

# From miracle fruit to transgenic tomato: mass production of the taste-modifying protein miraculin in transgenic plants

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**Abstract** The utility of plants as biofactories has progressed in recent years. Some recombinant plant-derived pharmaceutical products have already reached the marketplace. However, with the exception of drugs and vaccines, a strong effort has not yet been made to bring recombinant products to market, as cost-effectiveness is critically important for commercialization. Sweet-tasting proteins and taste-modifying proteins have a great deal of potential in industry as substitutes for sugars and as artificial sweeteners. The taste-modifying protein, miraculin, functions to change the perception of a sour taste to a sweet one. This taste-modifying function can potentially be used not only as a low-calorie sweetener but also as a new seasoning that could be the basis of a new dietary lifestyle. However, miraculin is far from inexpensive, and its potential as a marketable product has not yet been fully developed. For the last several years, biotechnological production of this taste-modifying protein has progressed extensively. In this review, the characteristics of miraculin and recent advances in its production using transgenic

plants are summarized, focusing on such topics as the suitability of plant species as expression hosts, the cultivation method for transgenic plants, the method of purifying miraculin and future advances required to achieve industrial use.

**Keywords** Recombinant miraculin production · Expression host · Transgenic tomato · Cultivation · Purification · Commercialization · Safety assessment

## Abbreviations

*35S* *Cauliflower mosaic virus 35S* promoter  
*NOS* *Nopaline synthase* terminator  
FW Fresh weight  
*HSP* *Heat shock protein 18.2* terminator from *Arabidopsis thaliana*  
PPF Photosynthetic photon flux

## Introduction

Various sweet-tasting proteins that have activity as sweeteners and taste-modifiers have been discovered. They are known as thaumatin, monellin, mabinlin, pentadin, brazzein, neoculin (curculin) and miraculin. Most have been identified in tropical plants (Kant 2005), and their original functions in these plants are not known. However, because of their sweet-tasting properties, they have attracted human 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).

Primarily in advanced countries, wealthy lifestyles and food satiety have increased the risk of adult disease from excessive sugar intake. Artificial sweeteners, such as

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saccharin, aspartame, sucralose and acesulfame-K, are used throughout the world 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 (Kant 2005). 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 using heterologous systems, such as bacteria, yeast, fungi and transgenic plants (Masuda and Kitabatake 2006). In particular, the production of sweet proteins, including thaumatin and monellin, by microorganisms has been attempted. Moreover, attempts have also been made to produce thaumatin using transgenic plants such as potatoes (Witty 1990), tomatoes (Bartoszewski et al. 2003), strawberries (Schestibratov and Dolgov 2005), pears (Lebedev et al. 2002), and cucumbers (Szwacka et al. 2002), whereas tomatoes and lettuce have been used to produce monellin (Peñarrubia et al. 1992). However, the recombinant protein accumulation levels have been quite low. Lamphear et al. (2005) produced recombinant brazzein in transgenic corn at a concentration of 4.3 mg/80 g and the field trial was performing by Applied Biotechnology Institute. However, none of these efforts, including the attempts with brazzein, have yet resulted in commercialization.

The mass production of miraculin, a taste-modifying protein, has also been attempted using heterologous organisms. Recently, amazing progress has been made using transgenic plants to mass-produce miraculin. In this review, we provide a general overview of the published studies and offer future assessments regarding commercial viability. This review could prove useful for the mass production of not only miraculin but also other sweet-tasting proteins using transgenic plants.

## Miraculin

Miraculin is a glycoprotein found in red berries known as miracle fruit (*Richadella dulcifica*; synonym *Synsepalum dulcificum*), produced by a tropical shrub native to West Africa. Miraculin itself is not sweet, but it has a taste-modifying activity and is capable of converting sour taste to sweet taste. After chewing the red berry 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 (Kurihara and Beidler 1968). Miraculin accumulates only in the miracle fruit and its production begins 6 weeks after pollination, or at the turning stage, when the fruit’s color changes from green to orange; the production peaks when the fruit is at the full-red stage (Fig. 1).

## Sweetness properties

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 the pH of the acid (Kurihara and Beidler 1969). The sweetening effect of a miraculin solution reaches its maximum level after being held in the mouth for approximately 3 min (Kurihara and Beidler 1969). A concentration greater than  $4 \times 10^{-7}$  M is required for maximum effect, and the sweetness corresponds to 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.

