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High-level accumulation of recombinant miraculin protein in transgenic tomatoes expressing a synthetic *miraculin* gene with optimized codon usage terminated by the native miraculin terminator

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Abstract

In our previous study, a transgenic tomato line that expressed the MIR gene under control of the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator (tNOS) produced the taste-modifying protein miraculin (MIR). However, the concentration of MIR in the tomatoes was lower than that in the MIR gene's native miracle fruit. To increase MIR production, the native MIR terminator (tMIR) was used and a synthetic gene encoding MIR protein (sMIR) was designed to optimize its codon usage for tomato. Four different combinations of these genes and terminators (MIR-tNOS, MIR-tMIR, sMIR-tNOS and sMIR-tMIR) were constructed and used for transformation. The average MIR concentrations in MIR-tNOS, MIR-tMIR, sMIR-tNOS and sMIR-tMIR fruits were 131, 197, 128 and 287 µg/g freshweight, respectively. The MIR concentrations using tMIR were higher than those using tNOS. The highest MIR accumulation was detected in sMIR-tMIR fruits. On the other hand, the MIR concentration was largely unaffected by sMIR-tNOS. The expression levels of both MIR and sMIR mRNAs terminated by tMIR tended to be higher than those terminated by tNOS. Read-through mRNA transcripts terminated by tNOS were much longer than those terminated by tMIR. These results suggest that tMIR enhances mRNA expression and permits the multiplier effect of optimized codon usage.

Keywords  Miraculin· Codon optimization· Miraculin terminator· Transgenic tomato· Read-through

Abbreviations  GUS, β-glucuronidase; MIR, miraculin; sMIR, synthesized MIR; NOS, nopaline synthase
Introduction

Plants provide many advantages for the production of valuable heterologous proteins over other production systems in terms of practicality, economy and safety (Twyman et al. 2003; Desai et al. 2010). In fact, the production and storage costs with plant systems are low compared to other systems such as mammalian cell culture and microbial fermentation (Desai et al. 2010). Plants are also a convenient system for large-scale production and have a lower risk of contamination by human pathogenic microorganisms (Giddings et al. 2000; Desai et al. 2010). Additionally, when target proteins are produced in the edible part of a plant, the protein can be consumed raw as an edible vaccine (Mason et al., 2002). However, the use of plants as expression hosts has several constraints. The primary limitation is the low level of protein accumulation (Daniell et al., 2001). Ways of improving transcription and translation levels include testing various promoters and terminators and optimizing codon usage.

The compatibility of the promoter with the host plant is crucial for high expression of the target gene (Twyman et al. 2003; Desai et al. 2010). However, the 3'-untranslated region and terminator of the mRNA also influence the expression level of the target gene by controlling RNA transcript termination and polyadenylation (Proudfoot 2004; Gilmartin 2005), and the efficiency of transcription is different with different types of terminators (Ingelbrecht et al. 1989; Nagaya et al. 2010). In fact, the expression level can change 60-fold depending on the terminator sequence used in a transient expression assay (Ingelbrecht et al. 1989).

The preferred codon usage varies significantly between different plant species (Murray et al. 1989). Therefore, when rare codons for a host plant are used in an introduced gene, the codon becomes the limiting factor in the translation process. This restriction is especially strong in the case of molecular farming in plants because the aim is mass production of the target protein. One of the strategies to increase translation efficiency is to modify the codons from the original sequence to more suitable ones in the host plant without changing the amino acid sequence (Gustafsson et al. 2004). By codon optimization, expression of the insect control protein gene cryIA(b) from Bacillus thuringienensis was increased up to 100-fold in transgenic tomato and tobacco (Perlak et al. 1991). Moreover, codon modification of the reductase gene (P450) from wheat increased its protein accumulation level in transgenic tobacco, demonstrating that preferred codon usage is different between plant species (Batard et al. 2000).

