The accumulation of recombinant miraculin is independent of fruit size in tomato

Azusa Ono1, Kyoko Hiwasa-Tanase2,3, Satoko Nonaka2,3, Hiroshi Ezura2,3,*

1Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan; 2Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan; 3Tsukuba-Plant Innovation Research Center, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
*E-mail: ezura.hiroshi.fa@u.tsukuba.ac.jp  Tel & Fax: +81-29-853-7263

Received July 13, 2020; accepted September 4, 2020 (Edited by K. Mishiba)

Abstract  The taste-modifying protein miraculin (MIR) has received increasing interest as a new low-calorie sweetener. In our previous study using the tomato variety ‘Micro-Tom,’ it was shown that in transgenic tomatoes in which MIR was expressed by using the cauliflower mosaic virus 35S promoter (p35S) and a heat shock protein terminator (tHSP) cassette (p35S-MIR-tHSP), higher levels of miraculin accumulated than when MIR was driven by the nopaline synthase terminator (tNOS) cassette (p35S-MIR-tNOS). ‘Micro-Tom’ is a dwarf tomato used for research and shows a low yield. To achieve high productivity of MIR, it is essential to improve the MIR accumulation potential by using high-yielding cultivars. In this study, we evaluate whether the high MIR accumulation trait mediated by the tHSP appears even when fruit size increases. A line in which the p35S-MIR-tHSP cassette was introduced into a high-yielding variety was bred by backcrossing. The line homozygous for MIR showed higher accumulation of MIR than the heterozygous line. Despite large differences in fruit size, the MIR level in the backcross line was similar to that in the p35S-MIR-tHSP line (background ‘Micro-Tom’). It was approximately 3.1 times and 4.0 times higher than those in miracle fruits and the p35S-MIR-tNOS tomato line 5B (‘Moneymaker’ background, which exhibits the highest miraculin productivity achieved thus far), respectively. These results demonstrate that the high MIR accumulation trait mediated by the tHSP appears even when fruit size is increased.

Key words:  fruit size, HSP terminator, miraculin, recombinant protein, transgenic tomato.

Miraculin (MIR) is a taste-modifying protein contained in miracle fruit (Synsepalum dulcificum), from the family Sapotaceae native to West Africa (Bouwer et al. 1968; Kurihara and Beidler 1968). Once exposed to MIR, the human tongue perceives sweetness in acidic conditions (Inglett et al. 1965). As MIR shows sweetness-inducing activity even at extremely low concentrations (Koizumi et al. 2011), it presents great potential as a new low-calorie sweetener, while sugar intake is increasing worldwide (Mattes and Popkin 2009). Nevertheless, the feasibility of its commercial application is restricted because natural miracle fruit exhibits low fruit productivity and originates in the tropics. Therefore, to realize the stable production of MIR, several attempts have been made to induce MIR accumulation in other organisms using gene recombination. Further studies have been conducted in tomato (Solanum lycopersicum) owing to its strong taste-modifying activity similar to that of MIR derived from miracle fruit (Sun et al. 2007) and the genetic stability of the MIR gene (Yano et al. 2010).

The transgenic tomato line 5B is one of the highest fruit-yielding tomato lines with miraculin accumulation generated to date. In this line, the MIR gene (AB512278) is expressed under the control of the cauliflower mosaic virus 35S promoter (p35S) and the nopaline synthase terminator (tNOS) of Agrobacterium tumefaciens (Sun et al. 2007; Yano et al. 2010) in the medium-sized tomato variety ‘Moneymaker.’ After that the development of this line, the terminator was optimized using the dwarf variety ‘Micro-Tom’ and the resulting transgenic tomato showed the highest MIR concentration achieved at that time (Hirai et al. 2011b). The heat shock protein 18.2 (A5g59720) terminator (tHSP) derived from Arabidopsis thaliana (Nagaya et al. 2010) is used in this line instead of tNOS, and its MIR accumulation level is approximately 10 times higher (955.0–1725.8 µg FW−1) than that of the p35S-MIR-tNOS line (102.4–176.4 µg FW−1) (Hirai et al. 2011b).

In recombinant protein production systems in plants, improving the productivity of the target protein by increasing the concentration of the target protein and the yield of the host plant leads to cost reduction in protein...
production. The p35S-MIR-tHSP tomatoes created by ‘Micro-Tom’ contributes to improved accumulation level of miraculin. However, ‘Micro-Tom’ is a dwarf tomato cultivar for horticultural ornamental or research use and are suitable for indoor cultivation, but the fruit size is small and less productive. Therefore, if it is intended to be grown in an outdoor facility such a greenhouse, the MIR accumulation trait needs to be transferred to a cultivar with more productive and larger fruit size. No commercial tomato varieties harboring the p35S-MIR-tHSP cassette have yet been developed. Hence, it is unclear whether the high MIR accumulation trait is maintained when fruit size is increased. In this study, we examined that whether fruit size affect MIR concentration.

