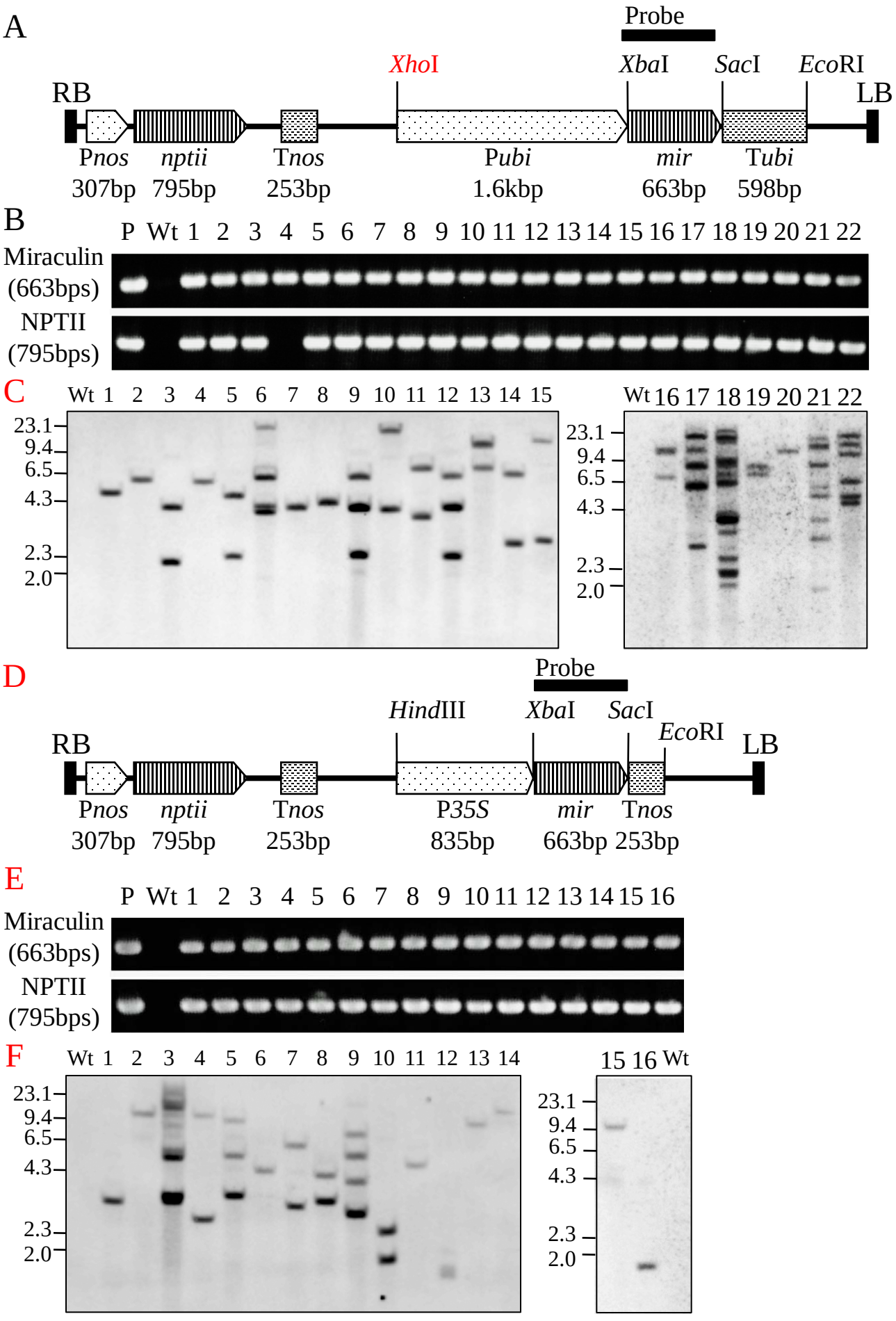

A
Probe
XhoI
XbaI
SacI
EcoRI
RB
LB
Pnos
nptii
Tnos
Pubi
mir
Tubi
307bp
795bp
253bp
1.6kbp
663bp
598bp
B
P Wt 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Miraculin
(663bps)
NPTII
(795bps)
C
Wt 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Wt 16 17 18 19 20 21 22
23.1
9.4
6.5
4.3
2.3
2.0
D
Probe
HindIII
XbaI
SacI
EcoRI
RB
LB
Pnos
nptii
Tnos
P35S
mir
Tnos
307bp
795bp
253bp
835bp
663bp
253bp
E
P Wt 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Miraculin
(663bps)
NPTII
(795bps)
F
Wt 1 2 3 4 5 6 7 8 9 10 11 12 13 14
15 16 Wt
23.1
9.4
6.5
4.3
2.3
2.0