Miraculin (MIR) is a glycoprotein in miracle fruit (Richardella dulcifica), a shrub originally from West Africa (Theerasilp and Kurihara 1988). It has the unique property of changing a sour taste into a sweet taste, although MIR itself is not sweet. It is possible to provide a safe yet appetizing diet for diabetic and dieting people who require a restricted diet by utilizing the taste-modifying behavior of MIR as an alternative low-calorie sweetener. Most important, this property of MIR can make dieting more appealing. However, despite its great potential, miracle fruit production is limited because it is a tropical plant. Efforts to produce recombinant MIR have succeeded using plants such as tomato, lettuce and strawberry as a host (Sun et al. 2006a, 2007; Sugaya et al. 2008). Among these species, tomato was the most suitable host for MIR production (Yano et al. 2010). The level of MIR accumulation remained steady over multiple generations, and the introduced MIR gene was stably inherited (Yano et al. 2010). In the studies, the MIR gene was driven by a cauliflower mosaic virus (CaMV) 35S promoter and terminated by the nopaline synthase (NOS) terminator, and the MIR content was < 1% of total soluble protein in the transgenic tomato fruit (Sun et al. 2007). In contrast, the MIR content in miracle fruit is around 10% of total soluble protein (Theerasilp and Kurihara 1988). This means that recombinant MIR protein is produced in miracle fruit more efficiently. Therefore, to improve transcript efficiency we isolated the MIR terminator and used it instead of the NOS terminator to produce MIR in transgenic tomatoes. In addition, the codon usage of the MIR gene was optimized to reflect frequently used codons in the tomato, and the effect on the translation process was evaluated.

Materials and methods

Isolation of the MIR terminator

Aliquots (4 µg) of genomic DNA isolated from miracle fruit leaves as described by Rogers et al. (1985) were digested with the restriction enzymes EcoRV, PvuII and ScaI, ligated with the specific sequence adapter using Ligaction high (TOYOBO, Osaka, Japan) and then were used as a template for polymerase chain reaction (PCR) amplification. To determine the MIR terminator sequences, PCR was performed using LA Taq (Takara-Bio Inc., Otsu, Japan) with an adapter-
specific primer (AP1) and MIR-specific primers (MIR1-1, MIR1-2), and then nested PCR was performed with the adapter-specific primer (AP2) and an MIR-specific primer (MIR2). The PCR products were ligated into pGEM®-T Easy vector (Promega, Madison, WI, USA) and sequenced. The primer sequences were adapter, 5'-GTAA TACGACTCACTA TAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3'; AP1, 5'-CCA TCCTAA TACGACTCACTA TAGGGC-3'; AP2, 5'-CTA TAGGGCACGCGTGGT-3'; MIR1-1, 5'-ACAACTCTGGGTGGACAAACGAAGCTGCCGTT-3'; MIR1-2, 5'-GGAGTTTCTCTCTCCGTCTA TGTCAAGAACCGGA TTG-3'; and MIR2, 5'-GCCGAA TCCGCTGCACTAAGCAGTGGTTT-3'.

Characterization of the MIR terminator

To assay the termination efficiency of different length MIR terminators, a transient assay was performed. MIR terminator fragments of 147, 278, 508 and 1085 bp were amplified by PCR using an added forward primer SacI restriction site and an added reverse primer EcoRI restriction site (Table 1, Fig. 1). Each MIR terminator fragment was used to replace the NOS terminator fragment in plasmid pBI121 by insertion between its SacI and EcoRI restriction sites. The resulting plasmids, named t147, t278, t508 and t1085, contained the β-glucuronidase (GUS) gene flanked by the MIR terminator and under control of the CaMV 35S promoter. Each plasmid and pBI121 as a control was transferred to Agrobacterium tumefaciens GV2260 (Deblaere et al. 1985) using the method of Shen and Forde (1989).

Sterilized tomato seeds (Solanum lycopersicum cv. Micro-Tom) were sown on Murashige and Skoog’s medium (1962) and cultured at 25°C with 16 h of light per day for ten days. Cotyledons of the seedlings were inoculated with plasmid-containing Agrobacterium and co-cultivated according to Sun et al. (2006b). After co-cultivation for three days, inoculated cotyledons were washed with sterilized water to remove the Agrobacterium and a GUS assay was performed according to the method of Jefferson et al. (1987) to evaluate the influence of the MIR terminators of different lengths on expression efficiency.

