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ORIGINAL ARTICLE

Production of marker-free tomato plants expressing the supersweet protein thaumatin II gene under the control of predominantly fruit-specific promoters

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Abstract

Despite the lack of evidence of the danger of genetically modified organisms the presence of marker and antibiotic-resistant genes in transgenic plants causes concern to consumers. Genetically modified plants with viral and bacterial genes are adopted by consumers, but with concerns; in addition, constitutive promoters have a number of disadvantages in industrial-scale cultivation of plants. In our study, we used the pMF vector system (Wageningen Plant Research, Wageningen, Netherlands), which combines inducible site-specific recombinase and a bifunctional selectable gene to obtain marker-free tomato plants. The gene of interest was the supersweet thaumatin II protein from the tropical plant *Thaumatococcus daniellii* under the control of tomato predominantly fruit-specific early-light inducible protein (ELIP) or *E8* promoters and tomato Rubisco terminator. The use of this gene in our laboratory allowed enhancing sweetness, as well as improving the taste characteristics of fruit such as apple, strawberries, carrots, tomatoes, and pears. By using different strategies of early and delayed selection we developed a protocol for obtaining fully marker-free tomato plants, which was checked by polymerase chain reaction and Southern blotting. The thaumatin II gene expression was confirmed by reverse transcription-PCR and western blotting analyses. The fruit of transgenic and marker-free tomato plants displayed a sweet taste. A quantitative comparative assessment of the level of expression of the thaumatin protein under the control of two promoters was carried out using enzyme-linked immunosorbent assay. Multiple and/or incomplete T-DNA inserts that often occur during transformation of Solanaceae greatly reduced the efficiency of the system used.

Key message

The strong tomato *ELIP* promoter provides a high level of expression of the supersweet thaumatin II protein gene in the fruit of marker-free tomato plants.

Keywords *Solanum lycopersicum* · E8 · Early-light inducible protein (ELIP) · Cytosine deaminase · R/RS recombination system · High expression level

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Abbreviations

5-FC	5-Fluorocytosine
ANOVA	One-way analysis of variance
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CaMV35S	Cauliflower mosaic virus 35S promoter
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9
Dex	Dexamethasone
ELIP	Early-light inducible protein
ELISA	Enzyme-linked immunosorbent assay
HSP	Heat shock protein
IAA	Indole-3-acetic acid

LBD	Ligand-binding domain
MS	Murashige and Skoog culture medium
PBS	Phosphate buffer saline
RS	Recombination site
SDS	Sodium dodecyl sulfate
TALEN	Transcription activator-like effector nucleases
TSP	Total soluble protein
ZFN	Zinc-finger nucleases

Introduction

Creation of plants with new traits that do not contain viral or bacterial genetic material is a modern approach that, owing to scientific and biotechnological progress, has become the reality of the 21st century. In addition, this approach meets growing biosafety requirements.

One of the main concerns of society related to genetically modified (GM) plants is the use of antibiotic resistance genes during genetic transformation. The need for such selective genes is further lost, but these DNA sequences remain forever in the transgenic plant genome. Another concern is the application of foreign genetic elements, especially viral regulatory sequences, for the expression of target genes in transgenic plants. It should be noted that these fears are largely unfounded and are promoted for economic reasons or by a lack of public awareness. Nevertheless, first, the opinion of most consumers cannot be ignored. Second, the restrictions stimulate the expansion of scientific research fields and the development of new technologies. For example, the actively developed technologies of genome editing, first of all, CRISPR/Cas9 system, which replaced ZFN and TALEN, created a real splash in the scientific community. The number of scientific publications devoted to plant genome editing increased from 19 in 2013 to 148 in 2017 (Miroshnichenko et al. 2019). However, the enthusiasm and optimism of many scientists declined after July 25, 2018, when the Court of Justice of the European Union ruled that organisms obtained by gene-editing technologies such as CRISPR/Cas9 are genetically modified organisms (GMOs) and fall under existing European law limiting the planting and sale of GM crops (Court of the European Union 2018). Thus, in our opinion, interest in the development of traditional transgenic approaches has largely disappeared following the spread of genome editing technologies, which remain relevant. This is especially true for cisgenic and intragenic plants.