Figure 2

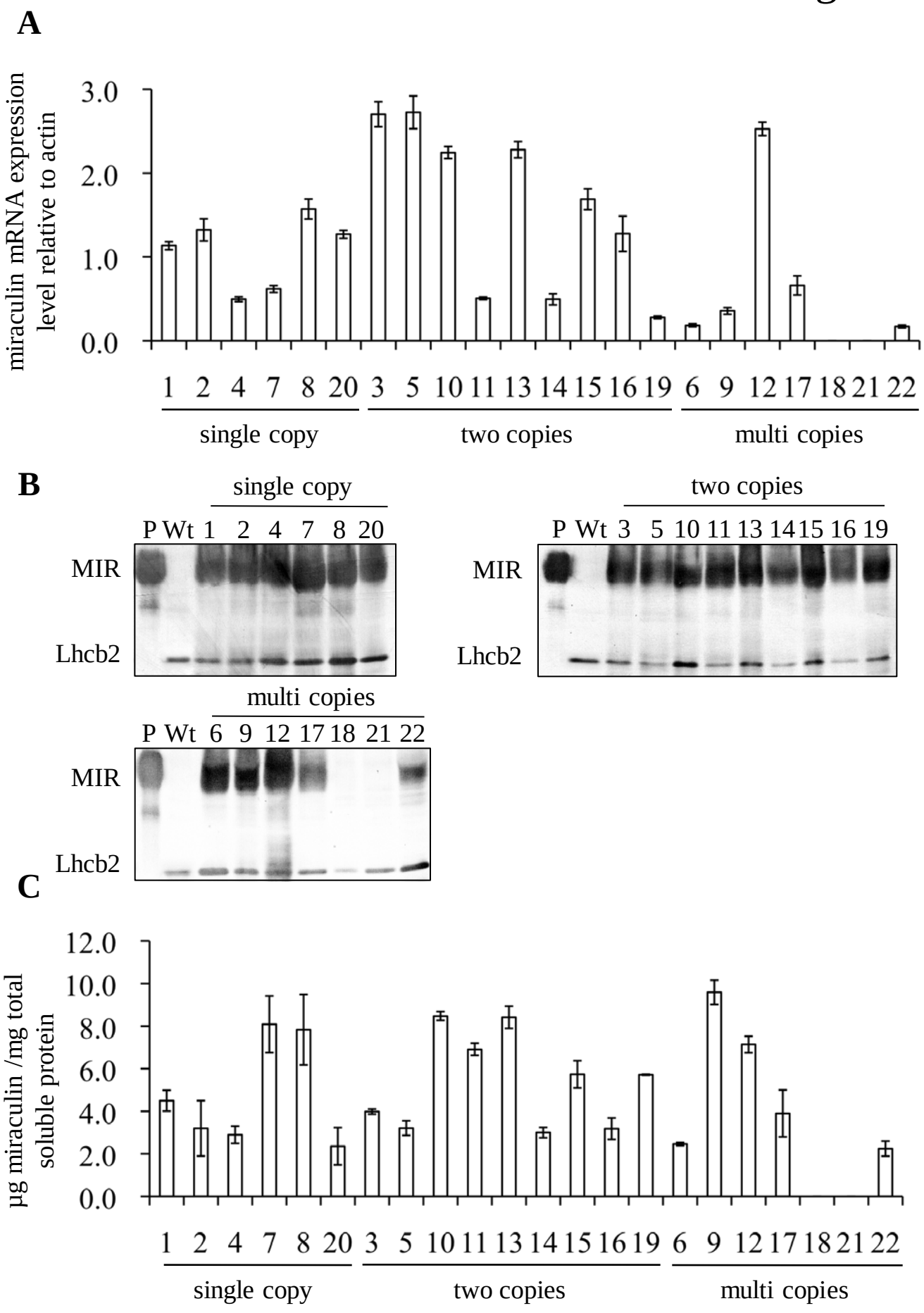

A
miraculin mRNA expression level relative to actin
3.0
2.0
1.0
0.0
1 2 4 7 8 20 3 5 10 11 13 14 15 16 19 6 9 12 17 18 21 22
single copy
two copies
multi copies
B
single copy
P Wt 1 2 4 7 8 20
MIR
Lhcb2
two copies
P Wt 3 5 10 11 13 14 15 16 19
MIR
Lhcb2
multi copies
P Wt 6 9 12 17 18 21 22
MIR
Lhcb2
C
µg miraculin /mg total soluble protein
12.0
10.0
8.0
6.0
4.0
2.0
0.0
1 2 4 7 8 20 3 5 10 11 13 14 15 16 19 6 9 12 17 18 21 22
single copy
two copies
multi copies

