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Modification of tomato taste in transgenic plants carrying a thaumatin gene from *Thaumatococcus daniellii* Benth

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With 4 figures

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Abstract

Fruit taste is an important component of fruit quality, but its genetic basis is complex, making it difficult to alter by plant breeding. Thaumatin is a sweet-tasting, flavour-enhancing protein produced by fruits of the African plant *Thaumatococcus daniellii* Benth. *Agrobacterium*-mediated transformation was used to produce two transgenic tomato lines expressing biologically active thaumatin in fruits. Transgenic tomato fruits from the T_2 plant generation were sweeter than the controls and possessed a specific aftertaste as determined by sensory evaluation. These results demonstrate that transgenic expression of thaumatin could be useful for modifying tomato fruit taste, especially in breeding lines possessing poor fruit taste, such as those carrying a *non-ripening* (*nor*) mutation.

Key words: *Lycopersicon esculentum* — *Agrobacterium tumefaciens* — fruit taste — gene transfer — transformation

Fruit quality is an important goal in tomato (*Lycopersicon esculentum* Mill.) breeding. One of the major parameters contributing to fruit quality is taste, with sugars and organic acids as major determinants (Stevens et al. 1977, Kader et al. 1978, Jones and Scott 1984). Other compounds also influence tomato taste, making it a complex and difficult-to-alter trial by breeding (Stevens 1979), and the taste of some tomato varieties has been criticized by consumers (Tigchelaar 1986). Transgenic approaches have been used to alter tomato fruit quality, for example, prolonged shelf life and firmness (Schuch et al. 1991, Kramer et al. 1992, Klee 1993, Poole 1993, Kramer and Redenbaugh 1994, James and Krattiger 1996), and enhanced phytonutrient content (Fraser et al. 2001, Mehta et al. 2002). Transgenic expression of the gene for the sweet protein monellin in tomato fruit has resulted in accumulation of monellin and altered fruit taste (Penarrubia et al. 1992).

Thaumatin is a sweet-tasting protein (207 amino acids) isolated from the fruit of the African plant katemfe (*Thaumatococcus daniellii* Benth.) (Van der Wel and Loeve 1972, Witty and Higginbotham 1994). In addition to its sweet taste, this protein enhances certain flavours while masking others, binding specifically with taste receptors (Van der Wel and Arvidson 1978). The function of thaumatin is not yet clear, although it has strong homology to thaumatin-like pathogenesis-related proteins (PRP-TL). Thaumatin extracted from plant fruits is commercially available and is used as a natural sweetener and flavour enhancer (Witty and Higginbotham 1994, Faus 2000). The thaumatin cDNA (931 bp without polyA tail) has been cloned and sequenced (Edens et al. 1982, Ledeboer et al. 1984). The expression of thaumatin in microorganisms to produce a recombinant protein for industrial applications only resulted in low yields reducing the economic feasibility of its large-scale production (Faus 2000). The thaumatin gene has been successfully expressed in plants. Witty (1990) introduced and achieved the expression of thaumatin in potato hairy roots using an *Agrobacterium rhizogenes*-based approach. Sensory assessment of hairy root extracts has shown new taste properties (sweet taste associated with aftertaste) (Witty and Harvey 1990). Transgenic cucumber expressing thaumatin has also been produced (Szwacka et al. 2002). The expression of thaumatin was confirmed at the protein level and by sensory evaluations of ripe cucumber fruits, and a sweet cucumber fruit taste was reported (Malepszy and Szwacka 2000, Szwacka et al. 2002). Rats fed on cucumber fruits showed no differences in growth and reproduction (Kosieradzka et al. 2001).

This study was performed to see if transgenic expression of thaumatin could be used to improve tomato fruit taste. Thaumatin was used because it can alter fruit taste, is approved for use by the food industry in many countries, and tomatoes do not normally produce this protein. Transgenic tomato lines were produced and the expression of the thaumatin gene influencing fruit taste was confirmed. This is the first report describing thaumatin-producing tomatoes.