## Protein structure

Miraculin is a glycoprotein consisting of 191 amino acid residues and two sugar chains 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 (Theerasilp and Kurihara 1988; Theerasilp et al. 1989). The nucleotide sequence encodes 220 amino acid residues, including a 29-amino acid signal sequence (Masuda et al. 1995). This indicates that mature miraculin protein is processed by post-translational modification at its N terminus. The pure

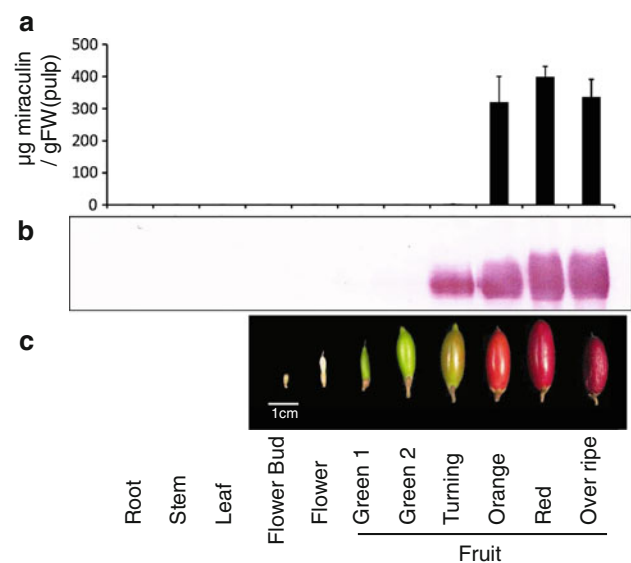


**Fig. 1** Native miraculin accumulation in the miracle fruit plant. Miraculin protein was extracted as described by Hirai et al. (2010a) after removal of the exocarp and seeds. The concentration was measured by ELISA (Kim et al. 2010b) (a), and the accumulation level was detected by Western blot analysis as described by Sun et al. (2007) (b). Developing and ripening miracle fruits are shown in the bottom photo (c). Vertical bars indicate the standard error from three replicates

state of native miraculin protein forms a tetramer; the crude non-reduced and denatured state forms a dimer (Igeta et al. 1991). Both the tetramer and native dimer forms of miraculin exhibit taste-modifying activity (Igeta et al. 1991), although the monomer form has no activity (Ito et al. 2007). Thus, miraculin dimerization, mediated by a covalent linkage at Cys-138, is crucial for its taste-modifying activity at acidic pH (Fig. 2).

#### Taste-modifying mechanism

Kurihara and Beidler (1969) 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 acid conditions. Some studies have revealed that miraculin does not possess taste-modifying properties as a monomer (Ito et al. 2007; Matsuyama et al. 2009). Additionally, mutagenesis and simulation analyses have shown that two histidine residues play an important role in its taste-modifying activity (Ito et al. 2007; Paladino et al. 2010) (Fig. 2). Moreover, the three-dimensional structure of the homodimer predicts a widely open conformation at acidic pH and a closed form at neutral pH (Paladino et al. 2008); it has been

suggested that the histidine residues are involved in this conformational change (Paladino et al. 2010). Neoculin, another taste-modifying protein, also exhibits an open conformation at acidic pH on the basis of crystal structure analysis and molecular dynamics simulation (Shimizu-Ibuka et al. 2006). However, the details of the mechanisms of where and how miraculin binds on the tongue and how it modifies sourness to sweetness are unknown.

Against these backgrounds, more recently Koizumi et al. (2011) succeeded in quantitatively evaluating the acid-induced sweetness of miraculin using a cell-based assay system at the molecular level. They also clarified that miraculin activated hT1R2–hT1R3, which is the human sweet taste receptor, when the pH decreased from 6.5 to 4.8 and suggested that miraculin bound to hT1R2 at the N terminus region. In near future the modifying mechanism from sourness to sweetness may also be clarified by the developed cell-based assay system for miraculin activity.

#### Miraculin production from miracle fruit

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 has a low proportion of fruit production after pollination. Furthermore, the taste-modifying activity is lost within 2–3 h after harvesting at room temperature (Kurihara and Nirasawa 1997). 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, as described below.

