

## Article

# An Endogenous, Flavor-Enhancing TRV/*Agrobacterium* System for Edible Tomato Fruits with the Sweet Protein Thaumatin II

Jiachun Chen <sup>1</sup>, Qizheng Liu <sup>1</sup>, Siyuan Guo <sup>1</sup>, Yitong Li <sup>2</sup>, Ruohan Chen <sup>3</sup>, Kexin Li <sup>1</sup>, Guangbin An <sup>1</sup>, Yuanrun Liu <sup>4</sup>, Zhengyue Hong <sup>1</sup>, Beixin Mo <sup>1</sup>, Xuedong Liu <sup>5,\*</sup> and Weizhao Chen <sup>1,\*</sup>

<sup>1</sup> College of Life Sciences and Oceanography, Shenzhen University, Shenzhen 518055, China; chenjiachun2022@email.szu.edu.cn (J.C.); liuqizheng2023@email.szu.edu.cn (Q.L.); guosiyuan2022@email.szu.edu.cn (S.G.); likexin2022@email.szu.edu.cn (K.L.); anguangbin2022@email.szu.edu.cn (G.A.); hongzhengyue2023@email.szu.edu.cn (Z.H.); bmo@szu.edu.cn (B.M.)

<sup>2</sup> School of Basic Medical Sciences, Medical School, Shenzhen University, Shenzhen 518055, China; liyitong2023@email.szu.edu.cn

<sup>3</sup> Institute for Advanced Study, Shenzhen University, Shenzhen 518060, China; chenruohan2022@email.szu.edu.cn

<sup>4</sup> School of Pharmacy, Medical School, Shenzhen University, Shenzhen 518055, China; liuyuanrun2023@email.szu.edu.cn

<sup>5</sup> National Citrus Engineering Research Center, Citrus Research Institute, Southwest University, Chongqing 400712, China

\* Correspondence: xuedong007@126.com (X.L.); cwz@szu.edu.cn (W.C.)

## Abstract

The rise in diabetes and obesity worldwide has created an urgent demand for low-sugar, nutrient-dense foods with appealing flavors. This study established an endogenous and “rapid validation–stable production” platform to enhance the flavor of edible tomato fruits by integrating two key technologies in the MicroTom cherry tomato: (1) TRV viral vector-mediated transient expression and (2) *Agrobacterium*-mediated stable genetic transformation. We employed the human sweet taste receptor TAS1R2 for in vitro functional validation and objectively demonstrated that tomato-derived recombinant thaumatin II exhibits receptor-binding activity equivalent to that of the native protein, overcoming the limitations of traditional sensory evaluation. Non-targeted metabolomic analysis (covering 1236 metabolites) confirmed that thaumatin II expression did not significantly alter the profiles of sugars, organic acids, or key flavor compounds in tomato fruits. This provides safety data supporting the development of “ready-to-eat sugar-substitute fruits.” Our strategy offers a solution and theoretical technical support for the development of low-sugar, high-nutrient foods.

**Keywords:** sugar-substitute fruit; natural sweetener; viral vector expression; plant biotechnology; TAS1R2; genetic transformation



Academic Editor: Wilfried Rozhon

Received: 19 August 2025

Revised: 19 September 2025

Accepted: 22 October 2025

Published: 24 October 2025

**Citation:** Chen, J.; Liu, Q.; Guo, S.; Li, Y.; Chen, R.; Li, K.; An, G.; Liu, Y.; Hong, Z.; Mo, B.; et al. An Endogenous, Flavor-Enhancing TRV/*Agrobacterium* System for Edible Tomato Fruits with the Sweet Protein Thaumatin II. *Horticulturae* **2025**, *11*, 1284. <https://doi.org/10.3390/horticulturae11111284>

**Copyright:** © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

Over the past two decades, excessive sugar consumption has emerged as a major contributor to the global surge in diabetes, cardiovascular diseases, and obesity [1,2]. Since the World Health Organization (WHO) issued its 2015 guidelines limiting free sugar intake, the demand for healthy, low-calorie, sugar-free foods has grown dramatically, with “sugar-free” becoming a key label in health-conscious diets [3]. Although artificial non-nutritive sweeteners (e.g., aspartame and sucralose) are widely used in beverages and processed

foods to meet sweetness demands, growing concerns persist regarding their potential carcinogenic risks [4] and environmental pollution. In contrast, plant-derived natural sugar substitutes, particularly natural sweet-tasting proteins, have gained prominence owing to their high sweetness, ease of digestibility into amino acids, and superior safety profile, making them promising candidates for use in beverages, snacks, and functional foods [5–9].

Among the known plant sweet proteins (e.g., thaumatin, monellin, brazzein, curculin, mabinlin, and pentadin), thaumatin II, sourced from the West African plant *Thaumatococcus daniellii* (Benth.), is notable for its excellent properties (Table 1). Mature thaumatin II consists of 207 amino acids folded into three domains, stabilized by eight disulfide bonds that confer remarkable structural rigidity. Notably, Domain III, a compact disulfide-rich region (residues 54–84) containing two critical residues (Lys67 and Arg82), has been identified as a key structural determinant of sweetness perception [10]. Kaneko and Kitabatake [11] demonstrated that even under extreme conditions (pH 2.0, 80 °C for 4 h), thaumatin II retains its sweet taste, highlighting its thermostability and acid resistance, which are crucial for food processing applications. Currently, thaumatin is FDA-approved as a safe sweetener with no reported genotoxicity, teratogenicity [12], or other hazards, and is commercially utilized in products including chewing gum and chocolate [13,14].

The large-scale production of thaumatin II for use as a food additive faces significant challenges. First, the natural source *T. daniellii* (Benth.) has stringent growth requirements, and attempts to cultivate it *ex situ* have been unsuccessful worldwide [15]. Second, the extraction process inevitably results in substantial yield loss. Given these limitations, recombinant DNA technology has been employed to enable heterologous expression of sweet proteins. In 1982, Edens et al. [16] observed that *Escherichia coli*-derived thaumatin II predominantly formed inclusion bodies, requiring denaturation and renaturation procedures that are time-consuming and yield low recovery rates. This inefficiency is attributable to the inability of prokaryotic systems to support the unique structure of plant-derived thaumatin II, which contains eight intramolecular disulfide bonds [17]. Alternative expression platforms, including *Bacillus subtilis*, *Streptomyces lividans* [18], *Aspergillus awamori* [19], and *Pichia pastoris* [20] have been used; however, critical bottlenecks persist, including inconsistent expression levels, low secretion efficiency, difficulties in optimizing process parameters [20], and complex purification protocols, all of which severely hinder commercial applications.

An alternative approach involves engineering crop plants as natural carriers, offering advantages such as low-cost raw materials, the capacity for complex protein folding and post-translational modifications, the potential elimination of downstream purification, and at the same time, provide a naturally sweeter-tasting food. Tomato (*Solanum lycopersicum*), a widely consumed fruit with a slightly tart flavor, is naturally low in sugar and has a low glycemic index. These attributes, combined with our sweetening enhancement, make tomato an ideal choice for bioengineering to achieve a sweet flavor experience while meeting the dietary needs of health-conscious individuals—particularly those who seek to manage blood sugar without compromising on taste. Two primary strategies can be employed to achieve this: transient transfection and transgenic technology. Viral vector systems have proven effective for high-level foreign protein expression and plant gene function studies [21]; however, no reports exist on the virus-mediated transient expression of thaumatin II in tomato. Furthermore, systematic evaluations of the effects of heterologous protein expression on critical nutritional components (e.g., sugars, organic acids, and flavor metabolites) in host plants remain scarce—a crucial consideration for developing “edible, low-calorie sweet fruit.”

Table 1. Comparative molecular characteristics of natural sweet proteins.

| Sweet Protein | Source                                  | Size (AA/MW)                     | Active Form                           | Variants                        | Sweetness Factor (Based on Weight)                                          | Stability                                                                                                                                             | Applications                                                                                             | References       |
|---------------|-----------------------------------------|----------------------------------|---------------------------------------|---------------------------------|-----------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|------------------|
| Thaumatococin | <i>Thaumatococcus daniellii</i> Benth.  | 207AA                            | Monomer                               | I, II, a, b, c <sup>(a)</sup>   | 3000 (licorice-like aftertaste)                                             | -Soluble<br>-Extreme acid/heat tolerance (80 °C, pH 2.0 for 4 h)<br>-Aggregates > 70 °C at pH 7.0                                                     | -FDA-approved natural flavor enhancer and high-intensity sweetener<br>-Approved in multiple countries    | [10,11,13,14,22] |
| Monellin      | <i>Dioscoreophyllum cumminsii</i> Diels | 45AA (A chain)<br>50AA (B chain) | Dimer (A + B)                         | -                               | 3000                                                                        | -Heat-labile (>50 °C)<br>-Low pH tolerance<br>-Solubility: Good                                                                                       | -Safety not fully evaluated<br>-Not approved as sweetener                                                | [23–26]          |
| Brazzein      | <i>Pentadiplandra brazzeana</i> Baillon | 54AA                             | Monomer                               | I, II, III                      | 2000 (Sucrose-like taste profile, sweetness potency varies by isoform [27]) | -Best thermostability among sweet proteins:<br>-No property loss after 2 h at 98 °C<br>-Stable at pH 2.5–8 (4 h at 80 °C)                             | -Traditionally used by indigenous peoples [28]<br>-Potential allergenicity concerns prevent approval [7] | [29,30]          |
| Curculin      | <i>Curculigo latifolia</i>              | 114AA                            | Monomer                               | -                               | 550 (taste-modifying)                                                       | -Good stability<br>-Taste-modifying activity remains after 1 h at 50 °C (pH 3–11)<br>-High thermostability:                                           | -Recognized as safe food additive in Japan [31]<br>-Not approved in US/EU [32]                           | [33,34]          |
| Mabinlin      | <i>Capparis masailai</i> Levi           | 33AA (A chain)<br>72AA (B chain) | Dimer (A + B)                         | I, II-a, III, IV <sup>(a)</sup> | 100                                                                         | -Mabinlin-II-a: Sweetness unchanged after 48 h at boiling point [35]<br>-Mabinlin-III/IV <sup>(a)</sup> : Sweetness unchanged after 1 h at 80 °C [36] | Not FDA-approved                                                                                         | [36,37]          |
| Pentadin      | <i>Pentadiplandra brazzeana</i> Baillon | -                                | Putative cross-linked brazzein analog | -                               | 500 (synergistic with saccharin to suppress bitter aftertaste)              | Stable at 100 °C for 5 h                                                                                                                              | -                                                                                                        | [7,38–40]        |