Codon modification of the MIR gene

The codon usage table for tomato (http://www.kazusa.or.jp/codon/index.html) was used to eliminate rare codons (less than 10%) in the MIR gene. ATTTA sequences, which are known to destabilize transcripts (Gutierrez et al. 1999), were also removed. Additionally, mRNA secondary structure formation was minimalized by original software of Invitrogen. The optimized MIR gene was synthesized by outsourcing (Invitrogen, Tokyo, Japan) and was cloned into pUCminusMCS. When the optimized MIR gene was synthesized, XbaI and SacI restriction sites were added to the 5' and 3' ends of the gene, respectively. Native MIR and synthetic sMIR share 73% identity at the nucleotide sequence level (Fig. 2).

Construction of plasmids and transformation into tomato

To evaluate the individual influences of the MIR terminator and the codon-modified MIR gene on MIR accumulation in tomato, we prepared four different constructs for introduction into tomato combining either MIR or sMIR with either the 508-bp MIR terminator (tMIR) or the NOS terminator (tNOS): MIR-tNOS, MIR-tMIR, sMIR-tNOS and sMIR-tMIR.

The native MIR gene was isolated from genomic DNA of miracle fruit leaves based on the published DNA sequence of MIR (GenBank accession number AB512278). A pair of specific primers (forward primer: 5'-TTTTTTTCTTAGATGAAGGAAATTAACACGCT-3', and reverse primer: 5'-TTTTTTGAGCTCAGCTTGAAGATACCGGTTTTTG-3') containing XbaI and SacI sites, respectively, was used to amplify the coding region of MIR. The amplification reaction using KOD-Plus (TOYOBO, Osaka, Japan) consisted of 9 for 3 min, 35 cycles of amplification (94°C for 15 sec, 56°C for 25 sec and 68°C for 50 sec) and a final extension at 68°C for 10 min. The PCR product was cloned into the XbaI and SacI sites of pBI121 after removing the GUS coding region via the restriction enzyme sites. The sMIR gene was subcloned from pUCminusMCS into pBI121 using the XbaI and SacI sites in the same way as with MIR. The MIR terminator (508 bp) used to assess the terminator as described above was cloned into the SacI and EcoRI sites of pBI121 containing MIR or sMIR after eliminating the NOS terminator fragment. In these constructs, MIR and sMIR gene expression are driven by the constitutive CaMV 35S promoter.
The constructed plasmids were transferred into *Agrobacterium* as described above. Using *Agrobacterium* with the introduced plasmid, tomato (*Solanum lycopersicum* cv. Micro-Tom) plants were transformed as described by Sun et al. (2006b, 2007).

**Genomic Southern blot analysis**

To confirm the copy number of the *MIR* or *sMIR* gene and the *neomycin phosphotransferase II* (*NPTII*) gene in transgenic tomato, genomic DNA was isolated using Maxwell® 16 DNA purification kits according to the manufacturer’s protocol (Promega, Tokyo, Japan). Isolated genomic DNA (10 µg) was digested using the restriction enzyme *XbaI* (which cleaves only once outside the *MIR* gene), separated by electrophoresis on a 1% agarose gel and then transferred to a Hybond-N+ nylon membrane (GE Healthcare, Tokyo, Japan). Hybridization was performed overnight in high-SDS buffer (50% deionized formamide (v/v), 5× SSC, 7% SDS, 2% Blocking Regent (Roche, Tokyo, Japan), 50 mM sodium phosphate (pH 7.0), and 0.1% N-lauroylsarcosine sodium salt (w/v)) containing a gene-specific DIG-labeled probe at 45°C. Probes were prepared with a PCR DIG Probe synthesis kit (Roche, Tokyo, Japan) following the manufacturer’s protocol. The hybridization signal was detected by chemiluminescence using CDP-Star (Roche, Tokyo, Japan) followed by exposure in the LAS4000mini Image Analyzer (Fujifilm Co. Ltd., Tokyo, Japan).

**Immunoblot analysis and enzyme-linked immunosorbent assay (ELISA)**

Tomato fruits were harvested during the red stage from *MIR* or *sMIR* and *NPTII* single-copy plants and ground to powder under liquid nitrogen. The protein was extracted as described previously by Hirai et al. (2010). Using the extracted protein, immunoblot analysis and ELISA were performed according to Sun et al. (2007) and Kim et al. (2010), respectively.

