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RESEARCH

Improving Flavour and Quality of Tomatoes by Expression of Synthetic Gene Encoding Sweet Protein Monellin

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Abstract Monellin a sweet-tasting protein exists naturally as a heterodimer of two non-covalently linked subunits chain A and B, which loses its sweetness on denaturation. In this study, we validated the expression of a synthetic monellin gene encoding a single polypeptide chain covalently linking the two subunits under T_7 and fruit-ripening-specific promoters in *Escherichia coli* and tomato fruits, respectively. Purified recombinant monellin protein retained its sweet flavour at 70 °C and pH 2. We developed 15 transgenic T_0 tomato plants overexpressing monellin, which were devoid of any growth penalty or phenotypic abnormalities during greenhouse conditions. T-DNA integration and fruit-specific heterologous expression of monellin had occurred in these transgenic tomato lines. ELISA revealed that expression of monellin was 4.5 % of the total soluble fruit protein. Functional analyses of transgenic tomatoes of T_2-5 and T_2-14 lines revealed distinctly strong sweetness compared with wild type. Monellin a potential non-carbohydrate sweetener, if expressed in high amounts in fruits and vegetables, would enhance their flavour and quality.

Keywords *Escherichia coli* · Monellin · Protein profiling · Thermostable · Transcript · Transgenic tomatoes

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Introduction

Globally, millions of people are affected by obesity and related problems, including type II diabetes, due to the consumption of high caloric food, which primarily comprises different sugars and/or carbohydrates. Monellin a naturally sweet protein as comprehended sweet by human taste receptor [1] is derived from West African berries of *Dioscoreophyllum cumminsii* and may be particularly beneficial to individuals clinically challenged for sugar intakes. Adding monellin as a flavour in the food could solve the problem to some extent. Thus, monellin has received sizeable attention from the food and beverage industry [2]. Previously, protein chemists have characterized monellin [3–5] and deciphered that its native structure is crucial for its sweet taste [6, 7]. However, production of this low-calorie natural sweetener is limited due to restricted habitats and due to its instability at high temperatures and acidic pH, causing loss of sweet flavour [8]. Linking the two subunits of this heterodimer can increase the stability of monellin [5, 9]. Structural studies have revealed that monellin comprising a single polypeptide exhibits identical conformation and sweetness in flavour as the double-chain monellin but stabler than the native counterpart [10].

Previous attempts to synthesize monellin via recombinant technology proved to be futile due to its low expression levels in plants [5]. Tomato (*Solanum lycopersicon* L.) being an essential vegetable crop is consumed in several forms especially raw. In the present study, we have synthesized monellin with expressions at high levels in prokaryotic (*Escherichia coli*) and eukaryotic systems (tomato fruits) for stable production of monellin protein. Results revealed that the expression of recombinant monellin protein was functional at different temperatures and pH values

in bacterial system. We generated T_2 transgenic tomato plants expressing monellin exclusively in fruits driven by fruit-specific E8 promoter. Transgene integration and transcript profiles were validated by PCR and RT-PCR. Western analysis for expression of monellin protein was performed for selected T_2-transgenic lines. Our approach would serve as a strategy to enhance the flavour and quality of transgenic vegetables in a healthy manner.

Materials and Methods

Plant Material and Bacterial Strains

Tomato (Var. Pusa ruby) seeds were procured from the National Seed Corporation (IARI), PUSA complex in New Delhi. Bacterial strains of *Escherichia coli* [*E. coli*; DH-5α, BL21 (DE3)] and *Agrobacterium tumefaciens* (*A. tumefaciens*; LBA 4404) were used for cloning and plant transformation. DH5α was used as a host strain for cloning, while BL21 (DE3) strains were used for protein expression.