One convenient approach for obtaining plants without marker genes is the inducible site-specific recombinases that, after selecting plants with the appropriate agent, allow the excision of an undesired DNA after induction. To date, three main systems based on site-specific recombinase have been well described. These are the Cre/lox system of the P1 phage (Dale and Ow 1991), the FLP/FRT recombinase system from *Saccharomyces cerevisiae* (Lyznik et al. 1996), and the R/RS recombinase system from *Zygosaccharomyces rouxii* (Onouchi et al. 1995; Sugita et al. 2000).

One of the latest schemes exploiting the R/RS recombinase system was developed in Wageningen Plant Research (Wageningen, Netherlands; Schaart et al. 2004). In this work to remove the selective genes from tomato genome we used site-specific recombinase belonging to the pMF vector. The main advantage of this system is the double selection. After *Agrobacterium*-mediated transformation, in the first stage, regenerants are selected using antibiotics such as kanamycin or hygromycin. Further chemical activation of the recombinase occurs, which cuts out the DNA sequences (containing the gene of the recombinase itself as well as selective genes) flanked by intact recombination sites (RS). The recombinase in the translational fusion with the ligand-binding domain (LBD) of the glucocorticoid receptor is in an inactivated state. Its activation is carried out after incubation of plant tissue in a solution of dexamethasone (Dex). At the second stage, selection on a medium with 5-fluorocytosine (5-FC) takes place. Plant tissues that have not undergone DNA excision do not give secondary regenerants, as the *codA* gene encoding cytosine deaminase is expressed. This enzyme converts non-toxic 5-FC into cytotoxic 5-fluorouracil, making it impossible to obtain chimeras owing to incomplete DNA elimination. This system was successfully applied on such cultures as strawberry, apple, and pear (Schaart et al. 2004; Vanblaere et al. 2011; Righetti et al. 2014; Krens et al. 2015; Timerbaev et al. 2019).

For people with diabetes and people who are prone to obesity, replacing sucrose with natural sweeteners, which are often low-calorie or non-caloric, is often the only option to avoid changes to their eating habits for medical reasons. Among the few sweet-tasting proteins, thaumatin is the most characterized, commercialized, and regulated (O'Brien-Nabors 2011; Garcia-Almeida et al. 2018). Thaumatin was isolated from the fruit of the african plant *Thaumatococcus daniellii* Benth (van der Wel and Loeve 1972). It has 3000 times more sweetness than sucrose and is about 100,000 times sweeter by molar ratio (Nikoleli and Nikolelis 2012). The perception of thaumatin sweetness is delayed to some extent—the sweet taste is retained for a long time and it leaves a slight liquorice-like aftertaste (Naim et al. 1986). Thaumatin is safe as a sweetener and does not cause any allergenicity or toxicity. It has been approved within the European Union since 1984 (food additive E957; Scientific Opinion on the safety of the extension of use of thaumatin 2015). Thaumatin also acts as a flavor enhancer and modifier in food applications. In addition, thaumatin II has antifungal activity (Vigers et al. 1992; Popowich et al. 2007) and is capable of performing protective functions in transgenic plants. The mature protein consists of a single chain

of 207 amino acid residues and has a molecular mass of 22 kDa (van der Wel and Loeve 1972). A wide range of cultures, including agricultural crops, expressing the thaumatin II gene has been developed so far, among them potato (Witty 1990), cucumber (Szwacka et al. 1996), pear (Lebedev et al. 2002), tomato (Bartoszewski et al. 2003), apple (Dolgov et al. 2004), strawberry (Schestibratov and Dolgov 2005), tobacco (Rajam et al. 2007), hyacinth (Popowich et al. 2007), carrot (Sidorova et al. 2013), and others.

The objectives of the above studies to modify taste or increase resistance were achieved owing to the high expression of thaumatin directed by the constitutive promoter. In the practical application of plant genetic engineering it is important to minimize the negative impact of the constitutive transgene expression on the plant itself and on the final consumer perception. This can be achieved by precise accumulation of the target protein in a particular plant organ or cell compartment using organ- or tissue-specific promoters. Besides, in the case of high expression as an effect of choosing the appropriate promoter, such plants can be considered as potential producers of recombinant thaumatin II instead of microorganisms.