The superscript ‘(a)’ indicates a subtype, following the original convention of the reference.

Transgenic crops play a pivotal role in bioreactor systems because of their mature protocols and stable inheritance. Firsov et al. [41] demonstrated that transgenic tomatoes can serve as effective biofactories for recombinant thaumatin (achieved recombinant thaumatin accumulation of 50 mg/kg fresh weight in transgenic tomatoes), capable of producing the protein in a functionally intact form. This work lays a solid foundation that the transgenic tomatoes can be engineered to accumulate recombinant thaumatin, functioning as a natural source of sweetness. It suggests that transgenic tomatoes could serve as thaumatin producers, potentially revolutionizing food industry applications by enabling direct consumption of sweet, low-sugar fruits and vegetables by diabetics and health-conscious consumers, moving beyond the mere additive use of microbial-extracted products. However, the current functional validation relies predominantly on subjective taste panels rather than biological assays. Although electronic tongues/noses have been developed for small-molecule sweeteners, their application in recombinant sweet protein detection remains unreported.

To address these critical gaps, we propose a strategy for thaumatin II production. Our innovation integrates a TRV viral vector-mediated transient system (see Supplementary Information, SI) with *Agrobacterium*-mediated stable transformation in a cherry tomato model (*S. lycopersicum* cv. MicroTom), establishing an efficient system combining two well-established technologies for rapid validation and stable production of thaumatin II. For functional assessment, we conducted the first reported in vitro human sweet taste receptor (TAS1R2) interaction analysis to objectively evaluate the sweetness activity of recombinant thaumatin II, thereby overcoming the limitations of sensory evaluation. Concurrently, untargeted metabolomic profiling of 1236 metabolites was used to systematically evaluate the impact on core nutritional and flavor components, providing critical data for developing safe, edible “sweetener fruits.” This approach demonstrates the feasibility of tomatoes as thaumatin II natural carriers, enabling direct protein consumption while bypassing costly, inefficient purification processes, drastically reducing production costs and environmental burdens to deliver a transformative solution for low-sugar, healthy food industries.

## 2. Materials and Methods

### 2.1. Plasmid Construction

The pTRV2 vector used in this study was designed by our laboratory (Shenzhen University, College of Life Sciences and Oceanography) for transient expression of foreign genes in Solanaceae crops and maintained in a  $-20^{\circ}\text{C}$  freezer. It is a shuttle vector containing a kanamycin resistance gene along with the right and left border sequences of the *Agrobacterium tumefaciens* Ti plasmid. This facilitates the transfer of target genes into tomato leaves via *Agrobacterium*-mediated transformation. The T-DNA fragments included the CaMV 35S promoter, TRV viral CP protein, and target gene expression cassette. The preprothaumatin II gene (derived from *T. daniellii*), fused to a C-terminal FLAG tag, was cloned into the expression frame and replicated along with the viral genome. The full sequence and vector map are provided in the Supplementary Information.

Another vector, pBWA(V)HS, was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). It contains the right and left border sequences of the *A. tumefaciens* Ti plasmid, as well as a HygR resistance gene driven by the enhanced CaMV 35S promoter. Successfully transformed explants grew normally in HygR-containing media. The preprothaumatin II gene was fused to a  $3 \times \text{HA}$  tag at the C-terminus for detection and purification. Oligonucleotides (synthesized using GenScript) were cloned downstream of the CaMV 35S promoter and linked to the NOS terminator.

Two constructs, TRV2\_CaMV35S\_Thaumatin II\_FLAG (Figure 1A) and pBWA(V)HS\_CaMV35S\_Thaumatin II\_3  $\times$  HA, were generated and transformed into

*Agrobacterium* GV3101 (Beyotime). Positive colonies were selected based on their resistance to kanamycin and hygromycin. In addition, pTRV1, a component of viral vector replication, was constructed and transformed into *Agrobacterium* GV3101. TRV2\_CaMV35S\_mCherry was used as a control for TRV2-mediated transformation, and wild-type MicroTom served as a control for transgenic experiments.

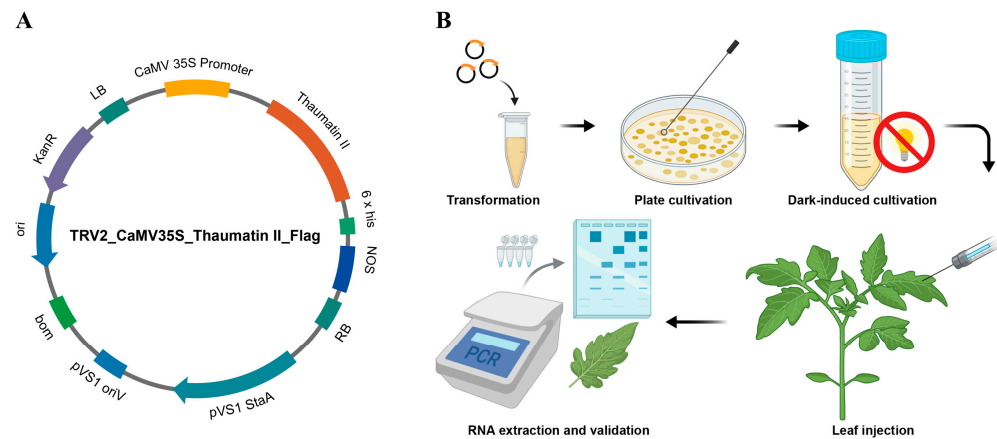


**Figure 1.** Establishment and validation of the method for transient transfection of tomato plants with viruses. (A). Schematic diagram of plasmid construction. (B). Transient infection process (lower panel).

## 2.2. Plant Materials

Cherry tomato seeds (*S. lycopersicum* cv. MicroTom) were provided by the Epigenetics Research Laboratory of Shenzhen University, China. *Agrobacterium* cells harboring the successfully transformed TRV2\_CaMV35S\_Thaumatococcus II\_FLAG plasmid were collected and resuspended in infiltration buffer (MMA: 10 mmol/L MgCl<sub>2</sub>, 10 mmol/L MES, and 100 mmol/L AS). The bacterial suspension was adjusted to OD<sub>600</sub> = 0.6 using a UV spectrophotometer and incubated at 25 °C for 3 h to induce gene expression. A 1 mL syringe was used to inject *Agrobacterium* cultures containing pTRV1 and pTRV2\_CaMV35S\_Thaumatococcus II\_FLAG into the lower leaves of four-leaf-stage plants. TRV2\_CaMV35S\_mCherry-transfected plants served as controls.

An *Agrobacterium*-mediated leaf explant transformation method was used for transgenic tomato plants. Cotyledons from two-week-old MicroTom seedlings were excised and inoculated onto callus-inducing MS medium (MS + 0.5 mg/L IAA + 1.0 mg/L 6-BA + 3% sucrose + 0.7% agar) and incubated in the dark at 25 °C to induce callus formation. An *Agrobacterium* suspension harboring pBWA(V)HS\_CaMV35S\_Thaumatococcus II was inoculated onto tomato tissue blocks overnight. Subsequently, the calli were transferred to hygromycin-supplemented medium for regenerant selection. Differentiated calli were then transferred to a differentiation medium (MS + 0.02–0.05 mg/L IAA + 1.0 mg/L 6-BA + 3% sucrose + 0.7% agar) and cultured under light at 25 °C. When shoots reached 2–3 cm in height, they were transferred to the rooting medium and cultured for an additional 10–15 days. Finally, the acclimatized plants were transplanted into soil. Wild-type MicroTom plants were used as controls.