Expression and Purification of Monellin Recombinant Protein

Monellin gene sequence comprising 294 bp was synthesized from Geneart®, including restriction sites. The gene was cloned in pET-28a (+) protein expression vector (with an N-terminal histidine-tag) by standard restriction digestion and ligation procedures (Fig. 1) and transformed into BL-21 DE3 bacterial strain. Positive colonies were verified by colony PCR using forward Nde I—F 5′-ATACTACA TATGGGAGAGTGG-3′ and reverse Not I—R 5′-TAAT GGGGCCGC TTATGGTGG-3′ monellin primers. The reactions were denatured at 94 °C for 2 min; followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 40 s and a final extension at 72 °C for 10 min. Recombinant *E. coli* harbouring monellin gene was grown in LB medium supplemented with 50 μg/ml kanamycin at 37 °C. As the absorbance (A_{600}) of the culture reached a value of 0.6, isopropyl-1-thio-b-D-galactopyranoside (IPTG; 1 mM) was added. Induced culture was harvested after 4 h, and cells were lysed by sonication. Clarified bacterial lysate was fractionated on SDS polyacrylamide gels that were stained with Coomassie Blue to visualize protein bands. Recombinant monellin protein was purified to near homogeneity on a Ni–NTA column (Qiagen, Germany) chromatography following manufacturer's instructions. The purified protein was dialyzed against 50 mM NaH_2PO_4 (pH 8.0) for further analysis. Protein concentration was calculated by measuring absorbance at 280 nm. The concentrated protein was freeze dried, and the purified recombinant monellin protein's sweetness was assessed using taste test.

The purified monellin was dissolved at 100 μg/ml in buffers at different pH values, pH 2.5, 5.0, and 7.5. Each sample was incubated at 37, 50, 60, 70, and 80 °C for 15 min and cooled down to room temperature before testing.

Developing Monellin Polyclonal Antibodies

Monellin was expressed in *E. coli* for developing polyclonal antibodies against purified recombinant monellin protein. Purified recombinant monellin protein was

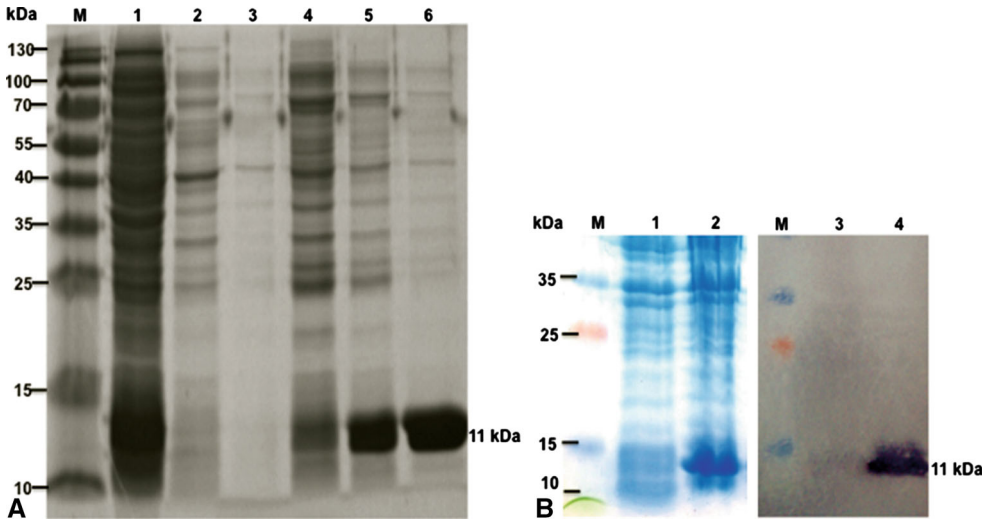

Fig. 1 **a** SDS-PAGE analysis of the recombinant single chain monellin expressed in *E. coli* BL21 (DE3) and its product of purification. Samples are mixed with SDS loading buffer and resolved in 15 % polyacrylamide gel. *Lane 1* supernatant, *Lane 2* pellet, *Lane 3–5* eluted fractions, *Lane 6* purified protein, *Lane M* protein marker. **b** Western blot analysis of recombinant single-chain monellin expressed in *E. coli* after purification. *Lane 1* uninduced and *Lane 2* induced fractions in PAGE; *Lane 3* and *Lane 4* uninduced and induced purified fractions of monellin protein in blot; *Lane M* prestained marker

emulsified with equal volume of Freund's complete adjuvant (Sigma Chemical Co., USA). Emulsified sample was injected subcutaneously into a New Zealand white rabbit. Rabbit was given two booster injections after a month's interval with recombinant monellin protein emulsified with Freund's incomplete adjuvant. Blood was collected after 2 weeks of second booster and serum was purified.

