Mass Production of the Taste-Modifying Protein Miraculin in Transgenic Plants

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Hiroshi Ezura and Kyoko Hiwasa-Tanase

Abstract

Miraculin is a glycoprotein that is found in red berries, which are known as a miracle fruit (*Richadella dulcifica*; synonym *Synsepalum dulcificum*) and are produced by a tropical shrub native to West Africa. Miraculin itself is not sweet, but it can convert a sour taste into a sweet taste. Due to its unique properties and potential use as an alternative sweetener, the mass production of miraculin is of interest. However, the plant has low fruit productivity, and there are limited natural sources of miraculin protein. Therefore, heterologous miraculin production based on genetic engineering techniques has been attempted using plants such as tomato, lettuce, and strawberry. The recombinant miraculin protein has been successfully expressed in transgenic tomatoes and lettuce in a genetically stable manner. In addition, a plant factory, which is a closed cultivation system and may be suitable for producing transgenic plants expressing recombinant miraculin, has been developed. Finally, a simple method for purifying miraculin from transgenic tomato fruits was established. In this chapter, we introduce the mass production of recombinant miraculin protein in transgenic tomatoes and lettuce.

Keywords

Miraculin • Taste-modifying protein • Tomato • Lettuce • Plant factory • Transgenic plant • Purification

Contents

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1 Introduction

Sweet-tasting proteins that have activity as sweeteners and taste modifiers have been discovered mostly in tropical plants [1]. These proteins include thaumatin, monellin, mabinlin, pentadin, brazzein, neoculin (curculin), and miraculin. Their original functions remain unknown. However, because of their sweet-tasting properties, they have attracted research attention. These properties are classified into three categories: the protein itself is sweet (thaumatin, monellin, mabinlin, pentadin, and brazzein), the protein induces sweetness (miraculin), or both (neoculin).

In many countries, wealthy lifestyles and food satiety have increased the risk of adult disease from excessive sugar intake. Artificial sweeteners such as saccharin, aspartame, sucralose, and acesulfame-K are used worldwide as low-calorie sweeteners to prevent chronic lifestyle diseases and to help control sugar consumption in diabetics; however, some of these compounds may have carcinogenic side effects [1]. Sweet-tasting proteins may be able to replace these artificial sweeteners because of their properties as natural, safe, and low-calorie sweeteners. Due to their high industrial potential, the mass production of these sweet-tasting proteins has been attempted by using heterologous biological production systems such as bacteria, yeast, fungi, and transgenic plants [2]. In particular, the production of sweet proteins, including thaumatin and monellin, by microorganisms has been attempted. Attempts have also been made to produce thaumatin using transgenic plants such as potatoes [3], tomatoes [4], strawberries [5], pears [6], and cucumbers [7], whereas tomatoes and lettuce have been used to produce monellin [8]. However, the recombinant protein accumulation levels in those transgenic plants are quite low. Lamphear et al. [9] produced recombinant brazzein in transgenic corn at a concentration of 4.3 mg/80 g, and a field trial was conducted. However, none of these efforts, including the attempts with brazzein, have yet resulted in the commercialization of these proteins as sweeteners.

The mass production of miraculin, which is a taste-modifying protein, has also been attempted using heterologous organisms. Recently, progress has been made using transgenic plants to mass-produce miraculin. In this chapter, we provide a general overview of the published studies and assess the future commercial viability of miraculin.

2 What Is Miraculin?

Miraculin is a glycoprotein that is found in red berries, which are known as miracle fruit (*Richadella dulcifica*; synonym *Synsepalum dulcificum*) and are produced by a tropical shrub native to West Africa (Fig. 1). Miraculin itself is not sweet, but it has a

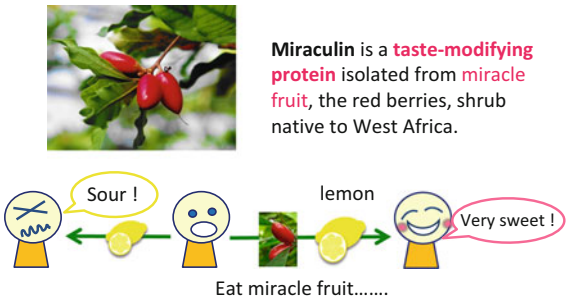

Fig. 1 What is miraculin? Miraculin by itself is not sweet, but it is able to turn a sour taste into a sweet taste

taste-modifying activity and is capable of converting a sour taste into a sweet taste. After chewing the miracle fruit, lemons taste as sweet as oranges. The name "miracle fruit" was derived from this unique and attractive property, and the isolated active substance was named miraculin [10]. Miraculin accumulates only in the miracle fruit, and its production begins 6 weeks after pollination, or at the turning stage, when the fruit color changes from green to orange; the accumulation peaks when the fruit is at the full-red stage.