Materials and Methods

Plant materials: *Lycopersicon esculentum* Mill. cultivar 'Beta', a tomato breeding line nor carrying the *non-ripening* (*nor*) mutation (Seroczynska et al. 1998), and line *ls* carrying the *lateral suppressor* (*ls*) mutation resulting in plants that do not produce or produce a very limited number of lateral shoots (Brown 1955) were used for transformation experiments. The seeds were provided by Dr A. Korzeniewska (Warsaw Agricultural University, Warsaw, Poland) and reproduced by self-pollination in an experimental field.

Binary plasmids: The binary plasmid pRUR528 (Szwacka et al. 2002) carrying the thaumatin II cDNA under the control of the 35S CaMV promoter was used. Plasmid pRUR528 was derived from pROK2 in which the *gus* gene coding sequence was replaced with the thaumatin cDNA as described by Szwacka et al. (2002). The T-DNA region of pRUR528 is shown in Fig. 1. As a positive control the binary plasmid pBI121 (Jefferson et al. 1987) carrying the 35S-gus gene construct (Clontech, Palo Alto, CA, USA) was used. Both binary plasmids possess the *nptII* gene conditioning kanamycin resistance. Plasmids were introduced into *A. tumefaciens* strain LBA4404 (Life Technol-

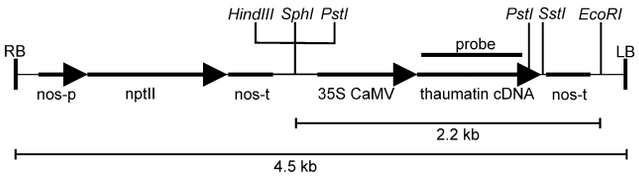

Fig. 1: T-DNA region of plasmid pRUR528. Size of *Eco*RI/*Hin*dIII restriction fragment and of the whole T-DNA are indicated in kilobasepairs (kb). RB = right border; LB = left border; nos-p = nopaline synthase promoter; nptII = neomycin phosphotransferase coding sequence; nos-t = nopaline synthase terminator; 35S CaMV = 35S cauliflower mosaic virus promoter

ogies, Rockville, MD, USA) by electroporation (Shen and Forde 1989).

Tomato transformation: Tomato transformation was performed according to McCormick et al. (1986) with modifications (Bartoszewski et al. 1996). Cotyledon explants from aseptically grown tomato seedlings were inoculated with *A. tumefaciens*. Putative transformed plants (T_0) were grown in the greenhouse and seeds were collected. About 35 seeds of each transgenic plant were surface-sterilized, planted on half strength MS medium (Murashige and Skoog 1962) with added kanamycin (75 mg/dm^3). After 2 weeks, kanamycin-resistant seedlings were scored and 8–10 kanamycin-resistant seedlings for each independent transgenic event were grown in a greenhouse. The seeds were collected from individual plants. About 120–200 seeds for each individual T_1 plant were planted on half strength MS medium with kanamycin and seedlings (T_2) were checked for kanamycin resistance. Based on this approach, homozygous T_1 plants were selected and progenies of those plants (T_2 generation) were used for determining thaumatin expression and for taste evaluation.

Flow cytometry analysis: Nuclei were extracted from young leaves of regenerated shoots. Freshly harvested leaves were chopped with a razor blade in nuclear extraction buffer according to the manufacturer's protocol (Partec, Münster, Germany). The samples were filtered through a nylon filter (pore size 40 μm), and the nuclei in the filtrate stained with 4,6-diamidino-2-phenolindole (DAPI). Flow cytometry was performed with a Partec CAII flow cytometer.

Polymerase chain reaction: Polymerase Chain reaction (PCR) was carried out in a reaction mixture (50 μl) consisting of 1 × PCR buffer (Fermentas, Vilnius, Lithuania), primers (1 μM each), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 1.25 units Taq DNA polymerase (Fermentas) as per the manufacturer's protocol. To amplify an 809-bp sequence for hybridization, pRUR528 plasmid DNA (1–5 ng) and the primers forward pT1 (TTC TCC TCC TCA CGC TCT CC) and reverse pT2 (AGC CAA TCC CCA CAC ACA TA) were used (Szwacka et al. 2002). To check transgenic plants for transgene presence, total genomic DNA (50–100 ng) was used as a template with the following primers: forward pT3 (GTC AAC CGC TGC TCC TAC ACC) and reverse pT4 (TCG GGC TGA AGT CCA TCG GCA). The primers pT3 and pT4 were designed to amplify a 333-bp fragment of thaumatin-coding sequence. The cycling conditions were as follows: initial denaturation 95°C for 5 min followed by 35 cycles: 95°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension of 72°C for 7 min.