Binary Vector Construction, Tomato Transformation and Regeneration of T_0 Monellin Transgenic Plants

Expression cassette (E8: monellin: polyA) that comprised monellin gene (Acc # JQ282905.1) driven by a tomato fruit-specific promoter (Acc: AF515784.1) was cloned in multiple cloning sites of the entry vector-1 (EV-1) and transferred to pMDC100 vector by site-specific homologous recombination. The recombinant plasmid harbouring the monellin cassette was mobilized into *A. tumefaciens* for tomato transformation. Two-day-old precultured cotyledonary explants (var. Pusa Ruby) on MS medium containing zeatin (0.5 mg/l) and acetosyringone (100 µM) were co-cultivated with the recombinant *A. tumefaciens* for 2 days. These infected explants were selected on kanamycin (75 mg/l) containing MS medium (B5 vitamins) with zeatin (1 mg/l), IAA (0.1 mg/l), carbenicillin (250 mg/l) and cefotaxime (250 mg/l) for 30 days. Regenerated T_0 generation shoots were transferred to MS (B5 vitamins), containing zeatin (0.5 mg/l), kanamycin (50 mg/l), carbenicillin (250 mg/l) and cefotaxime (250 mg/l) for elongation for 10 days. Finally, elongated shoots were transferred to half-MS (2.2 g/l of Duchefa's ready-to-use MS powder) rooting medium with kanamycin (50 mg/l). The plantlets after hardening were transferred to greenhouse maintained under controlled conditions for setting of seeds. The T_1 seeds from 15 putative T_0 lines were harvested, sterilized and germinated on half-MS medium containing kanamycin (50 mg/l) (Supplementary Fig. 1).

Evaluation of Transgene Integration by PCR

Representative T_1 progeny of five transgenic tomato lines that showed vigorous growth on MS medium supplemented with kanamycin (50 mg/l) were chosen for PCR analysis. Genomic DNA was isolated from T_1 transgenic tomato lines (4, 5, 7, 8 and 14) and control plants using CTAB buffer for PCR detection of NPTII and monellin integration. PCR was carried out using the following primers (NPTII F 5′-GGCTATTCGGCTATGACTGG-3′, NPTII R 5′-CAT GTGTCACGACGAGATCC-3′; Monellin-F 5′-ATA CTA CAT ATG GGA GAG TGG-3′, Monellin-R 5′-TAA TGG GGC CGC TTA TGG TGG-3′) in a PCR mixture (50 µl) containing template gDNA (200 ng), primers (150 ng), buffer (19), dNTPs (0.2 mM) and 2.5 U of Taq DNA polymerase. The PCR conditions were 94 °C, 30 s; 56 °C, 30 s and 72 °C, 40 s for 30 cycles.

RNA Extraction and RT-PCR Analysis

Total RNA was extracted from WT and transgenic tomatoes of the selected transgenic lines (T_2-5 and T_2-14) using Trizol method (Invitrogen). cDNA synthesis was carried out using the Superscript first-strand synthesis system (Invitrogen) with 200 ng total RNA. For each PCR amplification, 1 µl cDNA was used in a 20 µl reaction mixture. PCR was run for 30 cycles for tubulin and monellin genes. Optimization of the number of PCR cycles was achieved by testing the linear amplification with serially diluted cDNAs. We used the following primer pairs for amplification of tubulin and monellin, respectively; tubulin forward 5′-ATTCCAGGTTTGCCA CTCAC-3′ and reverse 5′-CACAGCCAATTTCCTCAG GT-3′; monellin forward 5′-ATA CTA CAT ATG GGA GAG TGG-3′ and reverse 5′-TAA TGG GGC CGC TTA TGG TGG-3′. PCR products were resolved on a 0.8 % Tris–acetate EDTA agarose gel containing ethidium bromide.