An important component in describing the classic taste of tomato is sugar. Sweet tomato varieties have always been valued by consumers and farmers. It is no secret that tasteless tomatoes have gradually flooded the markets of developed countries. Just recently, it was revealed that during tomato domestication and improvement aimed especially at increasing disease resistance, there is a substantial loss of genes and intense negative selection of genes (Gao et al. 2019). This explains, among other things, the deterioration of the tomato flavor during breeding. It can be assumed that increasing the sweetness of the tomato fruit and also giving a zesty liquorice-like aftertaste can attract buyers.

Considering all the above, in our work we used predominantly tomato-fruit-specific promoters—the classic *E8* promoter and the recently characterized strong *ELIP* promoter for the directed expression of thaumatin II protein in tomato (Timerbaev and Dolgov 2019). The objective of this study was to obtain marker-free tomato plants not containing functional nonplant DNA sequences expressing the supersweet protein thaumatin II gene predominantly in fruit.

Materials and methods

Binary vectors used for transformation

The binary vectors pMF-E8 and pMF-ELI using for *Agrobacterium*-mediated transformation of tomato plants are based on pMF1 vector (WUR, Wageningen; Schaart et al. 2004) containing recombinase R from the yeast *Z. rouxii* fused to the LBD of the glucocorticoid receptor and the bifunctional selection gene *CodA-nptII*, allowing the selection of plants by negative selection on 5-FC after removal of the undesirable region of the DNA from the genome. The common scheme of cloning steps and the creation of pMF-E8 plasmid was as described by Timerbaev et al. (2019). Both plasmids containing the coding sequence of the thaumatin II gene under the control of an 1118-bp *E8* gene promoter or 2165-bp early light-inducible protein (ELIP) gene promoter (Timerbaev and Dolgov 2019; GenBank accession no. MK867692) and a 402-bp fragment of tomato *rbcS3A* gene terminator. The scheme of binary vectors and the principle of the system are shown in Fig. 1.

Plant material and tomato transformation

Tomato (*S. lycopersicum* cv. Yalf) seeds were provided by Dr. G. Monakhos (Moscow Timiryazev Agricultural Academy, Moscow, Russia). *Agrobacterium*-mediated transformation of tomatoes was carried out according to McCormick

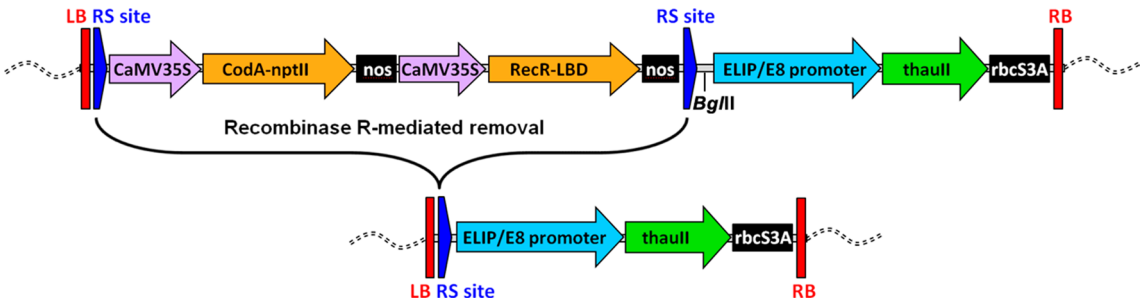

Fig. 1 Schematic representation of the T-DNA region of pMF-E8 and pMF-ELI vectors before and after excision of DNA sequences flanked by the recombination sites (Rs). LB, RB, left and right borders; CaMV35S, cauliflower mosaic virus promoter; CodA-nptII, translational fusion of cytosine deaminase and neomycin phosphotransferase II genes; nos, nopaline synthase terminator; RecR-LBD, translational fusion of recombinase R and ligand binding domain of glucocorticoid receptor genes; ELIP/E8 promoter, promoter fragment of *ELIP* or *E8* gene; thauII, coding region of thaumatin II gene; rbcS, ribulose-1,5-bisphosphate carboxylase/oxygenase gene terminator; *Bgl*II, position of the restriction site for which the DNA was digested for the Southern blot assay