**Quantitative reverse transcription-PCR (qRT-PCR) analysis**

The expression levels of the *MIR* and *sMIR* transcripts in the transgenic tomato plants were determined by qRT-PCR. Total RNA was isolated from red fruits that were the same fruits used for immunoblot analysis and ELISA using the RNeasy Plant Mini kit (Qiagen, Tokyo, Japan) with RNase-free DNase (Qiagen, Tokyo, Japan). The first-strand cDNA was synthesized from extracted total RNA (0.5 µg) using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Tokyo, Japan) with Oligo(dT)20 primer. Ten-fold diluted first-strand cDNA was used as a template for the reaction with SYBR Premix Ex Taq II (Takara-Bio Inc., Otsu, Japan) on the Thermal Cycler Dice Real Time System TP800 (Takara-Bio Inc., Otsu, Japan) following the manufacturer’s instructions. The qRT-PCR was subjected to 40 cycles of 95°C for 5 sec and 57°C for 30 sec. Relative quantification of *MIR* and *sMIR* gene expression was calculated by normalization to *Slubiquitin3* gene (GenBank accession number X58253) expression, which has been used as an internal control in tomato expression analysis (Hackel et al. 2006; Chincinska et al. 2008). Primer sequences were as follows: *MIR* forward, 5’- CCTGCAAAGTAAAA TGCGGAGA-3’ and reverse, 5’- AACTCGAATGCGAATGGTTATC-3’; *sMIR* forward, 5’- CCTGCAAAGTAAAA TGCGGAGA-3’ and reverse, 5’- AACTCGAATGCGAATGGTTATC-3’; and *ubiquitin* forward, 5’- CACCAAGCAAGGGAAGGTTTATC-3’; and reverse, 5’- TCAGCATTAGGGCACTCCTT-3’. The *MIR* and *sMIR* primer sets were designed at the same sequence position although these primer sequences were not completely consistent.

**Transcription termination of the *MIR* and *sMIR* transcripts harboring NOS or MIR terminators**

To evaluate the influence of different terminators on transcription termination, RT-PCR was performed with several sets of primers specific to each gene using the GoTaq® Green Master Mix (Promega, Tokyo, Japan). cDNAs synthesized for qRT-PCR were used as a template. The amplification reactions consisted of 95°C for 3 min, 35 cycles of amplification (95°C for 30 sec, 55°C for 30 sec and 72°C for 2.5 min) and a final extension at 72°C for 7 min. The primers used are described in Table 1. *MIR* or *sMIR* mRNA’s polyadenylation sites of each transgenic tomato from four different constructs and miracle fruit were detected using 3’-full RACE core set
(TAKARA, Tokyo, Japan) as described in the manual. Total RNA was used to synthesize single-strand cDNA and amplified with PCR using gene-specific primers containing BamHI or KpnI sites for cloning: BamHI-MIR, 5'-ACGGACGGATCCAAAGGAAGACGTGGTGTCGTCCTC-3'; KpnI-sMIR, 5'-ACGGACGTTACCTTTCATGCTTGAAGTGGAGAAG-3'. Clones confirmed the insert by colony PCR were analyzed the sequence with gene-specific primers: MIR-seq491F, 5'-AGATTGAGGAGTTTTGTGGTAGTGG-3', sMIR-seq491F, 5'-AGATTGAGGAGTTTGTTGCTCAGG-3'.

Results

Efficiency of GUS activity with MIR terminators of various lengths

We isolated a 1,953-bp fragment containing the MIR terminator, from which a sequence of 1085 bp was used to evaluate its terminator function. The sequence included three typical polyadenylation signals (AA TAAA) (Fig. 1a). Different length fragments of the terminator (146 bp, 287 bp, 508 bp and 1085 bp) were bound downstream of the GUS-coding region and transferred to tomato cotyledon explants (Fig. 1b). When the MIR terminator fragment was over 508 bp, transient GUS expression (observed as a blue spot of GUS activity) was stronger than with the NOS terminator (data not shown). Similarly, GUS activity (as determined based on 4-methyl umbelliferone (4MU) accumulation) relative to that with the NOS terminator was higher with the 508-bp and 1085-bp fragments and lower with the 146-bp and 287-bp fragments (Fig. 1c).