Protein Extraction and Immunoblot Analysis

Total soluble fruit proteins were extracted from ripened fruits of transgenic and wild-type lines. Proteins were fractionated on a 15 % SDS-PAGE and electroblotted onto a nitrocellulose membrane (Hybond-C membrane; Amersham Biosciences) according to Kaul et al. [11]. Detection of monellin polypeptide by anti-monellin polyclonal antibodies (1:5,000 dilutions) was carried out according to Krishnan and Natrajan [12]. The levels of monellin protein in transgenic tomato fruits were determined using quantitative ELISA analysis. The ELISA plates (Costar EIA/RIA 1 × 8 stripe well plate, USA) were read on Versa Max ELISA microplate reader (Molecular Devices, USA). Monellin protein fraction of the total soluble fruit protein was estimated by comparingwith standard concentrations of commercial monellin (Sigma).

Sensory Analysis of Sweet Protein in Transgenic Tomatoes

Sweetness assay (blind test) was performed [5] with modifications after 10 days of fruit ripening. For this assay, ripened fruits were used from control and transgenic seedless sections of fruit pericarp (1 g). Five test panellists were asked to taste fruit samples and to describe the fruit taste. Two sample fruits of T_2-5 and T_2-14 were given to the panellists.

Results

Purification and Western Blot Analysis Recombinant Monellin Protein from *E. coli*

In order to obtain large amounts of highly purified monellin protein, we have utilized the heterologous expression system to overexpress recombinant monellin protein in *E. coli*. Majority of the expressed recombinant protein obtained after IPTG induction was partitioned into the soluble fraction of *E. coli* lysate. Recombinant monellin protein was purified to near homogeneity from clarified lysate using Ni–NTA column chromatography. Molecular mass of recombinant monellin polypeptide on a SDS-PAGE (Fig. 1a) and western blot (Fig. 1b) was approximately 11 kDa. The purified monellin retained its sweetness even after incubation for 15 min at a range of temperature and pH i.e., 50–70 °C and at pH 2.0–7.5.

Construct Designing for Expression of Synthetic Monellin, Tomato Transformation, Inheritance of Transgenes and Molecular Characterization of Transgenic Lines

Plant transformation construct comprised the complete coding sequence of the synthetic monellin gene that was codon optimized for expression in tomato. The construct was designed as a transcriptional fusion between the ethylene-regulated fruit-specific E8 promoter from tomato and the coding sequence of monellin (Fig. 2a). Tomato cotyledons were transformed by co-cultivation with *Agrobacterium* culture harbouring the desired construct (E8: monellin) (Fig. 2a), according to the protocol as described in "Materials and Methods" section. Transgenic plants were regenerated from kanamycin-resistant shoots. Out of 100 co-cultivated cotyledons, 15 independent kanamycin-resistant T_0 plants were transferred to pots with soil in greenhouse under controlled conditions (Supplementary Fig. 1). The regenerated 15 putative T_0 transgenic plants were selfed individually, and T_1 seeds were harvested for further analysis. These seeds germinated and developed into T_1 seedlings on MS medium supplemented with kanamycin (50 mg/l) were used for PCR analysis. Approximately 85 % of the T_1 seeds germinated from each T_0 line. Amongst these 15 transformed lines that qualified the germination test and grew vigorously on kanamycin medium, five lines were chosen for further molecular analysis. PCR analysis of these five T_1 lines showed the expected amplification of 0.6 kb DNA fragment (Fig. 2b) corresponding to the integrated *NPTII* selection marker gene and 0.294 kb DNA fragment (Fig. 2c) pertaining to the integrated monellin gene. No such amplification was noticed in wild-type plants. The progeny of selected transgenic lines (T_1-5 and T_1-14) were grown for 2 weeks in the greenhouse for DNA isolation from individual seedlings for PCR amplification of E8: monellin cassette. These plants were scored for PCR-positive and -negative amplifications. In case of T_1-5 line, out of 35 plants 25 were PCR positive for monellin, whereas for T_1-14 out of 50 plants, 38 plants showed amplification for monellin, whereas 12 plants showed no amplification indicating that the segregation ratio was very near to single locus integration. Similarly, the other three transgenic lines namely, T_2-5, T_2-7 and T_2-8 were scored for positive and negative amplifications, and it was noted that 23 out of 30 plants, 26 out of 35 plants and 22 out of 28 plants showed positive amplifications, respectively, for monellin gene.