Miraculin is able to elicit sweetness from various acids, such as HCl, oxalic acid, lactic acid, formic acid, acetic acid, and citric acid; the sweetening effect is dependent on the sourness and pH of the acid [11]. The sweetening effect of a miraculin solution reaches its maximum level after being held in the mouth for approximately 3 min. A concentration greater than 4×10^{-7} M is required for the maximum effect, and the sweetness corresponds to that of a 0.4 M sucrose solution. The taste-modifying effect of miraculin can be sustained for more than 1 h, although it depends on the concentration of the miraculin solution.

Miraculin is a glycoprotein that consists of 191 amino acid residues and two sugar chains that are linked to Asn-42 and Asn-186. The molecular weight of the single polypeptide chain calculated based on its amino acid sequence and carbohydrate content (13.9%) is 24.6 kDa [12, 13]. The nucleotide sequence encodes 220 amino acid residues, including a 29 amino acid signal sequence [14]. This indicates that mature miraculin protein is processed by posttranslational modification at its N terminus. The pure state of native miraculin protein forms a tetramer, whereas the crude non-reduced and denatured state forms a dimer [15]. Both the tetramer and native dimer forms of miraculin exhibit taste-modifying activity [15], although the monomer form has no activity [16]. Thus, miraculin dimerization, as mediated by a covalent linkage at Cys-138, is crucial for its taste-modifying activity at acidic pH.

Kurihara and Beidler [11] suggested that the miraculin protein has two binding sites: one binds the taste-receptor membrane and the other reacts with sweetness receptors. The conformation of the protein changes under acidic conditions to induce sweetness. This speculation comes from results that show that the thresholds for salty, bitter, sweet, and sour tastes remain unaffected after holding miraculin in the mouth. Furthermore, holding miraculin on the tongue does not turn sourness into sweetness when gymnemic acid, which depresses sweetness, is held in the mouth after applying the miraculin solution. Thus, miraculin binds to membrane receptors

close to sweetness receptor sites and activates sweet receptors under acidic conditions. Some studies have revealed that miraculin does not possess taste-modifying properties as a monomer [16, 17]. Additionally, mutagenesis and simulation analyses have shown that two histidine residues play an important role in its taste-modifying activity [16, 18]. Moreover, the three-dimensional structure of the homodimer predicts a wide-open conformation at acidic pH and a closed form at neutral pH [19]; it has been suggested that the histidine residues are involved in this conformational change [18]. However, the details of the mechanisms of where and how miraculin binds on the tongue and how it modifies sourness to sweetness still require elucidation. Against this background, more recently, Koizumi et al. [20] quantitatively evaluated the acid-induced sweetness of miraculin using a cell-based assay system at the molecular level. They determined that miraculin activated the hT1R2–hT1R3 receptor, which is the human sweet taste receptor, when the pH decreased from 6.5 to 4.8 and suggested that miraculin binds to the hT1R2 receptor at the N-terminal region. In the near future, the taste-modifying mechanism from sourness to sweetness may also be clarified by the cell-based assay system for miraculin activity developed in that work.

Due to its unique properties and potential as a sweetener, the mass production of miraculin has been greatly anticipated. However, because it is a tropical plant, the miracle fruit plant cannot survive at less than 7 °C; additionally, it requires 3–4 years before it can bear fruit, and it has a low proportion of fruit setting after pollination. Furthermore, the taste-modifying activity is lost within 2–3 h after harvesting at room temperature [21]. Thus, there are limited natural sources of miraculin protein. Therefore, heterologous miraculin production based on genetic engineering techniques has been attempted using foreign hosts such as *Escherichia coli*, yeast, *Aspergillus oryzae*, and plants.