Production of transgenic tomato plants accumulating recombinant MIR protein

To assess the effects of codon optimization and use of the MIR terminator on MIR accumulation, four different constructs were created combining MIR or sMIR with the 508-bp MIR terminator sequence or the NOS terminator (MIR-tNOS, MIR-tMIR, sMIR-tNOS and sMIR-tMIR). In these constructs, MIR and sMIR gene expression are driven by the constitutive CaMV 35S promoter. Tomato cotyledons were transformed by infection with Agrobacterium containing the binary vector of each construct and then transformed tomato shoots were selected while rooting on medium including kanamycin. Kanamycin-resistant tomato lines were selected on selective medium, and the integration of the MIR gene in these plants was confirmed by Southern blot analysis after selection of diploids using the ploidy test and by investigation of MIR accumulation via western blot analysis. Eventually, seven to eleven independent transgenic tomato lines were obtained as single-copy MIR or sMIR plus NPTII plants for each of the four constructs (Fig. 3).

Accumulation of recombinant MIR protein in transgenic tomato fruit

The concentration of recombinant MIR protein was measured with ELISA (Fig. 4). When the MIR terminator was used, the concentration in MIR-tMIR fruits was 1.5 times as high as that in the control (MIR-tNOS) fruits. Moreover, the highest effect on MIR concentration was detected in sMIR-tMIR fruits, in which the MIR concentration was 2.2 times higher than that in control fruit. In contrast, the MIR level in sMIR-tNOS fruits was almost the same as that in MIR-tNOS fruits. Western blot analysis of MIR protein levels reflected the ELISA results and also revealed that the molecular size of the signal from recombinant MIR was coincident with that from MIR purified from miracle fruit (data not shown).

Expression of the MIR and sMIR genes in transgenic tomato fruit

MIR and sMIR mRNA expression levels were detected by qRT-PCR. These sequences had 73% nucleotide homology (Fig. 2). Therefore, we were not able to design a primer set of completely the same sequence for qRT-PCR. They had two mismatches in each of the forward and reverse primers. The two different primer sets were confirmed by amplification efficiency and characterization. When the NPTII primer set was used as a control, the standard curves of the MIR and sMIR primer sets exhibited almost the same slope and intercept as the standard curves of the NPTII primer set using either the MIR or sMIR plasmid as a template. Thus, these primer sets were used for qRT-PCR.
Transcription termination with different terminators

To assess the effects of these terminators, read-through lengths were analyzed using RT-PCR. Transcription read-through was detected at the end of the NOS terminator with the MIR-tNOS and sMIR-tNOS mRNAs (Fig. 6a), and some of transcripts were detected around the left border. On the other hand, utilization of the MIR terminator did not induce read-through, at least up to the end of the MIR terminator. Subsequently, two sets of primers were designed inside the region of the MIR terminator and used for detailed investigation of the lengths of MIR and sMIR transcripts. mRNA read-through was verified at 146 bp on the MIR terminator with the MIR-tMIR and sMIR-tMIR constructs (Fig. 6b), and some read-through was also detected at 287 bp.

We also identified the polyadenylation sites using 3’ rapid amplification of cDNA ends (RACE). The major sites were at 57, 151 and 166 bp in MIR-tNOS mRNA and, at 99 and 111 bp in MIR-tMIR mRNA with referring to bases downstream from the stop codon (Fig. 7). In sMIR-tNOS and sMIR-tMIR mRNAs, the major sites were almost at the same position in MIR-tNOS and MIR-tMIR, respectively. The sites of native MIR mRNA from miracle fruit were at 104 and 105 bp close to those of MIR-tMIR and sMIR-tMIR.

Discussion

The expression levels of the mRNAs tended to be stronger when they were terminated by the MIR terminator than by the NOS terminator: compare MIR-tMIR to MIR-tNOS or sMIR-tMIR to sMIR-tNOS (Fig. 5). In addition, the expression of MIR mRNA was approximately two times higher than that of sMIR mRNA when the terminator was the same.
through transcripts may contain an open reading frame (ORF) other than the target gene and sometimes unknown peptides from the ORF are produced (Rang et al. 2005). In the case of transgenic plants for human consumption, the existence of an additional peptide outside of the target protein is not suitable for commercial use. Read-through can also cause transcriptional interference of genes located downstream of the terminator (Ingelbrecht et al. 1991). Thus, the function of transcript termination is crucial not only for tuning expression levels but also for avoiding improper peptide production and transcriptional interference. In addition to these points, the MIR terminator is derived from an edible plant, the miracle fruit. Our results have provided one of the most useful terminators for commercial use.