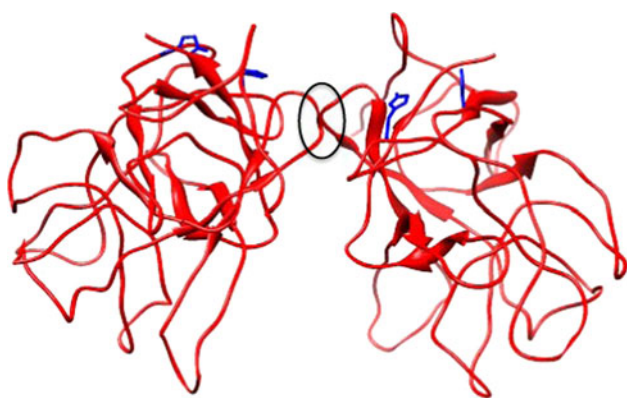


**Fig. 2** 3D protein structure model of miraculin homodimer. His residues are colored blue. Oval indicates the inter-chain disulphide bond (C138–C138). The molecular model was simulated as described by Paladino et al. (2008) but this model is not includes sugars. The molecular model of Paladino et al. (2008) shows that the sugar chains localized at the edges of the dimeric protein and suggest that sugar chains would not be involved in the taste-modifying activity

#### Hosts for the production of recombinant miraculin

To utilize its unique properties and to expand its market potential, heterologous production of miraculin has been attempted with various hosts (Table 1). 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 (Kurihara 1994). Recently, the production of recombinant miraculin was attempted again in *E. coli* by Matsuyama et al. (2009). They succeeded in producing 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 its 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 (Ito et al. 2007). In the *Saccharomyces*

**Table 1** Expression host used for recombinant miraculin production and the characterization

| Host                                     | <i>Escherichia coli</i> | <i>Aspergillus oryzae</i>              | <i>Saccharomyces cerevisiae</i> | Plant                      |                         |                             |
|------------------------------------------|-------------------------|----------------------------------------|---------------------------------|----------------------------|-------------------------|-----------------------------|
|                                          |                         |                                        |                                 | <i>Fragaria × ananassa</i> | <i>Lactuca sativa</i>   | <i>Solanum lycopersicum</i> |
| Miraculin yields                         | ND                      | 2.0 mg/L                               | 2.0 mg/L                        | 2.0 µg/g FW                | 43.5 µg/g FW            | 90.7 µg/g FW                |
| Relative activity (native miraculin = 1) | 0.17                    | 0.20 (after sugar chains were removed) | 0.20                            | ND                         | 1                       | 1                           |
| Glycosylation                            | –                       | +                                      | +                               | +                          | +                       | +                           |
| Genetic stability                        | ND                      | ND                                     | ND                              | + (35S/NOS) <sup>a</sup>   | –(35S/NOS) <sup>a</sup> | + (35S/NOS) <sup>a</sup>    |
| References                               | Matsuyama et al. (2009) | Ito et al. (2007)                      | Ito et al. (2010)               | Sugaya et al. (2008)       | Sun et al. (2006a)      | Sun et al. (2007)           |

ND no data

<sup>a</sup> 35S/NOS, *cauliflower mosaic virus 35S promoter/nopaline synthase terminator*

*cerevisiae* yeast 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 (Ito et al. 2010). 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 the types of sugar chains are also crucial for high and stable activity.

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. (2006a) expressed recombinant miraculin in transgenic lettuce (*Lactuca sativa*). They produced miraculin at a concentration of 43.5 µg/g fresh weight (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 (Sun et al. 2007). In transgenic strawberries (*Fragaria × ananassa*), which propagate vegetatively, recombinant miraculin accumulated at an extremely low level of 2.0 µg/g FW, although the accumulation level was stable in the vegetative progeny (Sugaya et al. 2008). 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 (Sun et al. 2007). Additionally, the accumulation and gene expression levels were genetically stable from T<sub>1</sub> to T<sub>5</sub> (Yano et al. 2010). 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. Tomato also has an advantage in the established *Agrobacterium*-mediated

transformation protocol using cotyledon explants from seedlings, and the transformation efficiency exceeds 40% of the explants (Sun et al. 2006b). The transformants are able to be obtained for 3 months after the seed germination. Subsequently, studies for the further improvement of miraculin production were conducted, as we discuss later.

### Improving recombinant miraculin accumulation in transgenic tomato fruit

It is imperative to improve the production of recombinant miraculin for future commercial use. Various approaches have been attempted to increase recombinant miraculin production using transgenic tomatoes.