## 2.3. RNA Extraction and RT-PCR Analysis

The tomato leaf tissue samples were frozen in liquid nitrogen and ground using a tissue grinder. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), followed by chloroform extraction (1:5 v/v). After a 5 min incubation, the mixture was centrifuged at 12,000 rpm and 4 °C for 15 min. The RNA-containing supernatant was precipitated with isopropanol, washed with 75% ethanol, and dissolved in diethyl

pyrocarbonate-treated water. cDNA was synthesized using the One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, Carlsbad, CA, USA) with random hexamer primers under the following conditions: 25 °C for 10 min, 42 °C for 15 min, and hold at 4 °C.

Gene-specific primers (Sangon Biotech, Shanghai, China) were used for PCR amplification.

For TRV2\_CaMV35S\_Thaumatococcus II:

Forward: 5'-TGGTAGCATTGAGTTTCGCA-3'

Reverse: 5'-GGGACATGCCCGGGCCTCGAG-3'

For pBWA(V)HS\_CaMV35S\_Thaumatococcus II:

Forward: 5'-TCAACCAGTACGGCAAGGAC-3'

Reverse: 5'-CAAGGGCAGTAGGGCAGAAA-3'

PCR conditions: 98 °C for 3 min; 35 cycles of 98 °C for 20 s, 56 °C for 20 s, and 72 °C for 40 s; followed by 72 °C for 5 min and a hold at 16 °C. The PCR products were separated on a 1.2% agarose gel and visualized under UV light.

#### 2.4. Protein Extraction and Detection

For total soluble protein extraction, tomato leaf and fruit samples were ground in liquid nitrogen. The resulting powder was resuspended in extraction buffer (Tris-HCl, pH 8.0, EDTA,  $\beta$ -mercaptoethanol, protease inhibitor, and glycerol). After 30 min of incubation at 4 °C, the suspension was centrifuged at  $12,000\times g$  and 4 °C for 20 min. The supernatants were collected for further analysis. Proteins were separated using 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue G250 (Shanghai Macklin Biochemical Technology Co., Ltd., Shanghai, China). For Western blotting, proteins were transferred to a nitrocellulose membrane, blocked with 5% BSA at 37 °C for 2 h, and probed with mouse anti-HA monoclonal antibody (1:2000, Abcam, Cambridge, UK) at 4 °C overnight. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:4000, Abcam), and proteins were detected using an ECL kit (Beyotime, P0018FM, Shanghai, China). Controls included mCherry-transformed plants for the viral infection experiments and wild-type MicroTom for the transgenic experiments.

#### 2.5. Thaumatococcus Purification and Co-Immunoprecipitation

Thaumatococcus was purified using an anti-HA-tagged magnetic bead kit (Beyotime, P2185S). Briefly, ground samples were lysed in buffer containing protease inhibitors, and the homogenate was centrifuged at  $14,000\times g$  and 4 °C for 5 min. The supernatant was concentrated using a 10 kDa ultrafiltration tube (Beyotime, FUF051). HA-tagged thaumatococcus was captured using magnetic beads and eluted for further analysis. To assess sweetness, purified thaumatococcus was incubated with the human sweet taste receptor protein TAS1R2. After washing, the coimmunoprecipitated products were eluted with SDS-PAGE buffer and verified by Western blot.

#### 2.6. Tomato Metabolomic Analysis

Metabolomic profiling was performed by Wuhan MetWare Biotechnology Co., Ltd. (Wuhan, China) [42], using a widely targeted metabolomics approach. The freeze-dried tomato tissues were ground using zirconia beads at 30 Hz (MM 400; Retsch, Haan, Germany). A total of 100 mg of powder was extracted with 1.0 mL of 70% methanol, sonicated for 30 min, and incubated overnight at 4 °C. After centrifugation at  $12,500\times g$  for 10 min, the supernatant was filtered (0.22  $\mu$ m, SCAA-104, ANPEL, Shanghai, China [43]) for UPLC-MS/MS analysis.

### 2.6.1. UPLC Conditions

Samples were analyzed on a Waters ACQUITY Premier HSS T3 column (1.8  $\mu\text{m}$ ,  $2.1 \times 100$  mm) at 40 °C with a flow rate of 0.30 mL/min and an injection volume of 2  $\mu\text{L}$ . Mobile phases: solvent A (5 mM ammonium acetate) and solvent B (acetonitrile). Gradient: 95% A to 5% A over 10 min, held for 2 min, returned to 95% A at 0.1 min, and equilibrated for 2.9 min.

### 2.6.2. MS Conditions

Data were acquired in DDA mode using MS-DIAL (version 4.9) software [44]. Parameters: sheath gas flow, 320 Arb; auxiliary gas flow, 40 Arb; temperature, 350 °C; ion spray voltage,  $\pm 3400/-3200$  V; and mass range, 85–1250 Da.

### 2.7. Statistical Analysis

Metabolomics data were normalized and unit-variance scaled prior to multivariate analyses. Unsupervised principal component analysis (PCA) was performed using the `prcomp` function in R (version 4.5.1) [45] following unit-variance scaling. Hierarchical clustering analysis (HCA) and Pearson correlation coefficient (PCC) calculations were performed using the `ComplexHeatmap` package. For differential metabolite analysis, fold changes (FC) were computed and statistical significance was assessed using Student's *t*-test (two-tailed) with Benjamini–Hochberg false discovery rate (FDR) correction; metabolites with  $\text{FDR} < 0.05$  and  $|\log_2\text{FC}| \geq 1$  were considered significant unless otherwise stated. Volcano/FC plots show  $\log_2\text{FC}$  and  $-\log_{10}(p\text{-value})$ . All tests were two-sided;  $p < 0.05$  was considered significant.

## 3. Results

### 3.1. Molecular Analysis of Transiently Transfected Plants with Viruses

To transiently express large amounts of thaumatin II in tomato leaves, a TRV2 plasmid carrying thaumatin II was constructed and transformed into *Agrobacterium*. Through kanamycin resistance screening and colony PCR analysis, the presence of an 849 bp amplified fragment of the inserted thaumatin II gene in *Agrobacterium* GV3101 was confirmed (Supplementary Information Figure S1). Successfully transformed *Agrobacterium* cells were induced and injected into four-week-old tomato leaves to obtain TRVT-35S plants. After 14 days, RNA and protein extracted from leaf tissue were subjected to RT-PCR and Western blot, respectively, to verify the transcription and expression of the thaumatin gene. As expected, an 849 bp amplified fragment was observed in the RT-PCR results (Supplementary Information Figure S1), and strong expression of a protein approximately 26.5 kDa was observed in the Western blot results (Supplementary Information Figure S1). This apparent molecular weight is higher than the theoretical mass of the unmodified polypeptide (~25.5 kDa, see GenBank accession number in Supplementary Information S2), which is consistent with the C-terminal  $3 \times \text{HA}$  tag (~3.3 kDa) included in the plasmid (see sequence in Supplementary Information S2) and the natural cleavage of its N-terminal signal peptide within the cells [39].

### 3.2. Metabolomic Analysis of Infected Tomato Plants

For metabolomic analysis, a widely targeted LC tandem MS (LC-MS/MS) method was used, and a total of 1236 annotated metabolites were identified in tomato fruit tissue, including 450 lipids and lipid-like molecules; 231 organic acids and their derivatives; 147 phenylpropanoids and polyketides; 121 organic heterocyclic compounds; 120 organic oxygen compounds; 60 benzene compounds; 54 nucleosides, nucleotides, and their analogs; 33 organic nitrogen compounds; 10 alkaloids and their derivatives; 4 homogeneous non-

metallic compounds; 4 lignans, neolignans, and related compounds; and 2 organic sulfur compounds. Based on the quality control analysis of these metabolites, the intra-group correlation coefficient (Pearson  $r$ ) was  $>0.998$  (Figure 2A), and the coefficient of variation (CV) of 75% of the metabolites was  $<0.3$  (Figure 2B), indicating that the data met high-quality standards. Principal Component Analysis (PCA) of the overall samples showed that the metabolic profiles of the three groups were clearly separated, with minimal intra-group variability (Figure 2C), indicating metabolic differences between the thaumatin group and the control group. Fold change (FC) analysis (Figure 2E) and functional pathway difference analysis (Figure 2D) revealed that the thaumatin II group and the control group differed significantly only in lipid and defense-related amino acid pathways, with  $|\log_2 \text{FC}| < 0.1$  for soluble sugars, organic acids, and major flavor precursors, providing direct evidence of the safety of “direct-edible sugar substitute fruits.” Additionally, metabolites with relatively large fold differences included phenylbutyrylglutamine (BYGN03669,  $\log_2 \text{FC} = 22.1$ ) and isoleucine (BYCP0082,  $\log_2 \text{FC} = 19.928$ ), which may originate from the activation of plant defense signaling pathways triggered by thaumatin. The downregulation of polyunsaturated fatty acids may result from a burst of reactive oxygen species (ROS) induced by thaumatin, thereby reducing membrane fluidity and decreasing pathogen spread. In summary, the results indicate that virus-mediated transient transformation of the thaumatin gene does not affect the nutritional components of tomatoes but instead triggers systemic defense activation in tomato plants.