Optimization of codon usage is frequently used when a gene introduced into a host plant is derived from another organism such as fungi (Xue et al. 2003; Peng et al. 2006), bacteria (Perlak et al. 1991) or animals (Rouwendal et al. 1997) because codon preferences are quite different in these kingdoms. In plants, it is also different not only between monocots and dicots but also between the nucleus and the plastid of the same plant (Batard et al. 2000; Kawabe and Miyashita 2003; Lin and Xue 2005). Therefore, modifying codons to suit the host may significantly improve the production of a target protein, especially when rare codons are used in the heterologous gene. We optimized the codons of the MIR gene to make it suitable for tomato. As a result, the production of recombinant MIR protein in sMIR-tMIR fruits was higher than that in MIR-tMIR fruits, although the expression of sMIR mRNA was low compared to that of MIR-tMIR, suggesting that the improvement in translation efficiency exceeded the decline in transcription efficiency (Fig. 4). Similar results were observed between sMIR-tNOS and MIR-tNOS fruits. The expression of MIR mRNA in MIR-tNOS was higher than that of sMIR mRNA in sMIR-tNOS, but the MIR productivity was almost the same. From another standpoint, the sMIR transcript levels were approximately half those of MIR using either the NOS or the MIR terminator; however, the impact on translation of codon optimization was higher when using the MIR terminator than when using the NOS terminator (Fig. 4, 5). These results imply that terminated mRNA by the NOS terminator causes a decline in translational efficiency.

In this study, the sMIR transcript levels of sMIR-tNOS and sMIR-tMIR fruits reduced compared to that of MIR. Some reports indicate that a sequence of 5'-UTR and the amino acid sequence of the first exon coding region influence transcription initiation, transcriptional efficiency and mRNA stability (Chiba et al. 1999; Gutiérrez et al. 1999; Suzuki et al. 2001; Matsuura et al. 2008). However, it is unknown if ORF sequences themselves have any effects on transcription. The unknown character of ORF sequences may be responsible for the decline in the amount of sMIR transcripts. Another possibility is that the secondary structure of mRNA may influence mRNA stability. The codons of the MIR gene were thoroughly modified. Therefore, there is a possibility that some changed sequences were critical for transcriptional efficiency and mRNA stability. Either way, further study is required to understand this mechanism. If the transcript level can be increased by further optimization of the MIR gene, higher production of recombinant MIR protein might be achieved using the MIR terminator.

In conclusion, we succeeded at producing transgenic tomatoes with recombinant MIR at concentrations up to 340 µg/g fresh weight by using the MIR terminator and codon optimization of the MIR gene, although the concentration was almost 100 µg/g fresh weight in our previous study (Sun et al. 2007; Hiraiz et al. 2010; Yano et al. 2010). Additionally, we suggest that the identified MIR terminator is useful for increasing the level of transcription, improving translational efficiency by codon modification and improving the quality of the mRNA. These factors play a key role in the final productivity.

Acknowledgments

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References


Figure captions

Fig. 1  Efficiency of gene expression with MIR terminators of various lengths in transfected tomato cotyledons. a Sequence alignment of the identified MIR terminator. Arrows indicate the positions and directions of the primers used for the transient assay. Double lines show the typical polyadenylation signal AA TAAA. b T-DNA region of pBI121 used for the transient assay. The various lengths of the MIR terminator and NOS terminator were fused to the GUS gene under control of the CaMV35S promoter. LB and RB, the left and right borders of the T-DNA region, respectively; pNOS, NOS promoter; NPTII, neomycin phosphotransferase gene; tNOS, NOS terminator; p35S CaMV 35S promoter. c GUS activity in tomato cotyledons with MIR terminators of various lengths. GUS activity is expressed as the amount of the reaction product 4-methyl umbelliferone (4MU) per ten pieces of tomato cotyledon segments. Vertical bars show the standard error from three replications. t147, 147-bp terminator of the MIR terminator; t278, t508 and t1085 are abbreviated in the same manner as t147.