Hybridization experiments: DNA was isolated from young leaves according to Dellaporta et al. (1984). For Southern blots, 10 μg of genomic DNA was digested with various restriction enzymes (Fermentas). After agarose gel electrophoresis, DNA was transferred to Zeta-Probe membranes (Bio-Rad, Hercules, CA, USA). RNA was isolated from tomato tissues (Mujer et al. 1996) and 20 μg was electrophoresed on a 1.2% formaldehyde-agarose gel and transferred

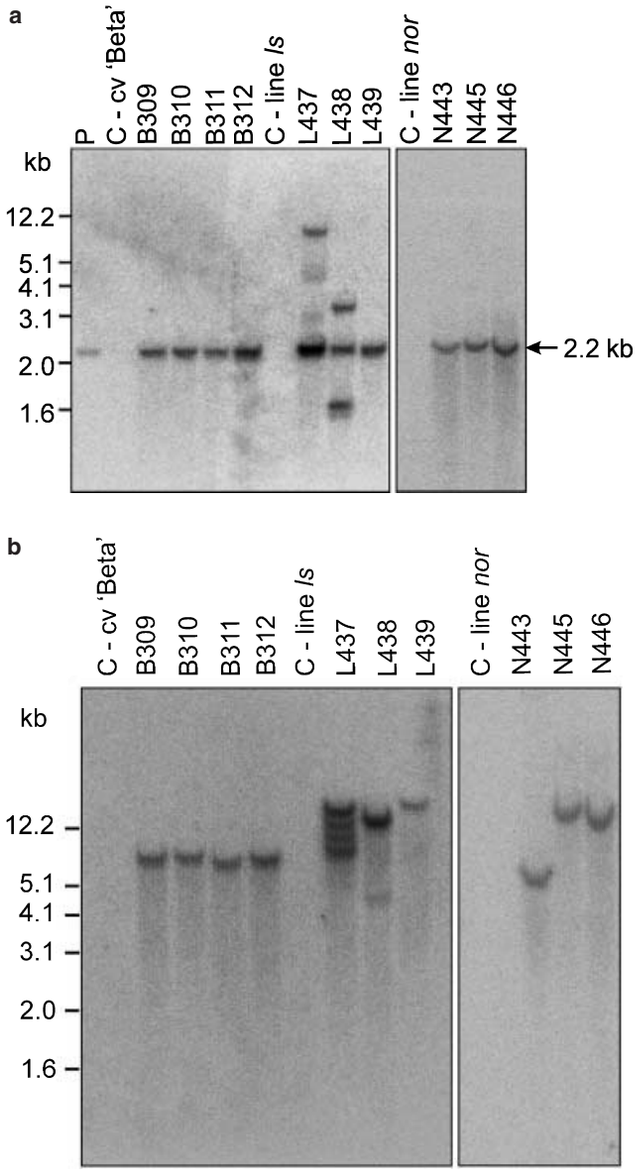

Fig. 2: Southern blot analysis of selected transgenic tomato plants. Genomic DNA was digested with restriction enzymes: a. *Eco*RI and *Hin*dIII, b. DraI. A ^{32}P-labelled 806-bp DNA fragment specific for the thaumatin coding sequence was used as a probe. Size marker is indicated in kilobasepairs (kb). P = plasmid pRUR528; C = non-transgenic plant; B309-B312 = transgenic plants from the cv. 'Beta'; L437-L439 transgenic plants from the line *ls*; N443, N445, and N446 = transgenic plants from the line nor; Exposure time = 48 h

to Zeta-Probe membranes. The membranes were hybridized overnight at 65°C with a ^{32}P-labelled probe (High Prime, Roche, Mannheim, Germany) and washed at high stringency as described by Bartoszewski et al. (2002). A PCR fragment (809 bp) corresponding to the partial thaumatin-coding sequence was used as a probe (Fig. 1). The membranes were exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, NY, USA).