#### Accumulation profile of recombinant miraculin in transgenic tomato fruit

Recombinant miraculin accumulates to high levels in the overripe fruit during fruit development when the miraculin gene is driven by the 35S promoter (Kim et al. 2010b). Among fruit tissues, 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 (Fig. 3). Why did recombinant miraculin accumulate to such an amount in the exocarp? The deduced amino acids of miraculin include an N-terminal signal sequence of 29 amino acids (Masuda et al. 1995). Miraculin protein is transported to and accumulates in the intercellular layer space of both miracle fruit and transgenic tomatoes (Hirai et al. 2010b). The cell size of the exocarp is considerably smaller than that of other fruit tissues such as the mesocarp (inside tissue of epidermis), the septa, the placenta and the 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 (Kim et al. 2010b). 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 (Kim et al. 2010b).

#### Modification of the gene cassette promoter and terminator

The promoter is an essential element for inducing target gene expression, determining the target tissue and achieving the desired expression time (Desai et al. 2010; Fluhr et al. 1986; Kaulen et al. 1986; Stougaard et al. 1987). There are multiple types of promoters 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 generally produces higher expression than tissue-specific promoters in various plant tissues and organs (Desai et al. 2010; Williamson et al. 1989). 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 (Desai et al. 2010; Twyman et al. 2003). In fact, the miraculin gene, when driven by the 35S promoter, was found to be silenced in transgenic lettuce (Sun et al. 2006a, 2007). Moreover, although gene silencing does not occur in transgenic strawberries using the 35S promoter, the production of miraculin is quite low (Sugaya et al. 2008). Unlike these transgenic plants, the 35S promoter works in transgenic tomatoes and increases miraculin production (Sun et al. 2007; Yano et al. 2010).

The tissue-specific *E8* promoter has been used to control the expression of the miraculin gene in transgenic tomatoes

(Hirai et al. 2011a). This promoter, which was first identified in cherry tomatoes (Deikman and Fischer 1988), activates transcription at the onset of tomato fruit ripening (Lincoln and Fischer 1988a, b). Miraculin gene expression in red tomato fruit was found to be significantly lower under control of the *E8* promoter than under the 35S promoter, and the miraculin content was also lower (Hirai et al. 2011a). This result indicates that the 35S promoter has higher transcriptional activity than the *E8* promoter in tomatoes (Table 2).

The 3'-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 post-transcriptional processes [such as capping, splicing and polyadenylation (Desai et al. 2010; Gilmartin 2005; Proudfoot 2004)] (Lin et al. 2009). In fact, difference of utilizing terminators has considerable effect on the gene expression level (Nagaya et al. 2010; Ingelbrecht et al. 1989). 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 total soluble protein (Hiwasa-Tanase et al. 2011; Kato et al. 2010; Sun et al. 2007). In contrast, the miraculin content in miracle fruit is around 10% of the total soluble protein (Theerasilp and Kurihara 1988). 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 (Hiwasa-Tanase et al. 2011). 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 when using the *NOS* terminator (Table 2).

Nagaya et al. (2010) assessed the capacity of termination to support increased transgene expression using several terminators derived from *Arabidopsis* genes, and they showed that the *heat shock protein 18.2* (*HSP*) terminator was the most effective at supporting high expression levels. In fact, application of the *HSP* terminator increased production of the B subunit of Shiga toxin 2e in transgenic lettuce at the T<sub>0</sub> generation to levels approximately 40 times higher than those obtained with the *NOS* terminator (Matsui et al. 2011). The *HSP* terminator has also been used for miraculin production in transgenic tomatoes (Hirai et al. 2011b). The miraculin accumulation level in the homozygous T<sub>1</sub> generation reached 1,726 µg/g FW, which is 10 times higher than that obtained with the *NOS* terminator, equivalent to 17.1% of total soluble protein (Table 2). In most cases using nuclear transformation, the accumulation level of the recombinant protein does not