To utilize its unique properties and expand its market potential, the heterologous production of miraculin has been attempted using various hosts. Recombinant miraculin produced in *E. coli* was found to have no taste-modifying activity, even though the protein was detected by SDS-PAGE and western blot analysis [22]. The production of the recombinant miraculin was also attempted in *E. coli* by Matsuyama et al. [17], who produced an active miraculin dimer. However, its activity was only one-sixth that of native miraculin. This result showed that the glycosylation of miraculin is crucial for protein folding and/or stability. An active form of recombinant miraculin was also produced using *A. oryzae,* which has glycosylation capacity, as a host strain [16]. In the *Saccharomyces cerevisiae* system, recombinant miraculin was produced after optimizing codon usage and the signal sequence, although the activity was only detected when the sugar chains were removed [23]. The activity of recombinant miraculin in both *A. oryzae* and *S. cerevisiae* was evaluated at one-fifth the concentration of native miraculin. These results suggest that not only is glycosylation important but that the types of sugar chains are also crucial for the high and stable activity of miraculin. In the beginning of recombinant miraculin production in transgenic plants, the expression cassette usually incorporated a cauliflower mosaic virus 35S (35S) promoter and a nopaline synthase (NOS) terminator. Sun et al. [24] expressed recombinant miraculin in transgenic lettuce (*Lactuca sativa*). They

produced miraculin at a concentration of 43.5 ug/g fresh weight (FW), and the resulting recombinant miraculin had a taste-modifying activity that was equal in strength to that of native miraculin. However, transgene silencing occurred in the progeny line of the transgenic lettuce [24]. In transgenic strawberries (*Fragaria x ananassa*), which propagate vegetatively, recombinant miraculin is accumulated at an extremely low level of 2.0 μg/g FW, although the accumulation level was stable in the vegetative progeny [25]. In contrast, transgenic tomatoes (*Solanum lycopersicum*) have produced recombinant miraculin at a level of 90.7 μg/g FW in fruit tissue, with activity equal to that of native miraculin [26]. Additionally, the accumulation and gene expression levels were genetically stable from the T1 to T5 generations [27]. These reports suggest that plants are a good platform for producing a biologically active form of miraculin. It may be that the plant-mediated glycosylations are similar to those of miraculin produced from miracle fruit. Furthermore, transgenic tomatoes were found to be the most suitable hosts for recombinant miraculin production from these plants. Tomatoes also have an advantage in the established *Agrobacterium*-mediated transformation protocol using cotyledon explants from seedlings, and the transformation efficiency exceeds 40% of the explants [28].

3 Expression of Recombinant Miraculin Protein in Transgenic Tomatoes

Recombinant miraculin protein was expressed in transgenic tomatoes [26]. After the successful expression of recombinant miraculin in transgenic tomatoes, various approaches have been attempted to increase recombinant miraculin production in transgenic tomatoes. Recombinant miraculin accumulates to high levels in the overripe fruit during fruit development when the miraculin gene is driven by the 35S promoter [29]. Among fruit tissues, the miraculin levels in the exocarp, or epidermis, were found to be extremely high, at 928 μg/g FW compared to below 110 μg/g FW in other tissues. Why did recombinant miraculin accumulate to such a high amount in the exocarp? The deduced amino acids of miraculin include an N-terminal signal sequence of 29 amino acids [14]. Miraculin protein is transported to and accumulates in the intercellular layer space of both miracle fruit and transgenic tomatoes [30]. The cell size of the exocarp is considerably smaller than that of other fruit tissues, such as the mesocarp, septa, placenta, and jelly. The amount of intercellular layer space is affected by the number of cells per fresh weight. In fact, the amount of miraculin per dry weight, which is less affected by cell size, hardly differs between the exocarp and other tissues [29]. Therefore, the authors speculate that the high miraculin accumulation in the exocarp is caused primarily by cell size, which influences the amount of intercellular layer space [29].

The promoter is an essential element for inducing target gene expression, determining the target tissue and achieving the desired expression time [31–34]. There are multiple types of promoters that are suitable for a variety of purposes, including constitutive, inducible, and tissue-specific expression. A constitutive 35S promoter from cauliflower

mosaic virus is frequently used to mass-produce recombinant proteins because it usually leads to higher expression than tissue-specific promoters in various plant tissues and organs [31, 35]. However, when the recombinant protein suppresses the growth or metabolism of the host plant in some tissues, the use of the constitutive promoter loses its advantage. Additionally, there have been some cases showing that gene silencing via co-suppression is caused by the utilization of a constitutive promoter. The compatibility of the promoter with the host plant is a crucial factor for the strong expression of a target gene [31, 36]. In fact, the miraculin gene, when driven by the 35S promoter, was silenced in transgenic lettuce [24]. Moreover, although gene silencing does not occur in transgenic strawberries using the 35S promoter, the production of miraculin is quite low [25]. Unlike these transgenic plants, the 35S promoter works in transgenic tomatoes and increases miraculin production [26, 27].