Western blot analysis: The protein extracts were prepared from powdered fruit pericarp (100 mg) homogenized in 600 μl of extraction buffer [0.1 M sodium phosphate (pH 7.6), 15 mM 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 15%

glycerol]. The homogenate was centrifuged at 3000 ***g*** for 15 min at 4°C and the supernatant was recentrifuged at 20 000 ***g*** for 15 min at 4°C, and taken for further analysis. Total protein was determined according to Bradford (1976) using Bio-Rad protein stain with bovine serum albumin as a standard. The same amounts of total protein (20 μg per lane) from each T_2 line were loaded, separated on 12.5% SDS-polyacrylamide gels (Laemmli 1970) and transferred to PVDF membrane (Bio-Rad) by semi-dry transfer (5 mA/m^2, 15 min). Rabbit anti-thaumatin polyclonal antibody (Szwacka et al. 2002) was used as a primary antibody (1 : 1000, v/v). An anti-rabbit IgG-alkaline phosphatase conjugate (Roche) was used as the secondary antibody (1 : 5000, v/v). Blots were developed with the NBT/BCIP staining solution. Commercially available thaumatin (Sigma, St Louis, MO, USA) was used as a positive control (200 ng per lane).

Analysis of fruit taste: Sweetness assay (blind test) was performed as described by Witty and Harvey (1990) and Szwacka et al. (2002) with modifications. For this assay, 6–7-week-old fruits were used. Seedless sections of fruit pericarp (1 g) were prepared. Three panellists (able to detect thaumatin in 10^{-8} M water solution) were asked to taste fruit samples and to describe fruit taste. Four samples were given to panellists (prepared from the transgenic lines N443, N445, N446 and the non-transgenic control). Taste tests were repeated three times.

Results

Approximately 1000 cotyledon explants were infected with *A. tumefaciens* carrying the construct pRUR528 with the thaumatin cDNA. Regenerated shoots were rooted on kanamycin-containing medium and checked for transgene integration by PCR (data not shown). Putative transgenic shoots were selected. Ploidy levels of selected shoots were determined using flow cytometry. Diploid and tetraploid shoots were identified (data not shown). A total of 18 transgenic plants (T_0) were PCR positive and diploid (11 plants for the variety 'Beta', four for the line nor, and three for the line *ls*). The plants were self-pollinated, and 17 produced seeds. Southern blot hybridization confirmed that single copies of the thaumatin gene had been integrated in the genome of 12 plants (Fig. 2a,b). Only two (L437 and L438) of 14 plants possessed more than one site of transgene integration. Growth and vigour of the transgenic plants did not differ from the non-transgenic control (data not shown), and the fruits were normal. The seeds were collected from all transgenic plants, although one plant (B307) showed low fertility.

For further analyses, three independent transgenic plants from line nor were chosen (N443, N445 and N446). Homozygous T_2 progenies were obtained and a single integration event was confirmed by Southern blot hybridization (data not shown). Transgene expression at the mRNA and protein level was evaluated with 6–7-week-old fruits. Northern blot analysis showed that two (N445 and N446) of the three chosen transgenic lines accumulated thaumatin mRNA (Fig. 3) and protein in the fruit (Fig. 4). Line N443 did not express thaumatin mRNA (Fig. 3) and had no thaumatin protein in the fruit (Fig. 4).

Fruit taste evaluations showed that lines N445 and N446 had a sweeter taste distinguishable from fruits of the control and line N443 (a transgenic line that does not express the thaumatin gene). All three panellists described the tomato pericarp of line N445 and N446 as sweeter than the controls. A specific aftertaste that lingered in the mouth after swallowing was noted for lines N445 and N446.