Figure 3

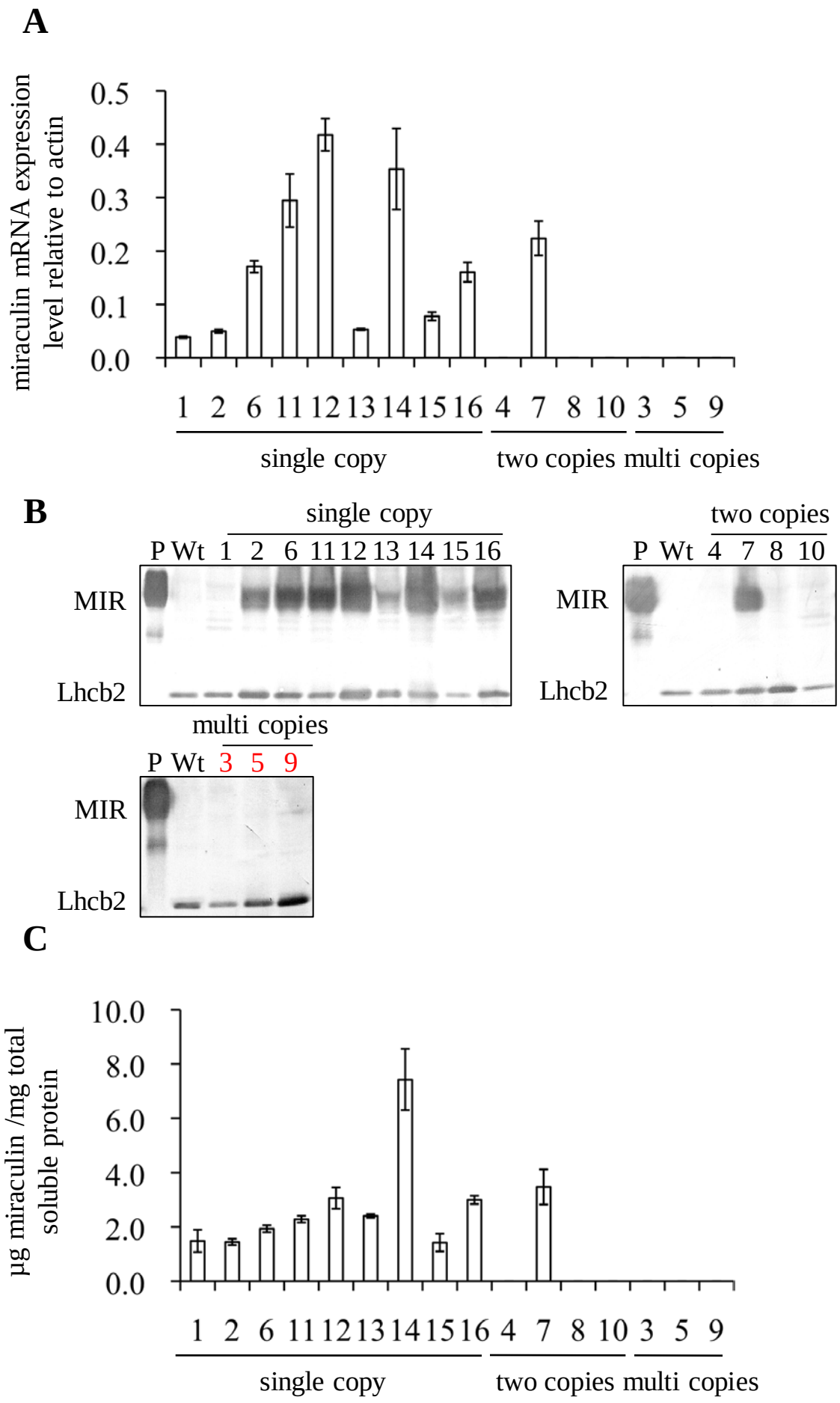

A
miraculin mRNA expression level relative to actin
0.5
0.4
0.3
0.2
0.1
0.0
1 2 6 11 12 13 14 15 16 4 7 8 10 3 5 9
single copy
two copies multi copies
B
single copy
two copies
P Wt 1 2 6 11 12 13 14 15 16
P Wt 4 7 8 10
MIR
Lhcb2
multi copies
P Wt 3 5 9
C
µg miraculin /mg total soluble protein
10.0
8.0
6.0
4.0
2.0
0.0
1 2 6 11 12 13 14 15 16 4 7 8 10 3 5 9
single copy
two copies multi copies