Functional Analysis of Transgenic Plants

Heterologous expression of monellin under the fruit-specific promoter (E8) did not show any phenotypic abnormalities either vegetative or related to fruit growth parameters in transgenic lines. RT-PCR was performed to examine monellin mRNA expression levels in ripened fruits from T_2 lines (Fig. 3a). Such amplification was not observed in WT tomatoes. The monellin transcript abundance was normalized against that of internal control tubulin. The maximum transcript levels were detected in lines T_2-5 and in T_2-14 at the exponential phase PCR cycle 24. Three independent experiments were performed with different biological replicates.

ELISAs using polyclonal antibody against monellin were used to detect the presence of monellin protein in transgenic and WT lines. Three independent experiments were performed, and each sample was measured in duplicate. The statistical errors were detected by standard deviations. The ELISA data were confirmed by immunoblot analysis by comparing the signal for each variant to that of purified monellin. Transgenic lines T_2-5 and T_2-14 showed maximum protein levels 60 μg/mg (±2.06) and 54 μg/mg (±2.58), respectively. Other transgenic lines also showed expressions of monellin, i.e. T_2-4 (51 ± 1.54), T_2-7 (47 ± 1.78), T_2-8 (45 ± 1.96). The results of the immunoblotting of total fruit protein extract (after the major protein Rubisco removed) from wild-type and transgenic plants expressing monellin were presented in Fig. 3c. Moreover, the immunoblot for loading control, i.e. actin was represented in Fig. 3d. There was no detectable monellin protein in untransformed wild-type fruit, thereby confirming the absence of the monellin transgene. Estimated yield of monellin/mg protein was 60 μg that corresponded to 4.5 % of the soluble proteins extracted from the transgenic tomato fruit expressing monellin. Fruit taste evaluations after 10 days of ripening showed that out of five transgenic lines T_2-5 and T_2-14 revealed distinguishable sweetness compared with the WT. All five panellists described the

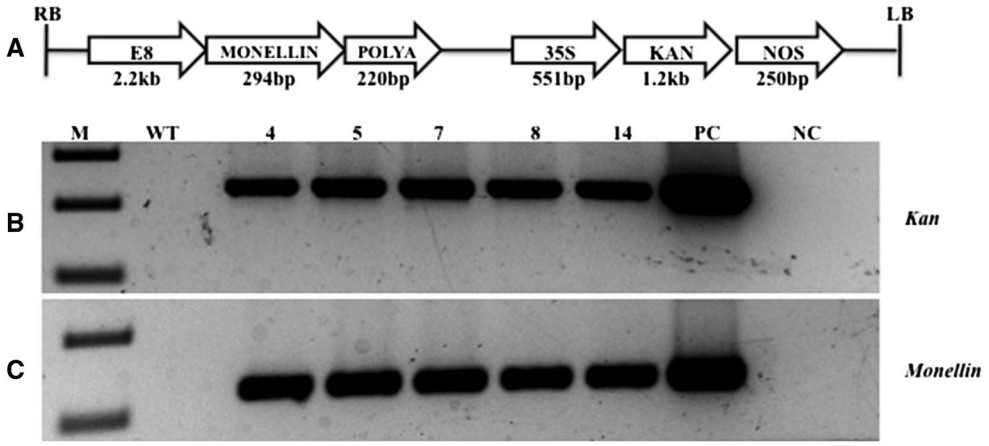

Fig. 2 **a** Schematic representation of the T-DNA region of binary vector pMDC100 used for tomato transformation showing synthetic monellin gene under the control of E8 fruit-specific promoter and the *kan* gene under the control of CaMV 35S promoter. **b** PCR amplification of 0.6 kb region of *kan* expression cassette and 0.294 kb region of transgenic monellin expression cassette from genomic DNA of T_1 tomato seedlings derived from different putative transgenic lines. PC stands for positive control (plasmid DNA template); NC stands for negative control (no DNA template); WT stands for wild-type tomato sample; *lanes 4*, *5*, *7*, *8*, and *14* represent putative transformed tomato samples; M stands for 1 kb DNA ladder