Fig. 2  DNA sequence comparison of the codon-modified sMIR coding region with the native MIR from miracle fruit. All codons of sMIR were optimized on the basis of the codon usage table for tomato. Arrows indicate the positions and directions of the primers used for qRT-PCR.

Fig. 3  Southern blot analysis of the MIR, sMIR and NPTII genes in transgenic tomatoes. DNA (10 µg) was digested with Xba I and detected with DIG-labeled probes from the coding sequences of MIR, sMIR or NPTII. Lane numbers show independent transgenic tomato lines (T0) with each of the different constructs: MIR-tNOS, MIR-tMIR, sMIR-tNOS, sMIR-tMIR. Tomato lines with the same number are clones. Upper and lower photos show the data for MIR or sMIR with the NPTII marker, respectively. M, marker (DNA Molecular Weight Marker II, DIG-labeled; 125, 564, 2,027, 2,322, 4,361, 6,557, 9,416, 23,130 bp).

Fig. 4  MIR concentrations in transgenic tomatoes. Recombinant MIR protein was extracted from three to five red-ripe fruits from independent transgenic plants and the concentration was measured by ELISA. The numbers under the columns show the independent transgenic tomato lines identified as in Fig. 3. The presented data under the construct names are the mean ± standard errors (SE). Alphabets indicate significant differences based on Tukey-Kramer’s Multiple Comparison test (P<0.01).

Fig. 5  Relative quantification of MIR and sMIR expression in transgenic tomatoes. MIR or sMIR mRNA was isolated from the same fruits used for Fig. 4, and the expression level was detected by real-time quantitative RT-PCR. The numbers under the columns show the independent transgenic tomato lines identified as in Fig. 3. The presented data under the construct names are the mean ± standard errors (SE). Alphabets indicate significant differences based on Tukey-Kramer’s Multiple Comparison test (P<0.01).

Fig. 6  Transcription termination of the MIR and sMIR transgenes. The cDNA samples used in Fig. 5 were analyzed by PCR using sets of primers (Table 1). The numbers (#1 to #4) show the independent transgenic tomato lines identified as in Fig. 3. Plasmid DNA was used as a positive control template. M, marker (Gene Ladder Wide 1, Nippon gene). a Detection of read-through transcripts with four different sets of primers. Small letters (a, b, c, d) above the photos show the primer sets described in Fig. 6c. b Detection of read-through transcripts from the MIR terminator. Small letters (e, f) above the photos show the primer sets described in Fig. 6c. c Primer design and size of PCR products. Numbers under the constructs show the nucleotide size of each region. The small letters on the left side (a to f) depict the sets of primers used and correspond to Fig. 6a, b. The size of the product from the MIR gene is indicated under each arrow and the size of the product from the sMIR gene is in parentheses. tNOS, NOS terminator; tMIR, MIR terminator; LB, left border.

Fig. 7  Polyadenylation sites of transcripts from MIR and sMIR transgenes and native MIR gene
of miracle fruit. 3'-RACE was performed using cDNA prepared from #1 and #2 transgenic lines for each construct. The resulting PCR products were cloned and sequenced. Polyadenylation sites were counted downstream from the transcriptional stop codon. The number of investigated clones was as follows: MIR-tNOS, 21 clones; MIR-tMIR, 21 clones; sMIR-tNOS, 30 clones, sMIR-tMIR, 30 clones, native MIR from miracle fruit, 22 clones.
Table 1  Sequences of oligonucleotides used in the study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>tMIR-start</td>
<td><strong>TCTAAGGAGCTC</strong>TTGGGTTTGGGGGTGGTTTTTCCA</td>
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<tr>
<td>tMIR-146R</td>
<td><strong>GCCAGTGAATTCT</strong>CGTACACGTCAGAAACACAACGCT</td>
</tr>
<tr>
<td>tMIR-287R</td>
<td><strong>GCCAGTGAATTCC</strong>ACACCTCTACCTTTGCTTTTCTTCAC</td>
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<tr>
<td>tMIR-508R</td>
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<tr>
<td>MIIR-start</td>
<td>CACCCAATCGGTTCTTGAC</td>
</tr>
<tr>
<td>MIIR-stop</td>
<td>TTAGAAGTATACGTTTTTGTTGAAACTCGAATG</td>
</tr>
<tr>
<td>sMIR-start</td>
<td>GAGCTACGATGCTTTTCTCTTAGC</td>
</tr>
<tr>
<td>sMIR-stop</td>
<td>TATCAGAAAGTGCAATCGACGC</td>
</tr>
<tr>
<td>tNOS-R</td>
<td><strong>TCCTAGTTTGCCTACATATTT</strong></td>
</tr>
<tr>
<td>tMIR-R</td>
<td><strong>CGTTCCTTTATGTTGTTCAAG</strong></td>
</tr>
<tr>
<td>LB-R1</td>
<td>ATTCAGGGCTCGCAACTG</td>
</tr>
<tr>
<td>LB-R2</td>
<td><strong>GGTGCCGTAAAGCACTAAATC</strong></td>
</tr>
</tbody>
</table>