Figure 4

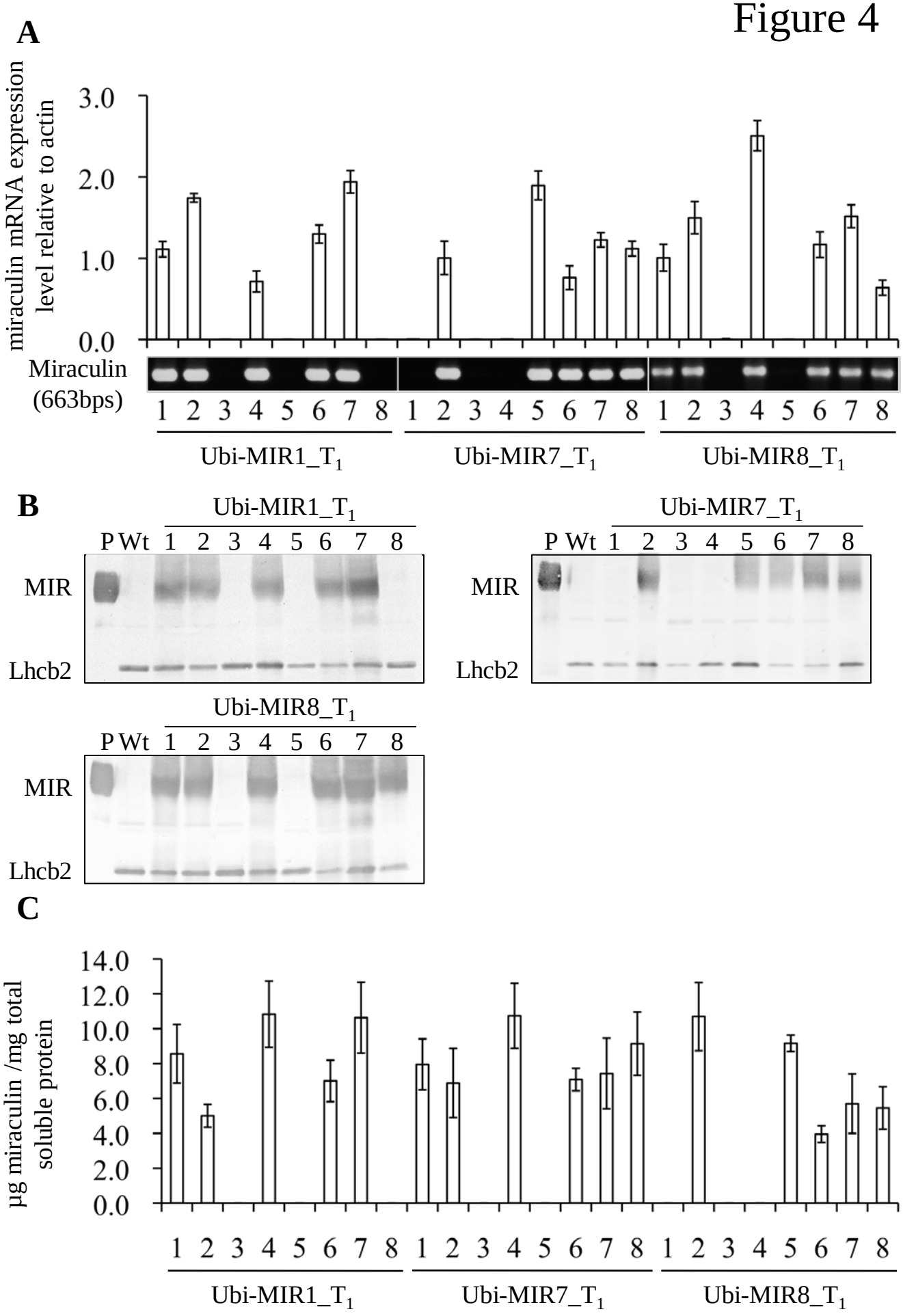

A
miraculin mRNA expression level relative to actin
3.0
2.0
1.0
0.0
Miraculin (663bps)
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
Ubi-MIR1_T1
Ubi-MIR7_T1
Ubi-MIR8_T1
B
Ubi-MIR1_T1
P Wt 1 2 3 4 5 6 7 8
MIR
Lhcb2
Ubi-MIR7_T1
P Wt 1 2 3 4 5 6 7 8
MIR
Lhcb2
Ubi-MIR8_T1
P Wt 1 2 3 4 5 6 7 8
MIR
Lhcb2
C
µg miraculin /mg total soluble protein
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
Ubi-MIR1_T1
Ubi-MIR7_T1
Ubi-MIR8_T1