The tissue-specific E8 promoter has been used to control the expression of the miraculin gene in transgenic tomatoes [37]. This promoter, which was first identified in cherry tomatoes [38], activates transcription at the onset of tomato fruit ripening [39, 40]. Miraculin gene expression in red tomato fruit was found to be significantly lower under the control of the E8 promoter than under the 35S promoter, and the miraculin content was also lower [37]. This result indicates that the 35S promoter has higher transcriptional activity in tomatoes than the E8 promoter does. The 30-untranslated region and terminator sequence are also crucial factors for regulating the expression level of target genes. These sequences influence the gene expression level and the efficiency of translation by controlling transcriptional termination and posttranscriptional processes [41]. In fact, the terminator has a considerable effect on the gene expression level [42, 43]. The NOS terminator from the Ti (tumor-inducing) plasmid of *Agrobacterium* is universally applied in various expression vectors when genes are transformed into plants. When the miraculin gene was driven by the 35S promoter and terminated by the NOS terminator in transgenic tomatoes, the miraculin content was 1–1.5% of the total soluble protein [26, 44, 45]. In contrast, the miraculin content in miracle fruit is approximately 10% of the total soluble protein [12]. Because miraculin protein is produced more efficiently in miracle fruit, the native miraculin terminator from miracle fruit was cloned and used to produce miraculin in transgenic tomatoes [44]. As a result, the accumulation levels of recombinant miraculin in transgenic lines using the miraculin terminator were 1.5 times higher per fresh weight than they were when using the NOS terminator. Nagaya et al. [42] assessed the capacity of termination to support increased transgene expression using several terminators derived from Arabidopsis genes and showed that the heat shock protein 18.2 (HSP) terminator was the most effective at supporting high expression levels. In fact, the application of the HSP terminator increased the production of the B subunit of Shiga toxin 2e in transgenic lettuce at the T0 generation to levels approximately 40 times higher than those obtained with the NOS terminator [46]. The HSP terminator has also been used for miraculin production in transgenic tomatoes [47]. The miraculin accumulation level in the homozygous T1 generation reached 1726 μg/g FW, which is ten times higher

than that obtained with the NOS terminator, equivalent to 17.1% of the total soluble protein. In most cases using nuclear transformation, the accumulation level of the recombinant protein does not exceed 1–2% of the total soluble protein [48–50]. Conversely, recombinant proteins derived from chloroplast transformation normally accumulate to 5–25% of the total soluble protein [51, 52]. It is difficult to use the chloroplast transgenic system for recombinant miraculin production because the protein requires posttranslational modifications such as glycosylation. However, the accumulation scale of recombinant miraculin in transgenic tomatoes using the 35S promoter/HSP terminator expression cassette matches the level obtained with chloroplast transformation.

Codon modifications are used to increase the translational efficiency of heterologous genes in their hosts. Preferred codon usage varies significantly among different organisms and even between plant species [53–59]. Therefore, this approach seeks to increase the accumulation of recombinant proteins by modifying their original codons to codons that are more suitable to the host without changing the amino acid sequence [60]. Codon optimization of the miraculin gene for tomatoes enhanced its translational efficiency more than twofold compared to that of the native miraculin gene in transgenic tomatoes [44]. The resulting accumulation of the codon-modified miraculin gene product exceeded that of native miraculin, and this effect was more pronounced when the miraculin terminator was used rather than the NOS terminator. These results indicate that it is important for productivity to both optimize codons and select a suitable terminator.