et al. (1986), with modifications. The seeds were sterilized for 7–8 min in 15% hypochlorite and transferred onto Murashige and Skoog (MS) medium (Murashige and Skoog 1962). After 2–3 weeks the cotyledons, hypocotyls, and leaves of young seedlings were cut and precultured in the dark on MS medium containing 5 mg/L 6-benzylaminopurine and 0.2 mg/L indole-3-acetic acid (IAA). Binary pMF-E8 and pMF-ELI vectors were introduced into the *Agrobacterium tumefaciens* strain AGL0 (Lazo et al. 1991) by electroporation. Tomato explants were inoculated in overnight bacterial suspension for 1 h on a shaker (150 rpm) and then placed on the hormone-free MS medium. Cocultivation was performed with light for 2 days. After washing, the explants were spread on MS medium supplemented with 1 mg/L zeatin and 0.1 mg/L IAA, 50 mg/L kanamycin, and 300 mg/L timentin for regeneration and selection of transformants. The resulting shoots were rooted in the hormone-free MS medium containing 50 mg/L kanamycin and 150 mg/L timentin. After adaptation tomato plants were grown in a greenhouse at 25 °C, with a 16-h photoperiod. A transgenic tomato cv. Yalf line expressing the thaumatin II gene under the control of the constitutive 35S promoter, obtained previously (Firsov et al. 2012), was used as a positive control in a western blot assay.

Screening of 5-fluorocytosine concentration for negative selection of marker-free plants

Before starting experiments to obtain marker-free tomato plants, we examined the effect of different concentrations of the 5-FC selective agent on tomato regeneration efficiency. Since cytosine deaminase (a product of a *codA* gene) converts nontoxic 5-FC to cytotoxic 5-fluorouracil this allows, after recombinase activation, selection of regenerants with eliminated DNA flanked by RS sites. To determine the threshold value at which the regeneration stops, we selected five concentrations of 5-FC from 50 to 250 mg/L. Hypocotyls and cotyledons of nontransgenic and transgenic plants were put onto regeneration medium with 5-FC (Sigma-Aldrich, St. Louis, MO). After two passages (about a month), we evaluated the regeneration frequency.

Obtaining of marker-free tomato plants

After plant transformation, two strategies were followed for the selection of marker-free transgenic tomato lines. In the early negative selection approach, after *Agrobacterium*-mediated transformation with pMF-E8 and pMF-ELI vectors, green, tight, and morphogenic kanamycin-resistant calluses were obtained. Selected calluses of about 1 cm^3 were transferred to the liquid medium, supplemented with 20 μM Dex to induce recombinase activity, for 1 h on a shaker (150 rpm). The calluses were transferred onto solid regeneration MS medium (1 mg/L zeatin and 0.1 mg/L IAA) supplemented with 20 μM Dex. After one night, cultivation explants were placed on the same medium supplemented with 250 mg/L 5-FC and the concentration of Dex reduced to 2 μM. Regenerants resistant to 5-FC were selected and rooted.

In the alternative, delayed selection strategy, after *Agrobacterium*-mediated transformation with pMF-E8 and pMF-ELI vectors stable transgenic plants were obtained. Tomato lines were analyzed by PCR, western blotting, and organoleptically. The seeds of self-pollinated plants from chosen lines were sterilized and germinated on MS medium containing 150 mg/L kanamycin. The hypocotyls and cotyledons of kanamycin-resistant plantlets were cut and treated with Dex as described above. Explants then were subjected to the second round of shoot regeneration on 5-FC selection medium. Selected shoots were analyzed by PCR and transferred to a greenhouse for further analysis.