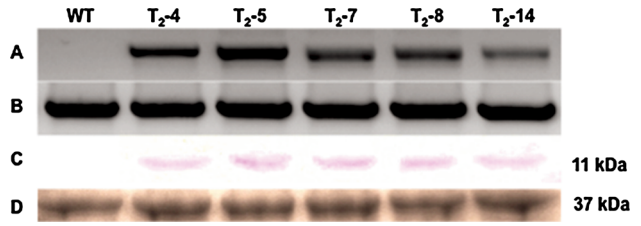

Fig. 3 Expression analyses of synthetic single-chain monellin protein in tomato transgenic lines. *a* Semi-quantitative RT-PCR analysis of monellin transcript abundance in transgenic and WT tomatoes. *b* The abundance of monellin transcript was normalized against that of tubulin transcript. *c* Protein blot analysis of monellin protein expression in transgenic tomato fruits using polyclonal antibodies against monellin. *d* Western blot for loading control using Anti-β actin antibodies. WT stands for wild-type tomatoes, T_2-4, T_2-5, T_2-7, T_2-8, T_2-14 represent transgenic tomato samples from different transgenic lines expressing synthetic monellin in fruits

sweetness of tomato pericarp of fruits from T_2-5 and T_2-14 transgenic lines as strongly distinguishable from the WT fruit. A specific, characteristic after-taste that lingered in the mouth after swallowing was noted for the fruits from lines T_2-5 and T_2-14. These observations suggested that transgenic tomatoes expressing monellin conferred sweetness in them.

Discussion

Intake of high caloric food causes obesity and related problems mainly due to consumption of refined sugars, which ultimately leads to higher probability of heart diseases, type II diabetes, sleep apnoea, certain types of cancer and osteoarthritis. The primary sources of these extra carbohydrates are sweetened beverages and other carbohydrate-rich foods. Although addition of artificial sugar-free sweeteners may replace sweetness, they still have some side effects. As a consequence, using monellin, a low-calorie, carbohydrate-free protein [13, 14] as natural sweetener, would be an ideal option. However, monellin protein purification is tedious and limited to restricted habitats. Moreover, the two subunits of monellin, i.e., chain A (45 amino acids forming three anti-parallel strands) and chain B (50 amino acids forming two β strands separated by an α-helix) are held together by non-covalent interactions, which leads to its instable nature [15]. To mitigate these problems, redesigning of native monellin as recombinant single-chain protein conferred enhanced stability at high temperatures and extreme pH and helped in retaining its sweet flavour [8]. Therefore, it is desirable to identify suitable monellin expression systems for large-scale production of this potential food supplement.

In this study, we have successfully demonstrated the stable and higher expression of a synthetic sweet protein monellin in prokaryotic (*E. coli*) and eukaryotic (tomato) systems than previously documented. The recombinant monellin protein is thermostable and retains strong sweetness over a range of temperatures (up to 70 °C) and extreme pH. Transgenic tomato plants reveal stable gene integration in T_1 generation as verified by PCR analysis. Transcript profiling of T_2 lines revealed enhanced monellin expression (Fig. 3a) that positively correlates to its protein expression profiles as deciphered by western blots (Fig. 3c)

of transgenic fruits from these lines. However, a remarkable sixfold increase in monellin yield was observed in the present study in comparison with previous reports [9, 16]. Ripe fruits of T_2-5 and T_2-14 transgenic lines elicit distinguishably strong sweetness than WT fruits as evidenced by RT-PCR and western blots. These two lines produced higher levels of monellin in their fruits in comparison with other T_2 lines. It has been shown previously that recombinant transgenic tomato fruits possess only mild sweetness, when treated with ethylene [9]. However, we found strong sweetness in the fruits of monellin expressing tomato transgenic lines without ethylene treatment after 10 days of fruit ripening. The enhanced expression of monellin in transgenic tomatoes leads to improvement in their flavour (sweetness) and quality (proteinaceous nature). In addition, these transgenic tomatoes with enhanced flavour and quality can be consumed by normal and sugar-challenged populations, throughout the globe.

Conclusions

We have synthesized a single-chain monellin gene and demonstrated its stable and high expression in heterologous systems not reported previously. The expression of monellin protein has improved the flavour and nutritional value of a local variety of tomato for enhanced nutrition. This strategy can be extrapolated to other vegetable crops for significant enhancement in flavour and nutritive value.

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