To produce recombinant proteins using plants, it is important to pay attention to not only the species but also the cultivar when selecting the genetic background. Kim et al. [61] created F1 hybrids from the transgenic tomato that accumulates miraculin (cv. "Moneymaker") by crossing with various tomato cultivars to elucidate the impact of genetic background on miraculin accumulation. The accumulation pattern in fruit tissues was unchanged; the protein is accumulated to high levels in the exocarp, followed by the mesocarp, just as in the original transgenic line. However, the accumulation of miraculin in fruits was different in the different genetic backgrounds. The ratio of fruit tissue to fruit weight is variable in tomato cultivars. As an example, the proportion of exocarp was 8.16% in "Micro-Tom," which was 5% higher than in the other varieties [29]. Therefore, miraculin accumulation could be affected by the ratio of each fruit tissue in the different genetic backgrounds because the accumulation levels differ depending on fruit tissues. The miraculin accumulation levels also differed in the exocarp and mesocarp among the hybrid lines [61]. Cell size and cell numbers per fresh weight are different even in the same exocarp, depending on the hybrid line, similar to the difference in fruit size. Such a different fruit structure is influenced by the final accumulation levels because cell numbers are roughly proportional to the intercellular layer space to which miraculin protein is transferred and stored. In addition, differences in harvesting time were found to affect miraculin accumulation in fruits in which the miraculin gene is driven by the 35S promoter; the miraculin levels in tomato fruits are higher at later harvesting times [37, 62]. Presumably, there are differences

in the fruit maturation times of these cultivars. In the case of miraculin in transgenic tomatoes driven by the 35S promoter, this factor is important for increasing the miraculin amounts. Moreover, crossbreeding transgenic tomatoes with other cultivars expands the capacity for modulating the production level of recombinant miraculin. One of the best examples of this strategy is the cross between the transgenic tomato that accumulates miraculin (cv. "Moneymaker") and "Micro-Tom," which is a miniature dwarf tomato. The miraculin concentrations in the crossed lines were 2.5 times higher than in the transgenic line as a parent [45].

Recombinant protein content can be increased by increasing the gene dosage given to the host plant. However, increasing gene dosage by delivering multiple gene copies does not always yield a good result because multi-copy transformants often trigger low levels of transgene expression or silencing [63, 64]. In contrast, using homozygous transgenes can increase production compared to using heterozygous transgenes. In fact, the expression levels in the exocarp and mesocarp in homozygous lines given a single copy of the miraculin gene were 4.7 and 1.5 times higher than those in heterozygous transgenic tomatoes, respectively [61]. The recombinant miraculin levels in the exocarp and mesocarp were two times higher in homozygous lines than in heterozygous lines. These results indicate that homozygosity of a single-copy gene promotes the production of recombinant miraculin better than does heterozygosity. A single-copy transgenic plant tends to be more stable in its transgenic expression than a plant with multiple transgene copies [65]. Thus, it is crucial for the stable production of recombinant miraculin to select a transgenic plant in which the gene was introduced as a single copy and to maintain its homozygous state.

The intracellular localization of recombinant heterologous proteins is an important determinant of their accumulation levels. The subcellular site in which a recombinant protein accumulates can have a profound effect on its proper folding, assembly, and posttranscriptional processing [36]. When a heterologous protein is not correctly folded or synthesized in a plant, the synthesized proteins are easily attacked by proteases [31]. Thus, suitable subcellular targeting contributes to protein stability and, eventually, to the yield of the recombinant protein [30, 66]. In the same manner, recombinant miraculin is secreted to the intercellular layer in transgenic tomato fruits and leaves by virtue of the signal sequence of the native gene [30]. Furthermore, transgenic tomatoes in which the miraculin gene is driven by the 35S promoter and terminated by the HSP terminator were found to accumulate recombinant miraculin at up to 1,725 μg/g FW, equivalent to 17% of the total soluble protein [47]. This accumulation level exceeds the 400 μg/g FW of miraculin protein that is produced in miracle fruit. Thus, the intercellular layer space appears to be one of the best compartments for the accumulation of recombinant miraculin in transgenic tomato fruit, although it leaves the possibility that other subcellular spaces are also suitable for recombinant miraculin accumulation. The taste-modifying activity of miraculin is relatively stable under acidic conditions [12]. The fact that the apoplastic pH of tomatoes declines from an average of 6.7–4.4 during fruit ripening might also be advantageous [67].

4 Expression of Recombinant Miraculin Protein in Transgenic Lettuce

Lettuce is a popular and easy-to-grow leafy vegetable that is 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 the mass production of target plants [68]. In a plant factory, it takes approximately 3 weeks from seed sowing to harvest lettuce under optimal growth conditions; therefore, lettuce can be harvested more than 20 times per year. Thus, if we can stably express a target protein of interest, lettuce will be an alternative platform for the mass production of recombinant proteins.