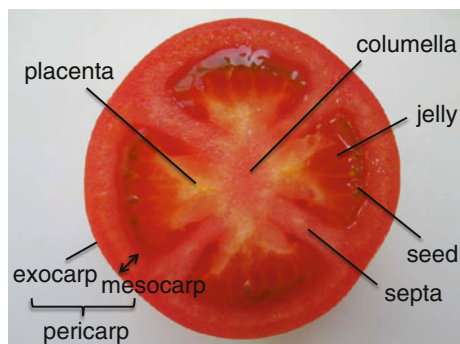


**Fig. 3** Transverse section of tomato fruit

**Table 2** Comparison of recombinant miraculin production in transgenic tomato fruits under different expression cassettes and cultivars

| Cultivar                             | Expression cassette (promoter/terminator) | Generation     | Introduced miraculin gene | Miraculin concentration (μg/g FW) | References                  |
|--------------------------------------|-------------------------------------------|----------------|---------------------------|-----------------------------------|-----------------------------|
| Moneymaker (56B)                     | 35S/NOS                                   | T <sub>5</sub> | Homo                      | 112                               | Yano et al. (2010)          |
| Micro-Tom                            | 35S/NOS                                   | T <sub>0</sub> | Hetero                    | 131                               | Hiwasa-Tanase et al. (2011) |
|                                      | 35S/MR                                    | T <sub>0</sub> | Hetero                    | 197                               |                             |
|                                      | 35S/NOS (sMIR)                            | T <sub>0</sub> | Hetero                    | 128                               |                             |
|                                      | 35S/MR (sMIR)                             | T <sub>0</sub> | Hetero                    | 287                               |                             |
|                                      | 35S/NOS                                   | T <sub>1</sub> | Homo                      | 176                               | Hirai et al. (2011b)        |
|                                      | 35S/HSP                                   | T <sub>1</sub> | Homo                      | 1,726                             |                             |
|                                      | E8/NOS                                    | T <sub>0</sub> | Hetero                    | ≈50                               | Hirai et al. (2011a)        |
| Moneymaker × Micro-Tom (cross no. 2) | 35S/NOS                                   | F <sub>7</sub> | Homo                      | 343                               | Kato et al. (2010)          |

*MIR* miraculin terminator, *sMIR* synthetic miraculin gene which was designed to optimize its codon usage for tomato, *HSP* heat shock protein 18.2 terminator from *Arabidopsis thaliana*

exceed 1–2% of total soluble protein (Leelavathi and Reddy 2003; Meyers et al. 2010; Staub et al. 2000). Conversely, recombinant proteins derived from chloroplast transformation normally accumulate to 5–25% of total soluble protein (Daniell 2006; Maliga 2003). It is difficult to use the chloroplast transgenic system for recombinant miraculin production because the protein requires post-translational 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.

#### Optimization of codons

Codon modifications are performed to increase the translational efficiency of heterologous genes in their hosts. Preferred codon usage varies significantly among different organisms and even between plant species (Kawabe and Miyashita 2003; Liu and Xue 2005; Murray et al. 1989; Peng et al. 2006; Perlak et al. 1991; Rouwendal et al. 1997; Xue et al. 2003). Therefore, this approach seeks to increase the accumulation of recombinant proteins by modifying their original codons to codons more suitable to the host without changing the amino acid sequence (Gustafsson et al. 2004). Codon optimization of the *miraculin* gene for tomatoes was found to enhance its translational efficiency more than twofold compared to that of the native *miraculin* gene in transgenic tomatoes (Hiwasa-Tanase et al. 2011) (Table 2). 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 both to optimize codons and to select a suitable terminator.

#### Adequacy of the cultivar (genetic background)

It is important to pay attentions not only to the species but also to the cultivar when selecting the genetic background in order to produce recombinant proteins using plants. Kim et al. (2010a) created F<sub>1</sub> 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 accumulated to high levels in the exocarp, followed by the mesocarp, just as in the original transgenic line. On the other hand, the accumulation levels of miraculin in fruits changed in the different genetic backgrounds. Ratio of each fruit tissues 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 (Kim et al. 2010b). Therefore, miraculin accumulation could be affected the ratio of each fruit tissues in the different genetic backgrounds because the accumulation levels differ depending on fruit tissues. Miraculin accumulation levels also differed in the exocarp and mesocarp among the hybrid lines (Kim et al. 2010a). Cell size and cell numbers per fresh weight is different even in same exocarp depending on the hybrid lines as same as the fruit size is difference. Such a different fruit structure is considerable to be 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 have an effect on

miraculin accumulation in fruits in which the *miraculin* gene is driven by the 35S promoter; miraculin levels in tomato fruits are higher at later harvesting times (Hirai et al. 2011a; Kato et al. 2011). 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 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 that in the transgenic line as a parent (Kato et al. 2010).

### Gene dosage

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 (Hobbs et al. 1990; Jorgensen et al. 1996). On the other hand, 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 found to be 4.7 and 1.5 times higher than those in heterozygous transgenic tomatoes, respectively (Kim et al. 2010a). 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 (Meyer 1996). 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.