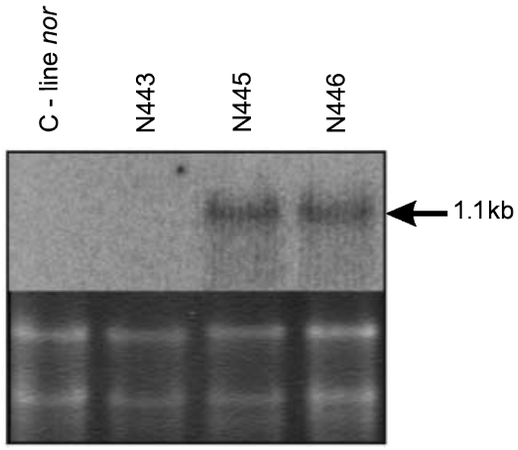

Fig. 3: Thaumatin mRNA accumulation in fruits of transgenic lines N443, N445 and N446. RNA transcripts were analysed by northern hybridization. A ^{32}P-labelled 809-bp DNA fragment specific for the thaumatin cDNA was used as a probe. Expected band 1.1 kb is observed for line N445 and N446. Ethidium bromide-stained rRNA was used as a control for equal loading of the RNA. Exposure time = 24 h

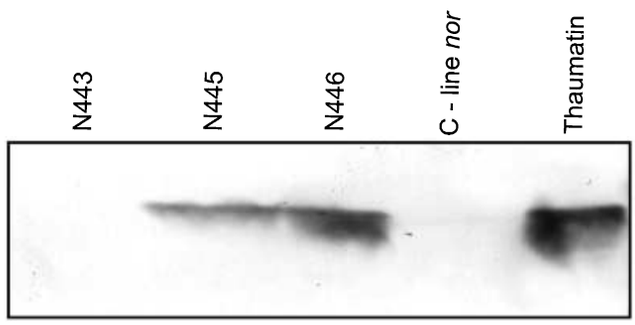

Fig. 4: Thaumatin accumulation in fruits of transgenic lines N443, N445 and N446. Total soluble proteins were analysed by western blot. A 22-kDa band was detected for lines N445 and N446. Commercially available thaumatin was used as a positive control. Twenty micrograms of total plant proteins and 200 ng of thaumatin were loaded per lane

Discussion

Other transgenic approaches have been successfully used to improve tomato fruit properties such as shelf life and firmness (Schuch et al. 1991, Kramer et al. 1992, Klee 1993, Poole 1993, Kramer and Redenbaugh 1994), increased provitamin A and lycopene content (Fraser et al. 2001, Mehta et al. 2002), and a sweeter fruit taste (Penarrubia et al. 1992).

Transgenic tomato lines expressing thaumatin have now been produced and the expression of thaumatin under the 35S CaMV constitutive promoter did not negatively influence plant morphology or growth (data not shown). Fruit evaluations revealed altered taste with clear differences. The main features of the transgenic fruits were a sweet taste and a liquorice aftertaste. Although the amount of thaumatin in the fruit of transgenic plants was not estimated precisely, the expression level was high enough to cause changes in taste in transgenic lines. Thaumatin increased fruit sweetness and generated a liquorice aftertaste lasting for a few minutes, which is one of the thaumatin features (Witty and Higginbotham 1994, Faus

2000) described for transgenic potato hairy roots (Witty and Harvey 1990) and cucumber fruits (Szwacka et al. 2002). Further, more sophisticated taste assays are necessary to determine if consumers would accept this new tomato fruit taste.

Tomato lines possessing the *nor* mutation are interesting for breeders because of delayed ripening, but they are not used in breeding programme, because of their poor fruit taste. Transgenic expression of thaumatin could be used to improve the taste of *nor* lines, making them more useful for breeding purposes but recent rejection of genetically modified organisms (GMOs) by consumers in European countries makes transgenic breeding somewhat questionable, leaving a need to breed better-tasting tomatoes by conventional methods. The lines that were generated in the present study are not useful for breeding purposes for technical reasons. The lines generated possess an antibiotic resistance gene (*nptII*) regulated by the constitutive promoter 35S CaMV. For breeding purposes lines that lack the antibiotic resistance genes and display fruit-specific expression of the thaumatin gene need to be obtained.

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