Recombinant miraculin protein was expressed first in transgenic lettuce [24]. In that study, miraculin was produced at a concentration of 43.5 μg/g FW, and the resulting recombinant miraculin had taste-modifying activity that was equal in strength to that of native miraculin. However, transgenic silencing occurred in the progeny line of the transgenic lettuce. Many attempts have been made to produce transgenic lettuce, and useful traits have been introduced into the crop [24, 69–72]. A high degree of transgene silencing in lettuce is one of the major barriers to commercializing transgenic lettuce.

For this reason, it is important to develop a strategy for stably expressing transgenes in lettuce. The promoter is a major factor influencing the level and stability of transgene expression. Curtis et al. [69] compared several promoter-GUS gene fusions in transgenic lettuce plants and found that the plastocyanin gene (petE) promoter exhibited higher expression than did the mannopine synthase gene (MAS) [73], hybrid 35S/Mas promoter 50 region (Mac) [74], or cauliflower mosaic virus (CaMV) 35S promoters in first seed generation (T1) plants. The choice of both the promoter and transgene constructs is important for long-term stable expression of transgenes in lettuce [71]. Unstable gene expression is also often related to the integration of multiple copies of the transgene in the plant genome [75], position effects [76], and the extent of DNA methylation in the transgene loci [77].

Ubiquitin is a small, highly conserved protein that consists of 76 amino acid residues and is found in all eukaryotes. The ubiquitins are encoded by gene families that contain two types of structures: polyubiquitin genes and ubiquitin extension protein genes [78, 79]. Both types of genes are translated as polyprotein precursors and then proteolytically processed to ubiquitin monomers [80]. Polyubiquitin genes are constitutively expressed in all types of plant tissues, with higher levels found in young tissues [81, 82]. Various promoters from ubiquitin genes have been tested for their potential use in driving the 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) [83–86].

To achieve stable miraculin expression in lettuce, we compared the level of miraculin accumulation between the CaMV 35S promoter/nos terminator cassette and the native lettuce ubiquitin promoter/terminator cassette in transgenic lettuce

[87]. Transgenic lettuce plants using the 35S promoter/nos terminator cassette showed almost complete silencing of miraculin gene expression in the T2 generation, whereas those using the native ubiquitin promoter/terminator cassette showed stable miraculin expression, even in the progenies. The 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 transgenic lines using the ubiquitin promoter expressed the miraculin gene in the T0 generation at a high level compared to using the 35S promoter. The expression of miraculin was also clear and stable in the T1 and T2 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. The effectiveness of the maize ubiquitin promoter was reported by Chen et al. [88, 89]. Chen et al. [88] reported that the 35S promoter-derived gene was silenced in the T1 generation of transgenic maize and that the maize ubiquitin promoter-derived gene was expressed in the T1 generation of transgenic maize. In this study, we have demonstrated the genetically stable expression of miraculin in transgenic lettuce, which suggests that lettuce would be an alternative platform for mass production of recombinant miraculin protein.

5 Production of Transgenic Tomato Fruits at a Plant Factory

To improve recombinant miraculin production and stabilize its quality, it is essential to develop a cultivation system for transgenic tomatoes. Tomatoes are usually cultivated in fields or greenhouses for commercial use. However, these environments depend a great deal on the season and weather conditions, such as temperature, the amount of solar radiation, and the photoperiod; these influence the fruit quality and yield. In contrast, a closed cultivation system (also called a plant factory) makes it possible to stabilize fruit yield and quality in a constantly controlled environment [90]. In fact, the transgenic tomato that expresses the miraculin gene produces recombinant miraculin more stably in a closed tomato cultivation system than in a netted greenhouse [68]. Additionally, the yield has been estimated at approximately 45 ton FW of fruits and 4 kg of recombinant miraculin per 1,000 m^2 of cultivated area in a year. Closed cultivation systems also have other advantages: the spread of transgenic plants and pollen to the external environment is easy to prevent and, at the same time, the plants are protected from disease and pests from the external environment. However, the limited space available for cultivation and the high operating costs become bottlenecks for its practical application. It is important to breed a plant that is suitable for cultivation in a plant factory [91]. To breed tomatoes suitable for a closed cultivation system with the aim of effective cultivation space utilization, a transgenic tomato line based on a normal cultivar was crossed with a dwarf tomato cultivar, “Micro-Tom,” that has a short life cycle and determinate-type growth [45]. In F2 plants, hybrids were selected by small plant size, miraculin accumulation, and determinate type; they were then propagated to the F6 or F7 generation by self-pollination. In the cross no. 2 line, it was not necessary to remove