### 3.3. Transgenics and Regeneration

After stable transformation of explants with the same gene cassette via *Agrobacterium*, hygromycin-resistant calli were generated on the selection medium within 6–7 weeks, and roots differentiated from the calli after 10 weeks of culture (Figure 3A). Explants that were not successfully inoculated did not survive in the selection medium. The transformation experiment yielded 33 regenerated plant lines (designated GMOT-35S lines). All 33 transgenic tomato lines grew normally, with no observable differences in growth or morphological characteristics between transgenic and non-transgenic tomato plants. For further characterization, all hygromycin-resistant lines derived from independent transformation events were grown in a greenhouse. After seedling formation, leaf samples were collected for DNA extraction and PCR, confirming the presence of the hygromycin gene in 30 regenerated plants, except for GMOT-35S-18, GMOT-35S-19, and GMOT-35S-26 (Figure 3B).

### 3.4. Characterization of Transgenic Plant Thaumatin II

To detect thaumatin II, amplification primers derived from the thaumatin II gene sequence were designed. Total RNA was extracted from leaf and fruit samples and subjected to RT-PCR. A 351 bp Thaumatin II gene fragment was amplified from the cDNA of GMOT-35S-1, GMOT-35S-2, GMOT-35S-3, GMOT-35S-5, and GMOT-35S-7 transgenic plants (Figure 4A). Among these, GMOT-35S-1, GMOT-35S-2, and GMOT-35S-3 showed clear bands and were selected for Western blot analysis. The results showed a single band corresponding to thaumatin II with a molecular weight of approximately 26.5 kDa, in both leaf and fruit samples. No comparable immunoreactive band was observed in the protein samples of the untransformed control group plants (Figure 4B). This indicates that recombinant thaumatin II was correctly expressed and folded, existing mainly as a soluble protein in tomato leaves and fruits.

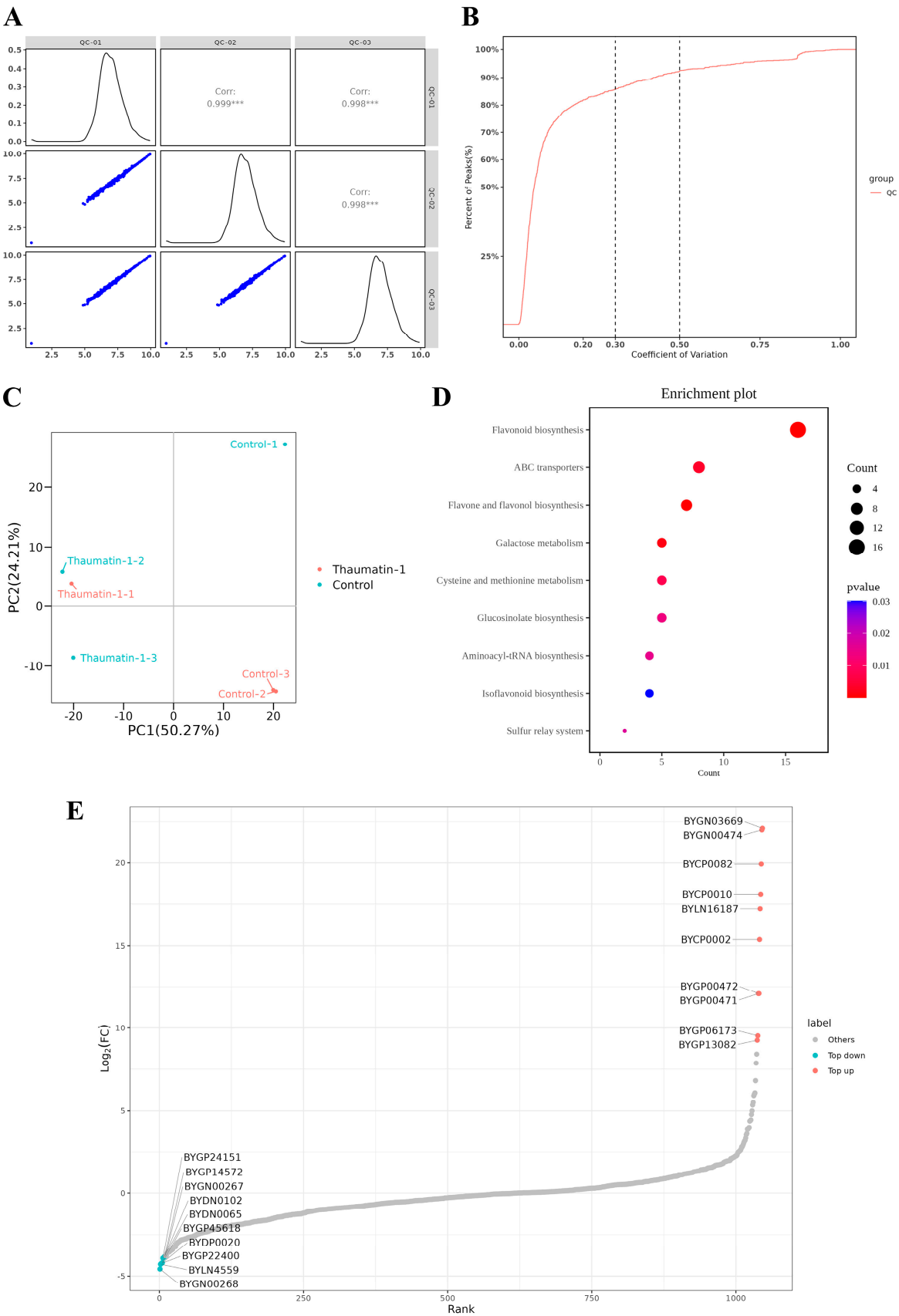


**Figure 2.** Metabolic analysis of virus-infected tomato plants. **(A).** Pearson correlation analysis of quality control (QC) samples. The squares in the lower left corner of the diagonal are scatter plots of

the correlation between corresponding QC samples, with horizontal and vertical coordinates representing metabolite content ( $\log_{10}$  normalized); each point corresponds to a single metabolite; the squares in the upper right corner of the diagonal represent Pearson correlation coefficients of the corresponding QC samples. Asterisks indicate statistical significance: \*\*\*  $p < 0.001$ . (B). Ratio of the standard deviation of the original data source to the mean of the original data source (coefficient of variation, CV) for QC samples. The empirical cumulative distribution function (ECDF) was used to analyze the frequency of occurrence of CV values for substances less than the reference value. The CV values corresponding to the two reference lines perpendicular to the X-axis are 0.3 and 0.5. Metabolites with CV values greater than 30% in the QC group were removed before subsequent differential analysis. (C). Principal component analysis (PCA) of mass spectrometry data for each sample group and QC samples. PC1 and PC2 represent the first and second principal components, respectively, and the percentages on the axes indicate their explanatory power for the dataset. (D). Bubble plot for functional pathway difference analysis. The x-axis represents the rich factor, which is the ratio of differentially expressed metabolites in a pathway to the total detected and annotated metabolites in that pathway; the y-axis represents the pathway name; point color reflects  $p$ -value significance, with redder points indicating more significant enrichment; the point size represents the number of differentially expressed metabolites. (E). Dynamic distribution map of differences in metabolite content between the experimental Group and the control group. The plot shows the top 20 metabolites, ranked by their FC value. The x-axis represents the cumulative number of substances arranged in ascending order of fold change, and the y-axis represents the logarithm of the fold change with base 2. Each point represents a metabolite, with red points representing upregulated metabolites and blue points representing downregulated metabolites. All top-ranked metabolites may either be upregulated or downregulated.

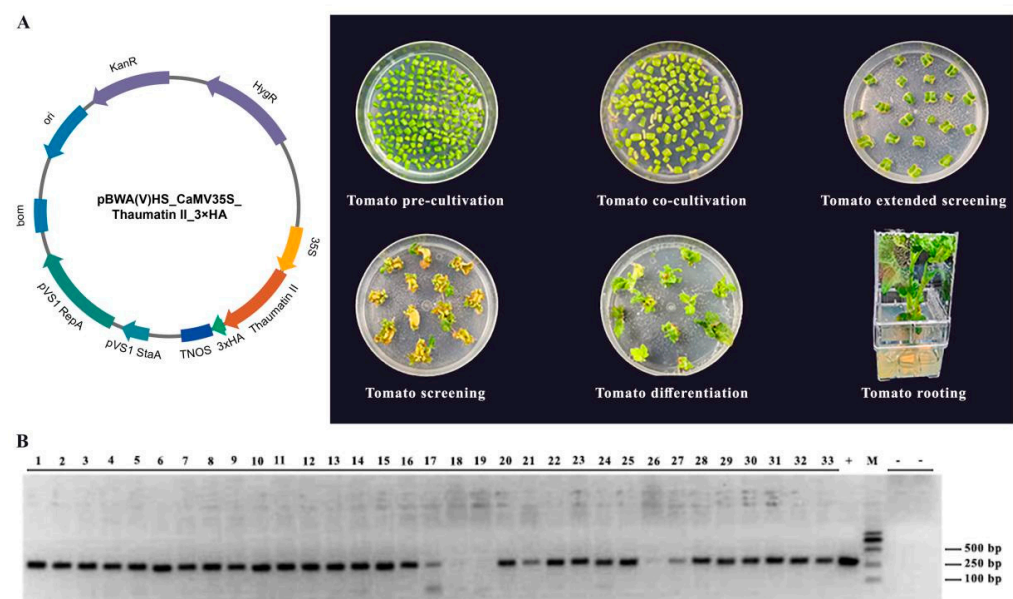


**Figure 3.** Establishment of transgenic tomato plants. (A). Construction of the expression vector and process from pre-cultivation of tomato seedlings to differentiation and rooting after infection. (B). PCR screening of leaves from the GMOT-35S transgenic tomato line using hygromycin as the target sequence.