Figure 5

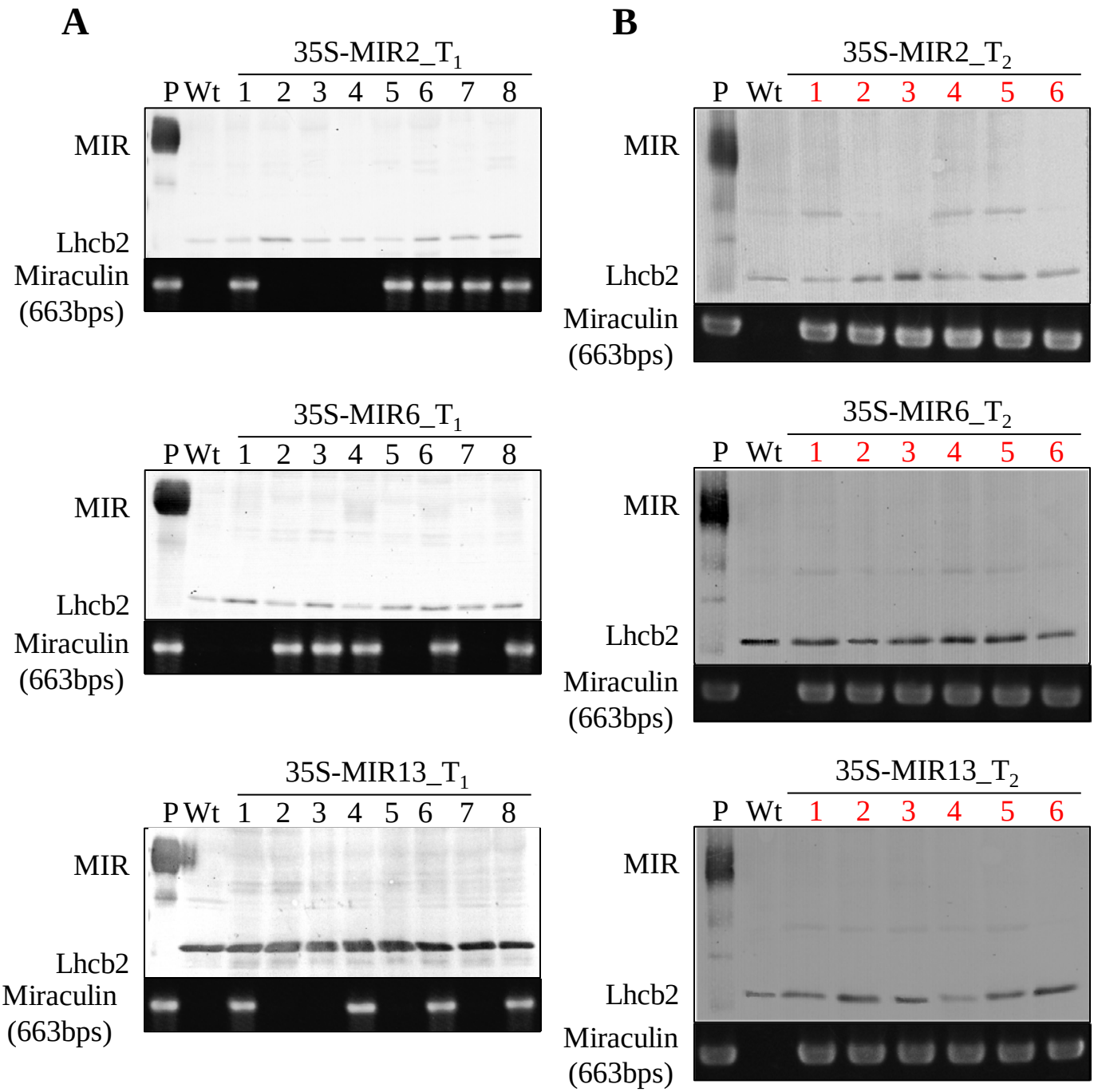