axial buds and leaves when cultivated in a two-layer closed cultivation system, in contrast to the original transgenic line. This growth habit helps reduce the labor costs. Additionally, this plant line has the potential to be grown in a three-layer cultivation system in the same space as a two-layer system because the plant height is significantly lower than in the other lines. The fruit yield of the cross no. 2 line in the two-layer cultivation system was equivalent to 45.9 ton FW per 1,000 m^2 of cultivation area in a year. The miraculin content in the tomato fruit significantly increased to 343 μg/g FW of pericarp (including exocarp and mesocarp), which is approximately 2.5 times higher than that of the original transgenic line, due to the effect of genetic background modification.

The high operating costs of closed cultivation systems mainly accrue from the electric bills arising from the artificial lighting and air conditioning. To reduce electricity costs, it is important to understand the effects of light intensity on both tomato fruit yield and the production of recombinant miraculin. The cross no. 2 line acquired some characteristics from the "Micro-Tom" dwarf tomato, such as its growth habit and its determinate type [45]. The ability to grow under lower intensity light was also gained by the cross no. 2 line. Normal-sized tomatoes cannot be cultivated under lower light conditions because low light makes processes such as flower-bud formation, flowering, photosynthesis, and healthy plant growth difficult. However, the cross no. 2 line grew normally even at a photosynthetic photon flux (PPF) of 100 μmol/m^2/s, as does "Micro-Tom," which can be cultivated under 80 μmol/m^2/s [62]. Kato et al. [62] evaluated the relationship between fruit yield and miraculin concentration under the different light conditions of PPF100, 200, 300, and 400 μmol/m^2/s and discussed the electricity costs of miraculin production. The miraculin concentrations were higher at low light intensity, but the fruit yield was higher at strong light intensity. Consequently, the miraculin production per unit area was highest at PPF300, but the miraculin production per unit of energy was best at PPF100. Thus, these results indicate that it is necessary to select a suitable light condition on the basis of market demand and the sales price of recombinant miraculin.

6 Purification of Recombinant Miraculin from Transgenic Tomato Fruits

The advantage of using an edible plant as a host lies in the fact that several processed and unprocessed types of recombinant proteins can be used, unlike in microbial and animal systems [36]. Thus, this practice can minimize purification costs.

Miraculin has a taste-modifying function that changes a sour taste into a sweet taste. This property of miraculin is helpful for preventing lifestyle diseases: it can be used as a low-calorie sweetener or as an additive for foods targeting diabetics because it removes the need for sugar. It might also find novel uses as a component of nutritionally enhanced snacks and drinks with the possibility of widespread consumer acceptance and new marketing opportunities. Additionally, recombinant miraculin could be used for safety assessment analysis and to identify the taste-modifying