A backcross breeding method was employed to transfer the high MIR accumulation trait of tomato associated with p35S-MIR-tHSP into a high-yielding variety (Figure 1). Among the p35S-MIR-tHSP tomato lines (‘Micro-Tom’ background) generated in our previous work (Hirai et al. 2011b), line #6 (T_3) and #26 (T_3) were selected as a donor parent owing to the retention of a single copy of MIR and high accumulation of MIR in the T_1 generation. As recurrent parents, the medium-sized and the large-sized Japanese cultivars ‘Natsunokoma’ and ‘Aichi First’ were selected. Line 5B (‘Moneymaker’ background, T_3), p35S-MIR-tNOS tomato line, generated in our previous work (Sun et al. 2007) was grown along with the F_1 and BC_2F_2 generations for the comparison of MIR accumulation. To compare fruit size, the non-transformed ‘Micro-Tom’ (accession number TOMPF00001), which was provided by University of Tsukuba through the National BioResource Project—Tomato of Ministry of Education, Culture, Sports, Science and Technology (MEXT), and ‘Natsunokoma’ and ‘Aichi First’ were cultivated as well. The approximate fruit size measured in our previous experiment were 3.1 ± 1.0 g fruit^{-1} in ‘Micro-Tom’, 52.7 ± 13.5 g fruit^{-1} in ‘Natsunokoma’, 165.5 ± 71.4 g fruit^{-1} in ‘Aichi First’ and 78.5 ± 33.3 g fruit^{-1} in line 5B (cv ‘Moneymaker’), respectively.

During the seedling-rearing period, all tomatoes were grown in a closed cultivation system as described by Kim et al. (2010a). Thereafter, the growth of line #6, line #26, the F_1 generations and ‘Micro-Tom’ was continued in a closed cultivation system; after the BC_1F_1 generation, stocks containing MIR and NPT II were screened by genomic PCR according to the methods described by Sun et al. (2007). In the BC_2F_2 generation, the segregation ratio test was performed by using Yates’s correction for continuity, and it was confirmed that there was no significant difference from the theoretical value.

A homo–hetero test was conducted in the BC_2F_2 generation. The position of transgene insertion in the tomato genome was identified via genome walking using a Straight Walk® kit (BEX) according to the manufacturer’s instructions and sequence analysis in an unknown region adjacent to the transgene. Plants homozygous and heterozygous for MIR were confirmed by genomic PCR using two sets of primers: one set of primers was designed to target the tomato genome in a region flanking the transgene insertion site, which amplified the right border region if the transgene was present; the other set was designed to target the tomato genome in a region flanking the transgene insertion site, which amplified the tomato genome if the transgene was not present. Samples in which bands were obtained with both sets primers were identified as heterozygous, and samples in which bands were obtained only with the former set were identified as homozygous.

The MIR accumulation levels in red-ripe fruits were determined through western blot analysis and ELISA as described by Sun et al. (2007) in the F_1 and BC_2F_2 generations, respectively. In the F_1 generation analysis, line #6, line #26 and line 5B were evaluated for comparison. Native miraculin was purified from the berries of S. dulcificum as described previously (Duhita et al. 2011). In the analysis of BC_2F_2 generation resulting from line #6×‘Natsunokoma’, homozygous (BC_2F_2:

![Flowchart of the breeding on transgenic tomato. BC_1F_2 and BC_2F_2 plants were screened for plant size, fruit size and form of petals and calyx after confirming of MIR gene. After confirming genetic zygosity of MIR, BC_2F_2 plants of line #6×‘Natsunokoma’ were cultivated in the netted greenhouse and the ripened red fruits were analyzed for MIR accumulation.](image-url)
Figure 2. MIR accumulation in transgenic tomato fruits in the F1 generation. (A) western blot analysis of MIR protein in extracts from fruit tissues. Protein sample were extracted from 0.1 mg fresh weight (FW) samples, separated by SDS-PAGE and blotted onto PVDF membrane. The membrane was hybridized with antibodies to MIR. 5B, p35S-MIR-INOS tomato line ('Moneymaker' background, T1); #6, p35S-MIR-HSP tomato line ('Micro-Tom' background, T1); #6×AF, the F1 generation resulting from line #6×'Aichi First'; #6×NK, the F1 generation resulting from line #6×'Natsunokoma'; P, purified native MIR from miracle fruit. (B) MIR concentration in red fruit of F1, 5B, and line #6, suggesting that the high MIR accumulation was maintained even when the fruit size was large. Similar results were observed within the each of the lines. Under the control of p35S, it was previously reported that MIR accumulation was evaluated in the BC2F2 lines of #6×'Natsunokoma' after the confirmation of homozygosity or heterozygosity for the MIR gene. The maximum and minimum fruit sizes differed by approximately 3-fold in each BC2F2 (homo) and BC2F2 (hetero) line and line 5B, but no significant decrease in MIR levels was observed even when the fruit size increased (Figure 3A). In addition, the fruit size of BC2F2 was sufficiently large, similar to that of 'Natsunokoma' that was grown in this experiment and was quite a bit larger than that of 'Micro-Tom' (Figure 3C). The difference was approximately 10 times in fruit weight. However, the MIR concentration of BC2F2 (homo) was 561.1 μgFW⁻¹, which was similar to that of line #6 (541.6 μgFW⁻¹); approximately 1.2 times that of BC2F2 (hetero) (482.8 μgFW⁻¹); and approximately 5.0 times that of line 5B (107.5 μgFW⁻¹) (Figure 3B).

The increased MIR concentration of homozygotes compared to heterozygotes might have been caused by gene dosage. In the comparisons between BC2F2 (hetero) and BC2F2 (homo); BC2F2 (homo) and line #6 (homo); and F1 (hetero) and line #6 (homo), the rates of the increases in the homologous lines compared to the heterologous lines were similar, regardless of the differences in cultivation environment and genetic background. A previous study reported that MIR mRNA expression and MIR accumulation in a homozygous MIR line were both higher than those in a heterozygous line (Kim et al. 2010a). Our results also suggested that the gene dosage of MIR influences the MIR accumulation level.

In a previous study, Hirai et al. (2010) showed that MIR accumulates in the intercellular space in transgenic tomato and miracle fruit. Based on this, Kim et al. (2010b) concluded that a small cell size is primarily responsible for high MIR accumulation. There is a positive correlation between fruit weight and cell size in tomato (Cheniclet et al. 2005). Therefore, we hypothesized that as fruit weight increases, the cell size will increase, and MIR accumulation will decrease. However, despite these concerns, the MIR accumulation level in BC2F2 (homo) was similar to that in line #6, suggesting that the high MIR accumulation was maintained even when the fruit size was large. Similar results were observed within the each of the lines. Under the control of p35S, it was previously reported that MIR continues to accumulate throughout fruit development, with the highest MIR concentration occurring in the ripening stage (Kim et al. 2010b). Moreover, in studies investigating the effects of light intensity (Kato et al. 2011) and promoter (Hirai et al. 2011a; Kurokawa et al. 2013) differences on MIR accumulation, it was reported that the length of the MIR accumulation period affected the MIR accumulation level. These results show that a longer fruit development period under the control of
p35S increases the final MIR content. In this study, the fruit development period of the 'Micro-Tom'-background tomato lines was much shorter than that of the backcrossed lines, in accord with the report of Saito et al. (2011) indicating that the life cycle of 'Micro-Tom' is shorter than that of other tomato cultivars. These results suggested that one of the reasons for the lack of change in MIR accumulation with changes in fruit size is that larger fruits exhibit a longer developmental period than smaller fruits and are able to continue producing MIR for a longer period.

The exocarp is a tissue that is prone to MIR accumulation, and the MIR concentration tends to increase in varieties in which the exocarp tissue accounts for a high proportion of fruit weight (Kim et al. 2010a). That is, the difference in the genetic background affects MIR accumulation. However, in this study, the MIR concentration did not change depending on the genetic background. The proportion of each tissue may have been similar between the tested cultivars. As a result, there may have been no change in the amount of MIR accumulation among the tested lines with different genetic backgrounds.

It has been reported that MIR purified from recombinant tomato exhibits sweetness-inducing activity equivalent to that of purified MIR from miracle fruit (Sun et al. 2007). Miracle fruit contains approximately 180 µg gFW⁻¹ of MIR, as evaluated by Sun et al. (2007), and the line 5B, which shows the highest MIR productivity achieved thus far, contains approximately 130 µg gFW⁻¹. In comparison, the BC₂F₂ (homo) line accumulated approximately 3.1 times and 4 times as much MIR as miracle fruit and line 5B, respectively. The production ability of tomatoes per unit area is significantly higher than that of miracle fruits. The potential for MIR production in transgenic tomato may provide much higher productivity than that in miracle fruits. We propose that the production of tomatoes showing MIR accumulation via the introduction of p35S-MIR-HSP into high-yielding varieties will provide an attractive MIR production platform with the highest MIR productivity to date.