To estimate the yield, the collected samples were weighed during the sampling process. The thaumatin II protein content was then determined using a thaumatin II-specific ELISA kit (Hnybio, Shanghai, China, Catalog No. HB212-SH), following a procedure that included enzyme conjugation, incubation, and washing steps. Through this we could estimate the production level, and the results indicated an approximate yield of 0.252 mg/kg. We note the yield difference compared to that of Firsov et al. [41] (50 mg/kg), which primarily stems from the difference in research objectives. Their aim was to maximize protein production,

hence the use of a large-fruited, high-biomass cultivar ('Yalf') and a process optimized for protein extraction and purification. In contrast, we selected 'MicroTom' for its short growth cycle and well-characterized flavor background, making it highly suitable for rapid screening and functional characterization of flavor traits. The extraction buffer we employed was primarily designed for organelle studies, which may also have limited the complete release and quantification of the target protein.

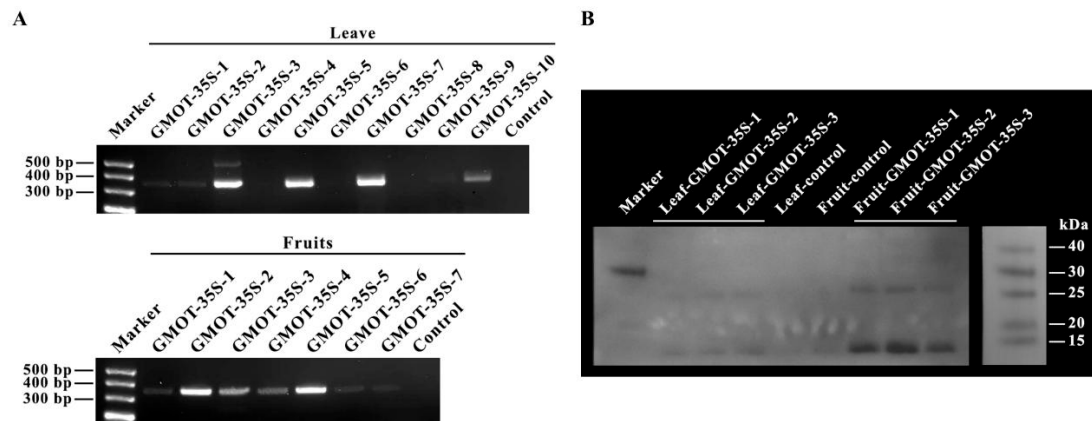


**Figure 4.** Molecular verification of transgenic tomato thaumatin II. (A). RT-PCR results for RNA from leaves (**upper**) and fruits (**lower**) of GMOT-35S transgenic tomato plants. (B). Western blot analysis of protein extracts from leaves and fruits of GMOT-35S transgenic tomato plants.

### 3.5. Interaction Analysis of Recombinant Thaumatin II-T1RS2

To explore the sweet taste characteristics of recombinant thaumatin II in a biological context, protein–protein interaction (PPI) predictions between thaumatin II and TAS1R2 and TAS1R3 were performed using AlphaFold3 [46] and HDOCK, respectively, and the key binding sites on thaumatin II that interact with the sweet taste receptor heterodimer were obtained (Figures 5 and 6). The results showed that in the AlphaFold model, the binding checkpoints of thaumatin II with TAS1R2 were ASP-21, ARG-79, TYR-99, LYS-163, GLY-162, and PRO-188, and the binding checkpoints with TAS1R3 were ARG-67, LYS-139, and PRO-141. In the HDOCK model, the binding checkpoints of thaumatin II with TAS1R2 were concentrated on ARG-79, TYR-95, and THR-160, whereas binding with TAS1R3 was located at GLN-42, all within the central region of the receptor. By analyzing the binding tightness (Tables 2 and 3), we conclude that TAS1R2 and TAS1R3 participate in the perception of thaumatin II's sweet taste in the form of a heterodimer. Among these interactions, thaumatin II exhibits a larger contact area and binding strength with TAS1R2.

Previous studies have shown that TAS1R2 can form non-functional homodimers when co-expressed with TAS1R3 in tissue culture cells, introducing significant confusion into the particle analysis. Additionally, TAS1R1 and TAS1R3 are involved in the perception of umami, whereas TAS1R2 and TAS1R3 are responsible for the perception of sweetness, indicating the functional or auxiliary properties of TAS1R3 (Figure 6A). To explore the sweetness of recombinant thaumatin II, immunoprecipitation was performed using the human receptor protein TAS1R2 and purified thaumatin II protein samples (from GMOT-35S-2 fruit). The obtained samples were subjected to Western blot analysis, and a significant band was detected between the immunoprecipitation product and purified thaumatin II at 26.5 kDa. Concurrently, the product contained His-tagged TAS1R2 at 66 kDa (Figure 6B), indicating that heterologous expression of thaumatin II can bind to TAS1R2, further confirming its sweet taste properties.

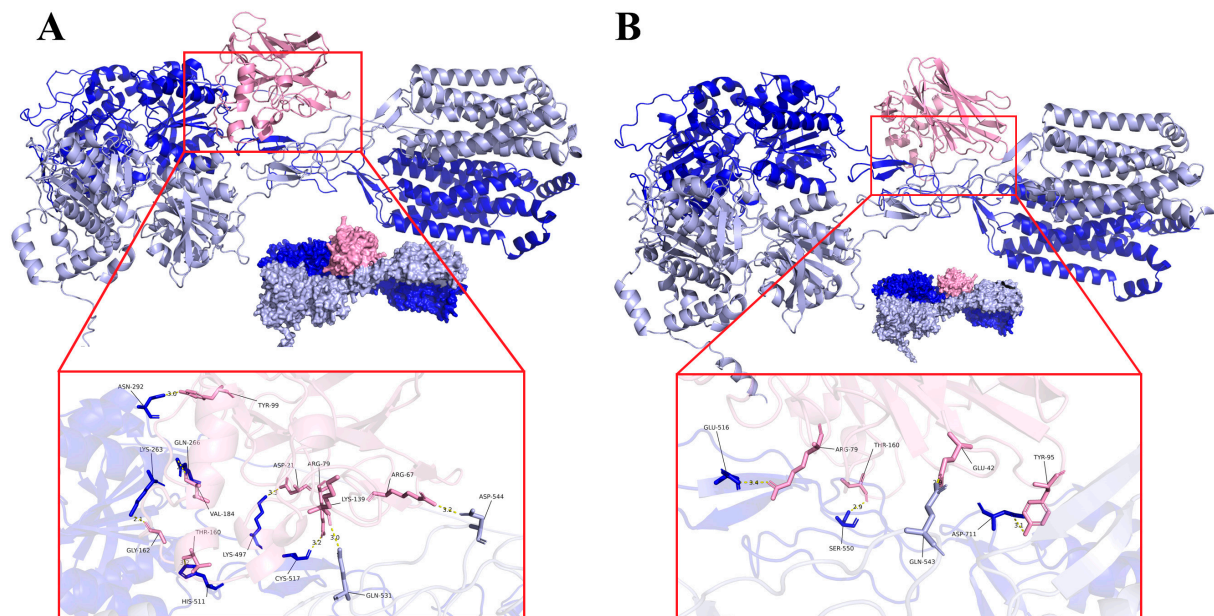


**Figure 5.** Protein–protein interaction predictions for thaumatin II with human sweet taste receptors and docking results (including the amino acid residues involved). (A). AlphaFold 3 predicted interface residues (key residues labeled). (B). HDock docking map and interface metrics. Interface areas and  $\Delta G$  values are shown in Tables 2 and 3. Blue: amino acid residues involved in T1R2 binding; light blue: residues involved in T1R3 binding; pink: residues involved in thaumatin binding.