### Miraculin localization

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 powerful effect on its proper folding, assembly and post-transcriptional processing (Twyman et al. 2003). When a heterologous protein is not correctly folded or synthesized in a plant, the synthesized proteins are easily attacked by proteases (Desai et al. 2010). Thus,

suitable subcellular targeting contributes to protein stability and, eventually, to the yield of the recombinant protein (Doran 2006; Twyman et al. 2003). Miraculin is secreted and accumulates in the intercellular layer in miracle fruit (Hirai et al. 2010b). In the same manner, recombinant miraculin is also secreted to the intercellular layer in transgenic tomato fruits and leaves by virtue of the signal sequence of the native gene (Hirai et al. 2010b). 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 total soluble protein (Hirai et al. 2011b). This accumulation level exceeds the 400 µg/g FW of miraculin protein produced in miracle fruit (Fig. 1; Table 2). 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 (Theerasilp and Kurihara 1988). The fact that the apoplastic pH of tomatoes declines from an average of 6.7–4.4 during fruit ripening might also be advantageous (Almeida and Huber 1999).

### Cultivation in a closed system

To improve recombinant miraculin production and to stabilize 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 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 (Morimoto et al. 1995). In fact, the transgenic tomato that expresses the *miraculin* gene (56B, ‘Moneymaker’) produces recombinant miraculin more stably in a closed tomato cultivation system than in a netted greenhouse (Hirai et al. 2010a). Additionally, the yield has been estimated at approximately 45 t FW of fruits and 4 kg of recombinant miraculin per 1,000 m<sup>2</sup> of cultivated area in a year (Table 3).

Closed cultivation systems also have other advantages: it is easy to prevent the spread of transgenic plants and pollen to the external environment, 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. To breed tomatoes suitable for a closed cultivation system with the aim of effective cultivation space utilization, the transgenic tomato 56B, ‘Moneymaker’ was

**Table 3** Comparison of transgenic tomato yield and recombinant miraculin production under different cultivation condition in closed cultivation systems

| Transgenic tomato | PPF ( $\mu\text{mol}/\text{m}^2/\text{s}$ ) | $\text{CO}_2$ concentration (ppm) | Finally planting density ( $\text{plants}/\text{m}^2/\text{layer}$ ) | Light period (h/day) | Days to harvest of all tomatoes (days after sowing) | Miraculin concentration ( $\mu\text{g}/\text{g}$ FW) | Number of layers (at same height) <sup>a</sup> | Fruit yield ( $\text{kg}$ FW/ $\text{m}^2/\text{year}$ ) | References           |
|-------------------|---------------------------------------------|-----------------------------------|----------------------------------------------------------------------|----------------------|-----------------------------------------------------|------------------------------------------------------|------------------------------------------------|----------------------------------------------------------|----------------------|
| 56B               | 450                                         | 600                               | 14.1                                                                 | 16                   | 109                                                 | 81                                                   | 2                                              | 35.5                                                     | Hirai et al. (2010a) |
|                   | 600                                         | 600                               | 14.1                                                                 | 12                   | 105                                                 | 82                                                   | 2                                              | 44.1                                                     |                      |
|                   | 600                                         | 600                               | 14.1                                                                 | 16                   | 104                                                 | 87                                                   | 2                                              | 46.3                                                     |                      |
| 56B               | 450                                         | 600                               | 13.3                                                                 | 16                   | 90                                                  | 140                                                  | 2                                              | 26.2                                                     | Kato et al. (2010)   |
| Cross no. 1       | 450                                         | 600                               | 26.7                                                                 | 16                   | 90                                                  | 367                                                  | 2                                              | 73.6                                                     |                      |
| Cross no. 2       | 450                                         | 600                               | 26.7                                                                 | 16                   | 104                                                 | 343                                                  | 2                                              | 45.9                                                     |                      |
| Cross no. 2       | 100                                         | 1,000                             | 44.4                                                                 | 12                   | 95                                                  | 472                                                  | 3                                              | 25.5                                                     | Kato et al. (2011)   |
|                   | 200                                         | 1,000                             | 44.4                                                                 | 12                   | 86                                                  | 362                                                  | 3                                              | 51.0                                                     |                      |
|                   | 300                                         | 1,000                             | 44.4                                                                 | 12                   | 84                                                  | 272                                                  | 3                                              | 83.1                                                     |                      |
|                   | 400                                         | 1,000                             | 44.4                                                                 | 12                   | 79                                                  | 211                                                  | 3                                              | 91.5                                                     |                      |