Acknowledgements

We thank the members of the Ezura Laboratory for helpful discussions. 'Micro-Tom' seeds (TOMJPF00001) were obtained from the National BioResource Project Tomato (NBRP-tomato) of the MEXT, Japan.

References

Bouwer JN, van der Wel H, Francke A, Hhenning GJ (1968) Miraculin, the sweetness-inducing protein from miracle fruit.
Cheniclet C, Rong WY, Causse M, Frangne N, Bolling L, Bordeaux VS, Ornon V (2005) Cell expansion and endoreduplication show a large genetic variability in pericarp and contribute strongly to tomato fruit growth. *Plant Physiol* 139: 1984–1994

Duhita N, Hiwasa-Tanase K, Yoshida S, Ezura H (2011) A simple method for purifying undenatured miraculin from transgenic tomato fruits. *Plant Biotechnol* 28: 281–286

Hirai T, Kim YW, Kato K, Hiwasa-Tanase K, Ezura H (2011a) Uniform accumulation of recombinant miraculin protein in transgenic tomato fruit using a fruit-ripening-specific E8 promoter. *Transgenic Res* 20: 1285–1292

Hirai T, Kurokawa N, Duhita N, Hiwasa-Tanase K, Kato K, Kato K, Ezura H (2011b) The HSP terminator of *Arabidopsis thaliana* induces a high level of miraculin accumulation in transgenic tomatoes. *J Agric Food Chem* 59: 9942–9949

Hirai T, Sato M, Toyooka K, Sun HJ, Yano M, Ezura H (2010) Miraculin, a taste-modifying protein is secreted into intercellular spaces in plant cells. *J Plant Physiol* 167: 209–215

Igeta H, Tamura Y, Nakaya K, Nakamura Y, Kurihara Y (1991) Determination of disulfide array and subunit structure of taste-modifying protein, miraculin. *Biochim Biophys Acta* 1079: 303–307

Inglett GE, Dowling B, Albrecht JJ, Hoglan FA (1965) Taste modifiers: Taste-modifying properties of miracle fruit (*Synsepalum dulcificum*). *J Agric Food Chem* 13: 284–287

Ito K, Asakura T, Morita Y, Nakajima K, Koizumi A, Shimizu-Ibuka A, Masuda K, Ishiguro M, Terada T, Maruyama J, et al. (2007) Microbial production of sensory-active miraculin. *Biochem Biophys Res Commun* 336: 407–411

Kato K, Maruyama S, Hirai T, Hiwasa-Tanase K, Mizoguchi T, Goto E, Ezura H (2011) A trial of production of the plant-derived high-value protein in a plant factory. *Plant Signal Behav* 6: 1172–1179

Kim YW, Hirai T, Kato K, Hiwasa-Tanase K, Ezura H (2010a) Gene dosage and genetic background affect miraculin accumulation in transgenic tomato fruits. *Plant Biotechnol* 27: 333–338

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

Koizumi A, Tsuchiya A, Nakajima K, Ito K, Terada T, Shimizu-Ibuka A, Briand L, Asakura T, Misaka T, Abe K (2011) Human sweet taste receptor mediates acid-induced sweetness of miraculin. *Proc Natl Acad Sci USA* 108: 16819–16824

Kurihara K, Beidler LM (1968) Taste-modifying protein from miracle fruit. *Science* 161: 1241–1243

Kurokawa N, Hirai T, Takayama M, Hiwasa-Tanase K, Ezura H (2013) An E8 promoter–HSP terminator cassette promotes the high-level accumulation of recombinant protein predominantly in transgenic tomato fruits: A case study of miraculin. *Plant Cell Rep* 32: 529–536

Mattes RD, Popkin BM (2009) Nonnutritive sweetener consumption in humans: Effects on appetite and food intake and their putative mechanisms. *Adv Clin Nutr* 89: 1–14

Nagaya S, Kawamura K, Shinuyu A, Kato K (2010) The HSP terminator of *Arabidopsis thaliana* increases gene expression in plant cells. *Plant Cell Physiol* 51: 328–332

Saito T, Arizumi T, Okabe Y, Asamizu E, Hiwasa-Tanase K, Fukuda N, Mizoguchi T, Yamaizumi Y, Aoki K, Ezura H (2011) TOMATOMA: A novel tomato mutant database distributing micro-tom mutant collections. *Plant Cell Physiol* 52: 283–296

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 Biotechnol J* 5: 768–777

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

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