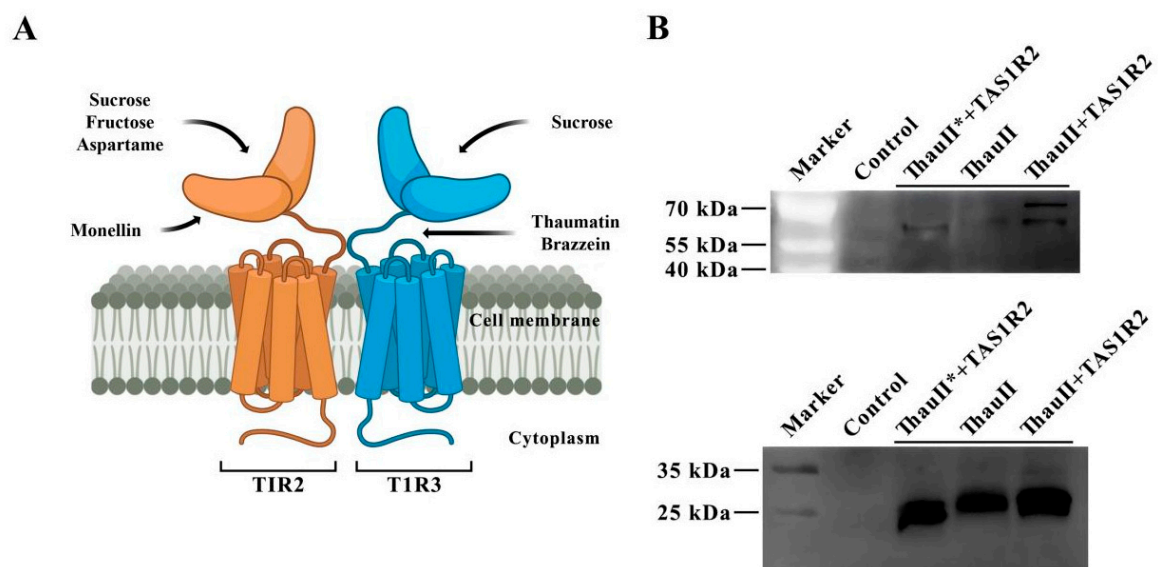


**Figure 6.** (A). Schematic diagram of sweet taste receptor binding with sweeteners. T1R2, type 1 taste receptor member 2; T1R3, type 1 taste receptor member 3 [47]. (B). Co-immunoprecipitation and Western blot analysis of human receptor protein TAS1R2 with purified thaumatin II protein samples. Lane 1: molecular weight marker; lane 2: protein extract from control tomato plant fruits; lane 3: positive control thaumatin II protein standard with TAS1R2; lane 4: purified thaumatin II protein from transgenic fruits; lane 5: TAS1R2 co-incubated with purified thaumatin II protein from transgenic fruits.

**Table 2.** Key amino acids related to docking and their binding strength with the protein, as predicted by AlphaFold.

| Thaumatococcus II | TAS1R2  | Distance (Å) | Interface Area (Å <sup>2</sup> ) | ΔiG (kcal/mol) |
|-------------------|---------|--------------|----------------------------------|----------------|
| SP-21             | LYS-497 | 3.3          | 584.3                            | −0.7           |
| ARG-79            | CYS-517 | 3.2          |                                  |                |
| TYR-99            | ASN-292 | 3.0          |                                  |                |
| LYS-163           | GLN-237 | 3.7          |                                  |                |
| GLY-162           | LYS-263 | 2.1          |                                  |                |
| PRO-188           | ASP-262 | 2.9          |                                  |                |
| Thaumatococcus II | TAS1R3  | Distance (Å) | Interface Area (Å <sup>2</sup> ) | ΔiG (kcal/mol) |
| ARG-67            | ASP-544 | 3.2          | 442.1                            | 0.1            |
| LYS-139           | GLN-531 | 3.0          |                                  |                |
| PRO-141           | BLN-531 | 3.0          |                                  |                |

Å(2) indicates the interface area; ΔiG indicates the solvation free energy gain upon interface formation.

**Table 3.** Key amino acids related to docking and their binding strength with the protein, as predicted by HDOCK.

| Thaumatococcus II | TAS1R2  | Distance (Å) | Interface Area (Å <sup>2</sup> ) | ΔiG (kcal/mol) |
|-------------------|---------|--------------|----------------------------------|----------------|
| ARG-79            | GLU-516 | 3.4          | 731.5                            | −0.1           |
| TYR-95            | ASP-711 | 3.1          |                                  |                |
| THR-160           | SER-550 | 2.9          |                                  |                |
| Thaumatococcus II | TAS1R3  | Distance (Å) | Interface Area (Å <sup>2</sup> ) | ΔiG (kcal/mol) |
| GLU-42            | GLN-543 | 3.2          | 56.0                             | 1.3            |

Å(2) indicates the interface area; ΔiG indicates the solvation free energy gain upon interface formation.

#### 4. Discussion

By integrating the transient expression system of the TRV viral vector with *Agrobacterium*-mediated stable genetic transformation, an efficient system for rapid validation and stable production was established. This approach enabled rapid functional assessment of the thaumatin II gene and preliminary optimization of production parameters. Ultimately, transgenic tomato lines (T-35S) stably expressing high levels of thaumatin II (approximately 26.5 kDa) in both the leaves and fruits were obtained. This streamlined production system eliminates the need for downstream purification steps, as thaumatin II can be directly obtained from edible fruit juice. These findings provide a strong technical foundation for scalable and controllable plant-based thaumatin II production, effectively overcoming the limitations of scarce natural resources and traditional microbial fermentation methods.

Ensuring that heterologous protein expression does not compromise the nutritional or flavor quality of the host crop is a prerequisite for developing edible sugar-substituting fruits. In this study, untargeted metabolomic analysis of 1236 fruit metabolites revealed that thaumatin II expression did not significantly alter the core nutritional profile of tomato fruits, including major sugars (glucose, fructose, and sucrose), organic acids (citric acid and malic acid), and most flavor-related compounds (e.g., phenylpropanoids and heterocyclic organic compounds). These results provide safety and quality data supporting the use of tomato fruits as direct carriers of thaumatin II, providing evidence for the feasibility of the “sugar-substituting fruit” concept. Notably, metabolomic profiling of transiently expressed lines also suggested that thaumatin II expression might enhance the plant’s immune defense response by increasing the levels of glutamine [48], isoleucine [49], and polyunsaturated fatty acids, implying potential stress resistance benefits. This aligns with previous findings that transgenic strawberry plants expressing thaumatin II exhibit resistance to *Botrytis*

*cinerea* [50]. Thus, metabolomic analysis may serve as a powerful tool for elucidating the disease resistance mechanisms of thaumatin II, warranting further investigation.

Considering the widespread use of sweeteners in daily life, the taste and safety of synthetic thaumatin II are critical. There is a growing market demand for high-quality, non-toxic sweeteners [51]. A lifecycle assessment of thaumatin highlighted the need to evaluate its contribution to sweetness, as its sensory properties differ from those of sugar (e.g., delayed onset of sweetness compared to sugar), and it is rarely used alone [52]. Previous studies have demonstrated that the binding of sweet proteins to TAS1R2 directly correlates with known sweetness perception mechanisms [53,54]. Molecular docking studies have shown that thaumatin II binds to the central region of the heterodimeric sweet taste receptors TAS1R2 and TAS1R3, specifically to the cysteine-rich domain (CRD), whereas sucrose, aspartame, and sucralose interact with the extracellular N-terminal domain (NTD) of TAS1R1 [55]. This study identified key binding residues (Arg79 and Tyr99) and, for the first time, employed the human sweet taste receptor TAS1R2 for in vitro functional evaluation of plant-derived recombinant thaumatin II. Rather than relying solely on subjective sensory evaluations, our study adopted a receptor-based molecular approach, which provides direct and reproducible evidence of sweetness activity at the biological level. Co-immunoprecipitation experiments confirmed that thaumatin II purified from transgenic tomatoes specifically bound to TAS1R2. This objective biological validation provides reliable scientific evidence that recombinant thaumatin II exhibits sweetness activity comparable to that of the native protein, overcoming the limitations of human sensory tests and conventional sweetness evaluation methods (e.g., concentration-based assessments) [56]. Thus, this approach offers a novel strategy for detecting sweetness. Nevertheless, we acknowledge that receptor binding cannot fully substitute for in vivo taste perception, and future work will integrate sensory panel evaluations to complement the molecular findings.

Notably, the present study primarily focused on the establishment and functional validation of the transformation system and the initial characterization of T0 plants. While we have successfully demonstrated proper expression and correct processing of thaumatin II in T0 plants through molecular and functional assays, the analysis of transgene stability and inheritance patterns in subsequent generations remains an essential next step. Additionally, untargeted metabolomics, while comprehensive, may overlook subtle relevant metabolic changes and more definite evidence of causal relationships between the transgene and observed metabolic shifts is needed. Future work will involve cultivating the T1 progeny from the selected homozygous T0 lines to perform segregation analysis and assess the inheritance of the transgene. This will be crucial for confirming the development of a true-breeding, stable transgenic line suitable for scalable agricultural applications. Furthermore, targeted metabolomics is needed to further characterize the system.

The tomato-based plant expression system developed in this study represents a paradigm shift in thaumatin II production. Its core advantage lies in enabling consumers to ingest functional thaumatin II directly by eating tomato fruits, bypassing the costly, complex, and protein-loss-prone downstream purification processes associated with traditional extraction and microbial fermentation methods. This significantly reduces production costs and minimizes chemical use and waste generation. The cherry tomato cultivar MicroTom, with its compact fruit size, short growth cycle, and well-established genetic manipulation system, is an ideal chassis. This strategy can be extended to other Solanaceae crops (e.g., common tomatoes and peppers) as well as berry fruits, offering diverse “sweet yet low-/no-sugar” dietary options for diabetics, obese individuals, and health-conscious consumers. This approach transcends the current limitation of adding purified sweet proteins only to processed foods, enabling comprehensive “sugar-free” dietary solutions.