PPF photosynthetic photon flux

<sup>a</sup> It means the number of installation available layers on the height of closed cultivation system

crossed with a dwarf tomato ('Micro-Tom') that has a short life cycle and determinate-type growth (Kato et al. 2010). In  $F_2$  plants, hybrids were selected by small plant size, miraculin accumulation and determinate type; they were then propagated to  $F_6$  or  $F_7$  by self-pollination. In cross no. 2 (selected between two lines named cross no. 1 and cross no. 2), it was not necessary to remove axial buds and leaves when cultivated in a two-layer closed cultivation system, in contrast to cross no. 1 and 56B. This growth habit helps to reduce 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 cross no. 2 in the two-layer cultivation system was equivalent to 45.9 t FW per 1,000  $\text{m}^2$  of cultivation area in a year (Table 3). The miraculin content in the tomato fruit significantly increased to 343  $\mu\text{g}/\text{g}$  FW of pericarp (including exocarp and mesocarp), which is approximately 2.5 times higher than that of 56B, due to the effect of genetic background modification. This effect was confirmed by the 1.9-fold higher weight ratio of the exocarp in cross no. 2 than in 56B, and no. 2's high miraculin concentration in the mesocarp relative to that of 56B.

The high operating costs of closed cultivation systems mainly accrue from the electric bills due to 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. Cross no. 2 acquired some characteristics from the 'Micro-Tom' dwarf tomato, such as its growth habit and

its determinate type (Kato et al. 2010). An ability to grow under lower intensity light was also gained by cross no. 2. Normal-size tomatoes, such as the 56B line, could not be cultivated under lower light conditions because low light makes processes such as flower-bud formation, flowering, photosynthesis and healthy plant growth difficult. However, cross no. 2 grew normally even at photosynthetic photon flux (PPF) 100  $\mu\text{mol}/\text{m}^2/\text{s}$ , as does 'Micro-Tom', which can be cultivated under 80  $\mu\text{mol}/\text{m}^2/\text{s}$  (Kato et al. 2011). Kato et al. (2011) evaluated the relationship between fruit yield and miraculin concentration under the different light conditions of PPF100, 200, 300 and 400  $\mu\text{mol}/\text{m}^2/\text{s}$  and discussed the electricity costs of miraculin production. Miraculin concentrations were higher at low light intensity, but 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.

#### Purification of recombinant miraculin from tomato fruit

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 (Twyman et al. 2003). 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 in that it can be used as a low-calorie sweetener or as an additive for foods targeting diabetics, as 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 should be used for safety assessment analysis and to identify the taste-modifying mechanism of miraculin. Therefore, industrial uses of recombinant miraculin from transgenic tomatoes encompass the consumption of unprocessed raw fruit and processed fruit, 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 (Brouwer et al. 1968; Giroux and Henkin 1974; Kurihara and Beidler 1968). However, these methods cannot remove the polyphenolic coloring agent and lead to a reduction of the taste-modifying activity due to the use of a carbonate extraction buffer (pH 10.5). In 1988, Theerasilp and Kurihara presented a method in which NaCl solution was used for crude extraction from the miracle berry, and then purification was achieved via CM-Sepharose ion-exchange chromatography and ConA-Sepharose 4B affinity chromatography. Armah et al. (1999) also developed a method using NaCl extraction buffer and then 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 (Duhita et al. 2009). This method utilizes four histidine residues 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, reducing purification loss and costs. In addition, the method is able to purify the active form of miraculin free from the denatured form because the IMAC column binds only proper miraculin that has maintained the three-dimensional structure of its homodimer (which elicits its sweetness-inducing activity) and does not bind monomeric miraculin, the inactive form. However, recombinant miraculin purification from tomato fruit is more difficult than 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 pure miraculin (Duhita et al. 2011). Recombinant miraculin has also been purified using the method of Theerasilp and Kurihara (1988) 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 (Sun et al. 2007). 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.