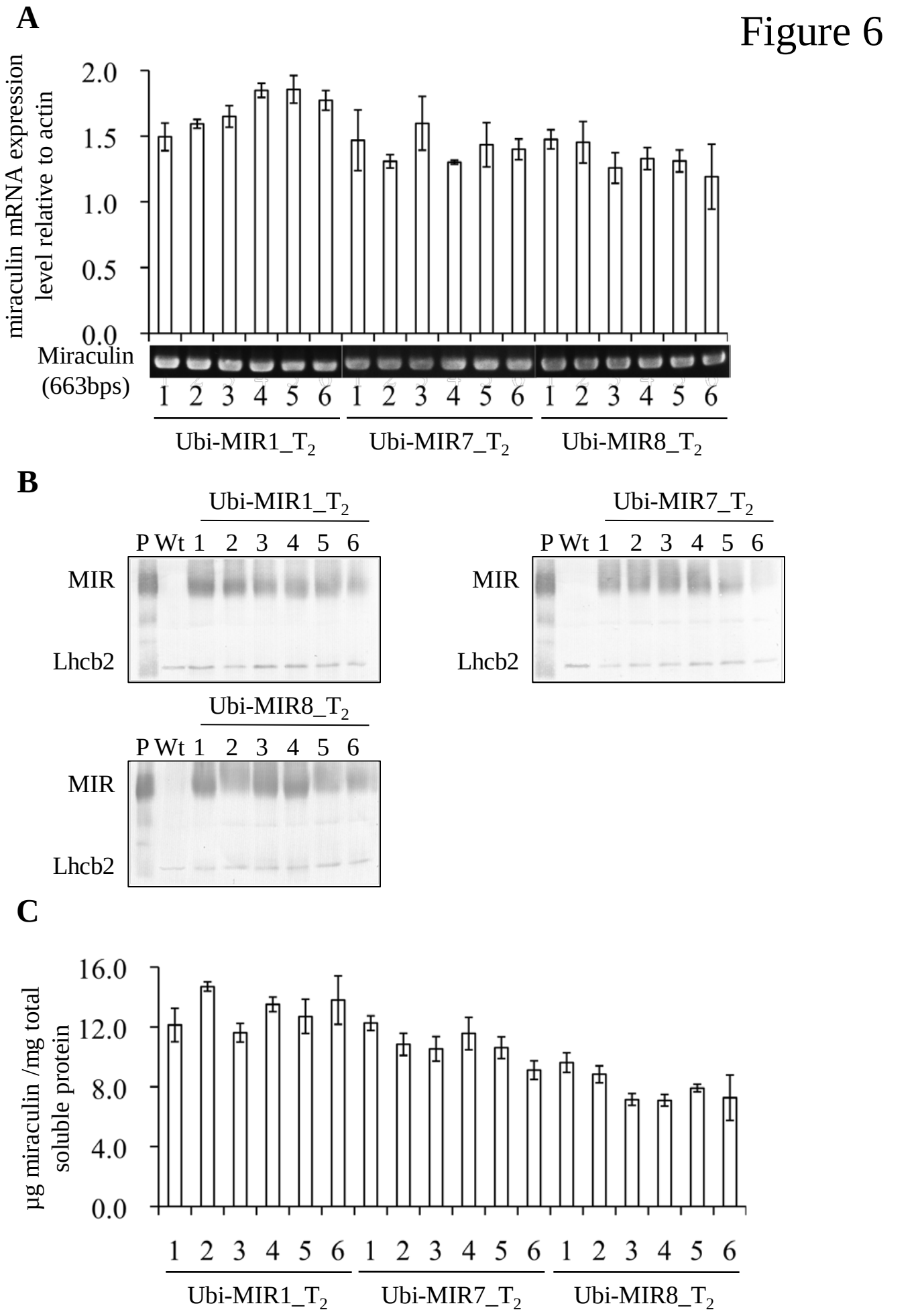