mechanism of miraculin. Therefore, the industrial uses of recombinant miraculin from transgenic tomatoes encompass the consumption of unprocessed raw fruit and processed fruit and its use as a food additive and as a reagent for research and development. To address this wide variety of uses, it is important to develop methods of purifying recombinant miraculin from transgenic tomatoes. Techniques for purifying miraculin from miracle fruit have been developed since 1968 [10, 92, 93]. However, these methods cannot remove the polyphenolic coloring agent and lead to a reduction in the taste-modifying activity due to the use of a carbonate extraction buffer (pH 10.5). In 1988, Theerasilp and Kurihara [12] presented a method in which a NaCl solution was used for crude extraction from the miracle fruit, followed by purification via CM-Sepharose ion-exchange chromatography and ConA-Sepharose 4B affinity chromatography. Armah et al. [94] also developed a method using a NaCl extraction buffer and hydrophobic interaction chromatography (Butyl-S-Sepharose 6 FF, Phenyl Sepharose 6 FF), ion-exchange chromatography (SP-Sepharose FF), and gel filtration chromatography (Sephacryl S-100); the authors received a US patent in 1999 (patent number 5886155). These methods permit the isolation of miraculin from polyphenolic pigments. Recently, a single-step purification system using nickel-immobilized metal-affinity chromatography (IMAC) was developed [95]. This method utilizes four histidine residues that are exposed on the surface of dimeric miraculin, as with a histidine tag. It is much simpler than previous methods, and the reduction in column steps makes the process less time-consuming, thus reducing the purification loss and costs. In addition, the method can purify the active form of miraculin free from the denatured form because the IMAC column binds only miraculin that has maintained the three-dimensional structure of its homodimer (which elicits its sweetness-inducing activity) and does not bind monomeric miraculin, which is the inactive form. However, recombinant miraculin purification is more difficult from tomato fruit than it is from miracle fruit because tomato fruit contains a wide variety of additional proteins. Therefore, this purification requires one additional column, an ion-exchange column (CM-Sepharose), for the production of highly purified miraculin [96]. Recombinant miraculin has also been purified using the method of Theerasilp and Kurihara [12] by the addition of molecular weight column chromatography (Sephacryl S-200 HR), but the method is time-consuming and has a low recovery rate due to the number of purification steps [26]. Although the purification method using IMAC is efficient compared to the other methods, in the future, it will require further development to be used on an industrial scale.

7 Conclusions

Transgenic plants are presumably the most suitable hosts for recombinant miraculin protein production with respect to posttranslational modifications such as glycosylation. A scheme for the mass production of recombinant miraculin protein using transgenic plants is summarized in Fig. 2. The selection of host plants; optimization

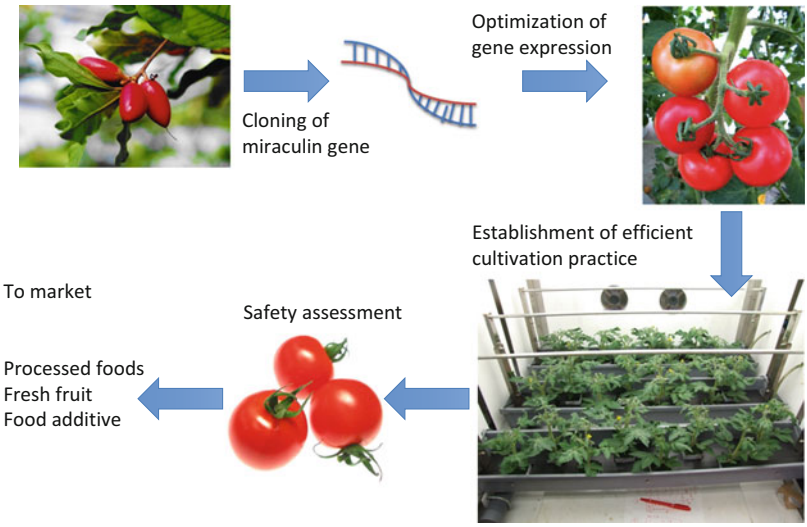

Fig. 2 A scheme for the effective and efficient mass production of recombinant miraculin using transgenic plant, a case of tomato

of expression vectors, especially promoters and terminators for each plant; and establishment of standard cultivation protocol for the transgenic plants are crucial for effective and efficient production of recombinant miraculin. In particular, the transgenic tomato has many advantages, including genetic stability, good accumulation levels, and good fruit-bearing capacity, and it is readily available to procure as a raw food. In addition, a newly produced transgenic lettuce was found to be able to stably accumulate recombinant miraculin in progeny lines using the ubiquitin promoter and terminator. Compared to the transgenic tomato, transgenic lettuce could also have some advantages such as scalability, a short life cycle, and an established cultivation system at a plant factory. Thus, it might be profitable as a processed food and purified powder. Future studies will assess the safety of recombinant miraculin: it is essential to investigate aspects such as its toxicity, allergenicity, digestibility, thermal stability, insertion position in the host genome, and processing status. Thermal stability of the recombinant miraculin protein is also crucial information for optimization and industrial use. Furthermore, scaling up the cultivation process and scaling up and improving the purification process are important for reducing the final cost. The commercialization of recombinant miraculin as a product will become a reality through these sustained development efforts in the near future.

Due to its unique properties and potential, recombinant miraculin proteins produced in transgenic plants such as tomatoes and lettuce will be an alternative sweetener that can help prevent chronic lifestyle diseases and control sugar consumption in diabetics.

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