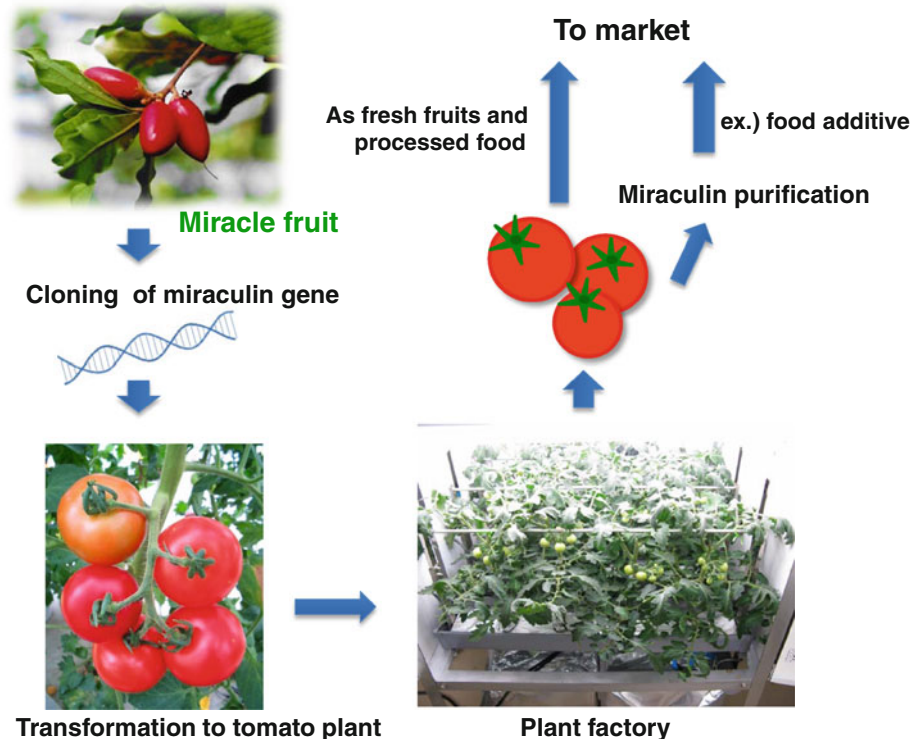
### Safety assessment

The safety of transgenic plants and their recombinant products are assessed at various points before coming to the market and being cultivated on an agricultural scale, although depending on the country, the legal systems have slight differences regarding requirements. In Japan, food safety assessments are mainly confirmed by biodiversity evaluation and safety as food products. One aspect of these assessments is performed by comparing the transgenic plant to the untransformed host plant on the basis of global standard. In the case of transgenic tomatoes accumulating recombinant miraculin, assessments of environmental effects and food safety are currently underway. The evaluation items for food safety assessment mainly include information pertaining to the original plant used as the transgenic host, the safety of the introduced gene and its use as a food (i.e., allergenicity, toxicity and harmful materials).

Metabolomics analysis has been performed in an attempt to use objective substantial equivalence to assess transgenic tomatoes accumulating recombinant miraculin (Kusano et al. 2011). This approach detects tomato fruit compounds with 86% coverage based on the metabolites listed in the LycoCyc database for tomatoes, and the analysis indicated that >92% vary within an acceptable range between the transgenic tomato and the untransformed host cultivar. Although as a new approach to prove safety, considerable data accumulation regarding metabolites and analyzed samples are essential to gain the reliability of this assessment, it might be useful as a first attempt for substantial equivalent evaluation at this stage. The multi-platform metabolomics has exhaustive information but the system could not detect complicated secondary metabolites such as tomatine, which is known as a toxic glycoalkaloid of tomato plant. Additional approach for confirmation of the food safety is necessary to detect the existence and quantity of tomatine and the other known toxic materials like solanine and chaconine that are included in potato, the relative species of tomato.

Generally, digestibility of non-allergenic proteins in simulated gastric fluid is higher than allergenic proteins

**Fig. 4** Scheme for the mass production of recombinant miraculin using transgenic tomato fruit



(Takagi et al. 2003). The digestibility of natural miraculin from miracle fruits and the recombinant miraculin from the transgenic tomato is in progress.

### Future directions and prospects

Transgenic plants are presumably the most suitable hosts for recombinant miraculin protein production with respect to post-translational modifications such as glycosylation. 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. More recently, a newly produced transgenic lettuce line was found to be able to stably accumulate recombinant miraculin in progeny lines using the ubiquitin promoter and terminator (Hirai et al. 2011c). Although it might be difficult to use as a raw food, 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 such aspects 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 more optimization of 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 (Fig. 4).

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