## 5. Conclusions

Our results indicate that a TRV/*Agrobacterium* dual-route transformation approach can be effectively applied to MicroTom tomato for the production of the sweet protein thaumatin II. This system provides a new pathway for low-cost, sustainable thaumatin II production and represents an advancement in healthy sweetener applications through its “sugar-substituting fruit” format. This approach demonstrates promising potential for applications in the health food industry. Future studies will be necessary to evaluate long-term transgene stability over multiple generations and to assess agronomic performance through field trials under practical growing conditions. Further research may also explore tissue-specific expression strategies to enhance production efficiency and reduce metabolic burden.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/horticulturae11111284/s1>. Figure S1: Molecular characterization of recombinant thaumatin II in tomato. (A) Colony PCR verification of *Agrobacterium tumefaciens* GV1301 transformed with the pTRV2\_CaMV35S\_ThaumatinII\_Flag plasmid. Lanes show independent colonies with PCR products confirming successful plasmid construction. (B) RT-PCR analysis of total RNA from leaves of tomato TRVT-35S line after transient transfection, demonstrating transcriptional expression of the transgene. (C) Western blot analysis for detecting the Thaumatin-3×Flag fusion protein in total protein extracts from leaves of the tomato TRVT-35S line, confirming protein expression and size. Figure S2: Full-length gel images for colony PCR. Uncropped gel images related to the colony PCR results presented in Figure S1A. Figure S3: Full-length gel and blot images for RT-PCR and Western blot. Uncropped gel images for RT-PCR (related to Figure S1B) and full membrane exposures for Western blot (related to Figure S1C). Sequence S1: Complete plasmid sequence of pTRV2\_CaMV35S\_ThaumatinII\_Flag. The full nucleotide sequence of the binary vector constructed and used in this study for both transient expression and stable transformation. Detailed experimental procedures and raw data are provided here. The original dataset underlying the displayed figures can be downloaded here.

**Author Contributions:** Conceptualization, J.C., B.M. and W.C.; methodology, J.C., Q.L., R.C. and X.L.; validation, J.C., Q.L. and S.G.; formal analysis, J.C. and R.C.; investigation, J.C., Q.L., S.G., Y.L. (Yitong Li), K.L. and G.A.; resources, X.L. and B.M.; data curation, J.C., Y.L. (Yitong Li), Y.L. (Yuanrun Liu) and Z.H.; writing—original draft preparation, J.C. and Q.L.; writing—review and editing, S.G., Y.L. (Yitong Li), R.C. and W.C.; visualization, S.G.; supervision, B.M., X.L. and W.C.; project administration, B.M. and W.C.; funding acquisition, B.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by Natural Science Foundation of China (Grant No. 32270595) to Beixin Mo; the SZU 2035 Excellence Research Program (Grant No. 2023B001) of Shenzhen University to Beixin Mo; the Undergraduate Academic Competition Project of Shenzhen University (Grant No. 803-0000341111) to Weizhao Chen and the Special Funds for the Cultivation of Guangdong College Students’ Scientific and Technological Innovation (“Climbing Program” Special Funds; Grant No. pdjh2025bc180) to Jiachun Chen.

**Data Availability Statement:** The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding authors.

**Acknowledgments:** We gratefully acknowledge the Key Laboratory of Epigenetics of Guangdong Province and its research staff for their expert guidance.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## References

1. Arnone, D.; Chabot, C.; Heba, A.C.; Kökten, T.; Caron, B.; Hansmann, F.; Dreumont, N.; Ananthakrishnan, A.N.; Quilliot, D.; Peyrin-Biroulet, L. Sugars and Gastrointestinal Health. *Clin. Gastroenterol. Hepatol.* **2022**, *20*, 1912–1924.e7. [\[CrossRef\]](#)
2. Chew, N.W.S.; Ng, C.H.; Tan, D.J.H.; Kong, G.; Lin, C.X.; Chin, Y.H.; Lim, W.H.; Huang, D.Q.; Quek, J.; Fu, C.E.; et al. The global burden of metabolic disease: Data from 2000 to 2019. *Cell Metab.* **2023**, *35*, 414–428.e3. [\[CrossRef\]](#) [\[PubMed\]](#)
3. World Health Organization. *Guideline: Sugars Intake for Adults and Children*; World Health Organization: Geneva, Switzerland, 2015.
4. Artificial Sweeteners. Available online: <https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/artificial-sweeteners> (accessed on 1 August 2025).
5. Sun, Y.Y.; Liang, J.; Zhang, Z.R.; Sun, D.J.; Li, H.; Chen, L.X. Extraction, physicochemical properties, bioactivities and application of natural sweeteners: A review. *Food Chem.* **2024**, *457*, 140103. [\[CrossRef\]](#)
6. Kant, R. Sweet proteins—Potential replacement for artificial low calorie sweeteners. *Nutr. J.* **2005**, *4*, 5. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Farag, M.A.; Rezk, M.M.; Elashal, M.H.; El-Araby, M.; Khalifa, S.A.M.; El-Seedi, H.R. An updated multifaceted overview of sweet proteins and dipeptides as sugar substitutes; the chemistry, health benefits, gut interactions, and safety. *Food Res. Int.* **2022**, *162*, 111853. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Assadi-Porter, F.M.; Aceti, D.J.; Cheng, H.; Markley, J.L. Efficient production of recombinant brazzein, a small, heat-stable, sweet-tasting protein of plant origin. *Arch. Biochem. Biophys.* **2000**, *376*, 252–258. [\[CrossRef\]](#)
9. Chen, Z.J.; Cai, H.; Lu, F.P.; Du, L.X. High-level expression of a synthetic gene encoding a sweet protein, monellin, in *Escherichia coli*. *Biotechnol. Lett.* **2005**, *27*, 1745–1749. [\[CrossRef\]](#)
10. Masuda, T.; Okubo, K.; Baba, S.; Suzuki, M.; Tani, F.; Yamasaki, M.; Mikami, B. Structure of thaumatin under acidic conditions: Structural insight into the conformations in lysine residues responsible for maintaining the sweetness after heat-treatment. *Food Chem.* **2022**, *389*, 132996. [\[CrossRef\]](#)
11. Kaneko, R.; Kitabatake, N. Sweetness of sweet protein thaumatin is more thermoresistant under acid conditions than under neutral or alkaline conditions. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 409–413. [\[CrossRef\]](#)
12. Teratogenesis. Available online: <https://www.sciencedirect.com/topics/pharmacology-toxicology-and-pharmaceutical-science/teratogenesis> (accessed on 28 July 2025).
13. Kelada, K.D.; Tusé, D.; Gleba, Y.; McDonald, K.A.; Nandi, S. Process Simulation and Techno-Economic Analysis of Large-Scale Bioproduction of Sweet Protein Thaumatin II. *Foods* **2021**, *10*, 838. [\[CrossRef\]](#)
14. Zemanek, E.C.; Wasserman, B.P. Issues and Advances in the Use of Transgenic Organisms for the Production of Thaumatin, the Intensely Sweet Protein from *Thaumatococcus-Danielli*. *Crit. Rev. Food Sci. Nutr.* **1995**, *35*, 455–466. [\[CrossRef\]](#)
15. Kielkiewicz, M.; Gajc-Wolska, J.; Ślusarz, S.; Szwacka, M. Growth, development and yield of transgenic 35S-thaumatin II-expressing cucumber plants—Open field evaluation. *Sci. Hortic.* **2012**, *143*, 82–91. [\[CrossRef\]](#)
16. Edens, L.; Heslinga, L.; Klok, R.; Ledebor, A.M.; Maat, J.; Toonen, M.Y.; Visser, C.; Verrips, C.T. Cloning of cDNA encoding the sweet-tasting plant protein thaumatin and its expression in *Escherichia coli*. *Gene* **1982**, *18*, 1–12. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Daniell, S.; Mellits, K.H.; Faus, I.; Connerton, I. Refolding the sweet-tasting protein thaumatin II from insoluble inclusion bodies synthesised in *Escherichia coli*. *Food Chem.* **2000**, *71*, 105–110. [\[CrossRef\]](#)
18. Illingworth, C.; Larson, G.; Hellekant, G. Secretion of the sweet-tasting plant protein thaumatin by *Bacillus subtilis*. *Biotechnol. Lett.* **1988**, *10*, 587–592. [\[CrossRef\]](#)
19. Lombrana, M.; Moralejo, F.J.; Pinto, R.; Martín, J.F. Modulation of *Aspergillus awamori* thaumatin secretion by modification of bipA gene expression. *Appl. Environ. Microbiol.* **2004**, *70*, 5145–5152. [\[CrossRef\]](#)
20. Jahic, M.; Wallberg, F.; Bollok, M.; Garcia, P.; Enfors, S.-O. Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. *Microb. Cell Factories* **2003**, *2*, 6. [\[CrossRef\]](#)
21. Guo, G.; Li, M.-J.; Lai, J.-L.; Du, Z.-Y.; Liao, Q.-S. Development of tobacco rattle virus-based platform for dual heterologous gene expression and CRISPR/Cas reagent delivery. *Plant Sci.* **2022**, *325*, 111491. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Vanderwel, H.; Loeve, K. Isolation and Characterization of Thaumatin I and II, Sweet-Tasting Proteins from *Thaumatococcus-Daniellii* Benth. *Eur. J. Biochem.* **1972**, *31*, 221–225. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Ota, M.; Sawa, A.; Nio, N.; Ariyoshi, Y. Enzymatic ligation for synthesis of single-chain analogue of monellin by transglutaminase. *Biopolymers* **1999**, *50*, 193–200. [\[CrossRef\]](#)
24. Ogata, C.; Hatada, M.; Tomlinson, G.; Shin, W.C.; Kim, S.H. Crystal-Structure of the Intensely Sweet Protein Monellin. *Nature* **1987**, *328*, 739–742. [\[CrossRef\]](#)
25. Morris, J.A.; Martenson, R.; Deibler, G.; Cagan, R.H. Characterization of Monellin, A Protein That Tastes Sweet. *J. Biol. Chem.* **1973**, *248*, 534–539. [\[CrossRef\]](#)
26. Kim, S.H.; Kang, C.H.; Kim, R.; Cho, J.M.; Lee, Y.B.; Lee, T.K. Redesigning a Sweet Protein—Increased Stability and Renaturability. *Protein Eng.* **1989**, *2*, 571–575. [\[CrossRef\]](#)