A
35S-MIR2_T1
P Wt 1 2 3 4 5 6 7 8
MIR
Lhcb2
Miraculin
(663bps)
35S-MIR6_T1
P Wt 1 2 3 4 5 6 7 8
MIR
Lhcb2
Miraculin
(663bps)
35S-MIR13_T1
P Wt 1 2 3 4 5 6 7 8
MIR
Lhcb2
Miraculin
(663bps)
B
35S-MIR2_T2
P Wt 1 2 3 4 5 6
MIR
Lhcb2
Miraculin
(663bps)
35S-MIR6_T2
P Wt 1 2 3 4 5 6
MIR
Lhcb2
Miraculin
(663bps)
35S-MIR13_T2
P Wt 1 2 3 4 5 6
MIR
Lhcb2
Miraculin
(663bps)

Figure 6


A
miraculin mRNA expression level relative to actin
2.0
1.5
1.0
0.5
0.0
Miraculin (663bps)
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Ubi-MIR1_T2
Ubi-MIR7_T2
Ubi-MIR8_T2
B
Ubi-MIR1_T2
P Wt 1 2 3 4 5 6
MIR
Lhcb2
Ubi-MIR7_T2
P Wt 1 2 3 4 5 6
MIR
Lhcb2
Ubi-MIR8_T2
P Wt 1 2 3 4 5 6
MIR
Lhcb2
C
µg miraculin /mg total soluble protein
16.0
12.0
8.0
4.0
0.0
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Ubi-MIR1_T2
Ubi-MIR7_T2
Ubi-MIR8_T2