Restriction enzyme recognition sites in sequences are shown in bold font.
Fig. 1

(a) DNA sequences and transcriptional activity. (b) Schematic representation of the constructs. (c) GUS activity (pmol 4MU/hr/10 segments) in different transgenic lines.
Fig. 2

**MIR (AB512278)**

<table>
<thead>
<tr>
<th>MIR</th>
<th>sMIR</th>
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<tbody>
<tr>
<td>1 ATGAGGAAT TAAACTGCT CTCTCTCTCG TTCTCTTGCT GTCTCGATT GTTGCGAGCA 80</td>
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</tr>
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<td>2 ATGAGAAAAGC TAAACTGCT CTCTCTCTCG TTCTCTTGCT GTCTCGATT GTTGCGAGCA 90</td>
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<tr>
<td>121 GACAGACGGC TCGAGCAGG TACCCCGTGC TGTGTCCTCG GCAGCATGCG 180</td>
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<td>121 GTGAGAAGG TACCCCGTGC TGTGTCCTCG GCAGCATGCG 180</td>
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<td>181 GAGGCCTTTA CAGATTATCA TACCCCGTGC TGTGTCCTCG GCAGCATGCG 240</td>
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<td>181 GAGGCCTTTA CAGATTATCA TACCCCGTGC TGTGTCCTCG GCAGCATGCG 240</td>
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<tr>
<td>601 AGCGGTTCG GAGTACGCT CCTGATGATG GAGTACGCT CCTGATGATG GAGTACGCT 660</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3

Fig. 4
Fig. 5

Relative transcript level

|MIR-tNOS| MIR-tMIR| sMIR-tNOS| sMIR-tMIR|
---|---|---|---|
Mean ± SE| 2.0 ± 0.15a| 3.2 ± 0.34b| 0.7 ± 0.11c| 1.6 ± 0.20a|
Fig. 6

C

<table>
<thead>
<tr>
<th></th>
<th>MIR (sMIR)</th>
<th>tNOS</th>
<th>LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>569 bp (616 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>841 bp (888 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>1035 bp (1082 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>1145 bp (1192 bp)</td>
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<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>MIR (sMIR)</th>
<th>tMIR</th>
<th>LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>569 bp (616 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>1066 bp (1113 bp)</td>
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</tr>
<tr>
<td>c</td>
<td>1236 bp (1283 bp)</td>
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</tr>
<tr>
<td>d</td>
<td>1352 bp (1391 bp)</td>
<td></td>
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</tr>
<tr>
<td>e</td>
<td>721 bp (768 bp)</td>
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<tr>
<td>f</td>
<td>862 bp (909 bp)</td>
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</tbody>
</table>

**MIR-start (sMIR-start) / MIR-stop (sMIR-stop)**

**MIR-start (sMIR-start) / tNOS-R**

**MIR-start (sMIR-start) / LB-R1**

**MIR-start (sMIR-start) / LB-R2**

**MIR-start (sMIR-start) / tMIR-R**

**MIR-start (sMIR-start) / tMIR-146R**

**MIR-start (sMIR-start) / tMIR-287R**

**primer sets**
Fig. 7

![Graphs showing clones](image)

- **MIR-tNOS**
- **sMIR-tNOS**
- **MIR-tMIR**
- **sMIR-tMIR**

*Downstream size from the stop codon (bp)*