27. Lamphear, B.J.; Barker, D.K.; Brooks, C.A.; Delaney, D.E.; Lane, J.R.; Beifuss, K.; Love, R.; Thompson, K.; Mayor, J.; Clough, R.; et al. Expression of the sweet protein brazzein in maize for production of a new commercial sweetener. *Plant Biotechnol. J.* **2005**, *3*, 103–114. [\[CrossRef\]](#)
28. Zeece, M. Chapter six—Flavors. In *Introduction to the Chemistry of Food*; Zeece, M., Ed.; Academic Press: London, UK, 2020; pp. 213–250.
29. Izawa, H.; Ota, M.; Kohmura, M.; Ariyoshi, Y. Synthesis and characterization of the sweet protein brazzein. *Biopolymers* **1996**, *39*, 95–101. [\[CrossRef\]](#)
30. Ming, D.; Hellekant, G. Brazzein, a New High-Potency Thermostable Sweet Protein from *Pentadiplandra-Brazzeana*-B. *Febs Lett.* **1994**, *355*, 106–108. [\[CrossRef\]](#)
31. Bahadur, B.; Pal, A.K. Natural sweetening plants: A global review. *LS Int. J. Life Sci.* **2020**, *9*, 134–157. [\[CrossRef\]](#)
32. Tafazoli, S.; Vo, T.D.; Roberts, A.; Rodriguez, C.; Viñas, R.; Madonna, M.E.; Chiang, Y.-H.; Noronha, J.W.; Holguin, J.C.; Ryder, J.A.; et al. Safety assessment of miraculin using in silico and in vitro digestibility analyses. *Food Chem. Toxicol.* **2019**, *133*, 110762. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Yamashita, H.; Theerasilp, S.; Aiuchi, T.; Nakaya, K.; Nakamura, Y.; Kurihara, Y. Purification and complete amino acid sequence of a new type of sweet protein taste-modifying activity, curculin. *J. Biol. Chem.* **1990**, *265*, 15770–15775. [\[CrossRef\]](#)
34. Yamashita, H.; Akabane, T.; Kurihara, Y. Activity and stability of a new sweet protein with taste-modifying action, curculin. *Chem. Senses* **1995**, *20*, 239–243. [\[CrossRef\]](#)
35. Liu, X.; Maeda, S.; Hu, Z.; Aiuchi, T.; Nakaya, K.; Kurihara, Y. Purification, complete amino acid sequence and structural characterization of the heat-stable sweet protein, mabinlin II. *Eur. J. Biochem.* **1993**, *211*, 281–287. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Nirasawa, S.; Nishino, T.; Katahira, M.; Uesugi, S.; Hu, Z.; Kurihara, Y. Structures of heat-stable and unstable homologues of the sweet protein mabinlin. The difference in the heat stability is due to replacement of a single amino acid residue. *Eur. J. Biochem.* **1994**, *223*, 989–995. [\[CrossRef\]](#)
37. Nirasawa, S.; Liu, X.; Nishino, T.; Kurihara, Y. Disulfide bridge structure of the heat-stable sweet protein mabinlin II. *Biochim. Biophys. Acta (BBA)-Protein Struct. Mol. Enzymol.* **1993**, *1202*, 277–280. [\[CrossRef\]](#)
38. Wel, H.v.d.; Larson, G.; Hladik, A.; Hladik, C.M.; Hellekant, G.; Glaser, D. Isolation and characterization of pentadin, the sweet principle of *Pentadiplandra brazzeana* Baillon. *Chem. Senses* **1989**, *14*, 75–79. [\[CrossRef\]](#)
39. Faus, I. Recent developments in the characterization and biotechnological production of sweet-tasting proteins. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 145–151. [\[CrossRef\]](#)
40. Kashani-Amin, E.; Faraji, H.; Nouriyengejeh, S.; Ebrahim-Habibi, A. Structure-sweetness relationship of sweet proteins: A systematic review on “sweet protein” studies as a sub-group of “sweetener” investigations. *Mosc. Univ. Biol. Sci. Bull.* **2021**, *76*, 175–190. [\[CrossRef\]](#)
41. Firsov, A.; Shaloiko, L.; Kozlov, O.; Vinokurov, L.; Vainstein, A.; Dolgov, S. Purification and characterization of recombinant supersweet protein thaumatin II from tomato fruit. *Protein Expr. Purif.* **2016**, *123*, 1–5. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Wuhan MetWare Biotechnology Co., Ltd. Available online: <https://www.metware.cn/> (accessed on 25 June 2025).
43. ANPEL. Available online: <https://www.anpel.com.cn/> (accessed on 25 June 2025).
44. MS-DIAL Software. Available online: <https://lipidmaps.org/resources/tools/16?task=4.3> (accessed on 25 July 2025).
45. The R Project for Statistical Computing. Available online: <https://www.r-project.org/> (accessed on 30 July 2025).
46. Abramson, J.; Adler, J.; Dunger, J.; Evans, R.; Green, T.; Pritzel, A.; Ronneberger, O.; Willmore, L.; Ballard, A.J.; Bambrick, J. Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **2024**, *630*, 493–500. [\[CrossRef\]](#) [\[PubMed\]](#)
47. DuBois, G.E. Molecular mechanism of sweetness sensation. *Physiol. Behav.* **2016**, *164*, 453–463. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Yao, X.; Sui, X.; Zhang, Y. Amino Acid Metabolism and Transporters in Plant–Pathogen Interactions: Mechanisms and Implications. *Plant Cell Environ.* **2025**, *48*, 6086–6098. [\[CrossRef\]](#)
49. Bauer, S.; Mekonnen, D.W.; Geist, B.; Lange, B.; Ghirardo, A.; Zhang, W.; Schäffner, A.R. The isoleucic acid triad: Distinct impacts on plant defense, root growth, and formation of reactive oxygen species. *J. Exp. Bot.* **2020**, *71*, 4258–4270. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Schestibratov, K.A.; Dolgov, S.V. Transgenic strawberry plants expressing a thaumatin II gene demonstrate enhanced resistance to *Botrytis cinerea*. *Sci. Hortic.* **2005**, *106*, 177–189. [\[CrossRef\]](#)
51. Kawakami, C.A.; Selani, M.M.; Saldaña, E.; Pimentel-Filho, N.d.J.; Fontenele Domingues, M.A. Sensory dynamic profile and consumer acceptance of short-dough biscuits with reduced sucrose and thaumatin addition. *Food Res. Int.* **2025**, *200*, 115524. [\[CrossRef\]](#)
52. Suckling, J.; Morse, S.; Murphy, R.; Astley, S.; Boy, C.; Halford, J.C.G.; Harrold, J.A.; Le-Bail, A.; Koukouna, E.; Musinovic, H.; et al. Life cycle assessment of the sweetness enhancer thaumatin (E957) produced from *Thaumatococcus daniellii* fruit foraged from West Africa: The SWEET project. *J. Clean. Prod.* **2023**, *411*, 137226. [\[CrossRef\]](#)
53. Pallante, L.; Malavolta, M.; Grasso, G.; Korfiati, A.; Mavroudi, S.; Mavkov, B.; Kalogeras, A.; Alexakos, C.; Martos, V.; Amoroso, D.; et al. On the human taste perception: Molecular-level understanding empowered by computational methods. *Trends Food Sci. Technol.* **2021**, *116*, 445–459. [\[CrossRef\]](#)

54. Shi, Z.; Xu, W.; Wu, L.; Yue, X.; Liu, S.; Ding, W.; Zhang, J.; Meng, B.; Zhao, L.; Liu, X.; et al. Structural and functional characterization of human sweet taste receptor. *Nature* **2025**, *645*, 801–808. [[CrossRef](#)]
55. Ohta, K.; Masuda, T.; Tani, F.; Kitabatake, N. The cysteine-rich domain of human T1R3 is necessary for the interaction between human T1R2–T1R3 sweet receptors and a sweet-tasting protein, thaumatin. *Biochem. Biophys. Res. Commun.* **2011**, *406*, 435–438. [[CrossRef](#)] [[PubMed](#)]
56. Chen, W.; Wu, W. Molecular basis and detection technologies of sweetness. *Food Res. Int.* **2025**, *219*, 116965. [[CrossRef](#)]

**Disclaimer/Publisher’s Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.