Polymerase chain reactions

For PCR analysis, the genomic DNA from transgenic and control tomato plants was isolated using the GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA). Primary tomato transformants, as well as selected regenerants grown on medium with 5-FC, were studied in detail for the presence of target and selective genes and their regulatory elements by PCR analysis. The primary tomato transformants were checked for the presence of selective sequences and gene of interest using the following primer sets: RS site–35S promoter (RS site; 5′-CGA TTTGATGAAAGAATGAATTAATG-3′ and 5′-GTGTGT CGTGCTCCACCATG-3′), neomycin phosphotransferase II (NptII; 5′-TCTGATGCCGCCGTGTTCC-3′ and 5′-ATG CGCGCCTTGAGCCTG-3′), Recombinase R (RecR; 5′-ATGCGCAAGGAGGCAGGTCG-3′ and 5′-GCCACA CGGGAGACGCCTTC-3′), Thaumatin II (Thau; 5′-GCG CTGCCACCTTCGAGATCG-3′ and 5′-GCAGGTGAC GGTGGTTGGCT-3′). Putative marker-free lines were analyzed using primers specified for the cauliflower mosaic virus 35S promoter (35S prom; 5′-CTCCTCGGATTCCAT TGC-3′ and 5′-CCTCTCCAAATGAAATGAAC-3′), nopaline synthase terminator-specific primers (nos ter; 5′-GAT CGTTCAAACATTTGG-3′ and 5′-CCGATCTGTAACATA GATG-3′), RecR and NptII to check for the absence of the undesired DNA fragments and confirm the loss of selective genes' regulatory elements. The Thau gene primers and the ribulose-1,5-bisphosphate carboxylase/oxygenase gene terminator (rbcS ter) primers (5′-TCTAGAAAAACTAAT TGCC-3′ and 5′-GAGGGAGTAGTAGAGATAAG-3′) were used for confirmation of the presence of expression of the cassette gene of interest. Primers for a selective bacteria-resistant *nptIII* gene (5′-CGGACAGCCGGTATAAAGG-3′

and 5′-AGACAAGTTCCTCTTCGGGC-3′) were used to confirm the absence of a vector backbone in marker-free tomato lines.

Reactions were carried out using Phire Hot Start II DNA polymerase (Thermo) as follows: 1 cycle of 30 s at 98 °C, followed by 29 cycles of denaturation at 98 °C for 5 s, annealing 5 s, extension at 72 °C for 10 s, and one final cycle of 60 s at 72 °C. PCR analysis was performed using Taq polymerase (Thermo). The PCRs were carried out in a 10-µl reaction mix on SimpliAmp Thermal Cycler instrument (Thermo). PCR products were separated using 1.2% agarose gel electrophoresis, visualized with ethidium bromide under ultraviolet light and photographed.

Reverse transcriptase-polymerase chain reaction experiments

RNA samples were extracted from leaves of in vitro transgenic and control plants using GeneJET Plant RNA Purification Mini Kit (Thermo) and from red, ripe fruit (8–9 days after the breaker stage) according to Meisel et al. (2005). Two grams of fresh fruit tissue were ground in liquid nitrogen and subjected to extraction. Each sample was treated with DNAse (Thermo) and its concentration was measured spectrophotometrically. The cDNA was synthesized using Maxima H Minus Reverse Transcriptase (Thermo) according to the manufacturer's protocol using oligo(dT)18 primers. For each sample, 3 µg total RNA were taken for the reverse transcription reaction. For data normalization specific primers for the tomato housekeeping ribosomal protein L2 gene (rpl2; 5′-GGTGACCGTGGTGTCTTTGC-3′ and 5′-ACCAACCTTTTGTCCAGGAGG-3′; Fleming et al. 1993) were used. The thaumatin II specific primers (ThauII; 5′-GCGCTGCCACCTTCGAGATCG-3′ and 5′-GCAGGT GACGGTGGTTGGCT-3′), neomycin phosphotransferase II-specific primers (nptII; 5′-TCTGATGCCGCCGTGTTC C-3′ and 5′-ATGCGCGCCTTGAGCCTG-3′) and recombinase R (recR; 5′-ATGCGCAAGGAGGCAGGTCG-3′ and 5′-GCCACACGGGAGACGCCTTC-3′) were used to detect the transgene expression in obtained tomato lines. PCR analysis and electrophoresis were performed as described in the section "Polymerase chain reactions."

Southern blot analysis

Tomato genomic DNA (20 µg) was digested overnight at 37 °C with 60U *Bgl*II (see position on Fig. 1). The fragments were separated on 0.9% agarose gel and transferred to a positively charged nylon membrane Hybond N + (GE Healthcare, Little Chalfont, United Kingdom) by capillary blotting following the manufacturer's instructions. The DNA probes were constructed by PCR using plasmid pMF-ELI as the template. DNA probes of 725 bp (for *thauII* gene), 440 bp (for *nptII* gene), or 637 bp (for *RecR* gene) were labeled with alkaline phosphatase using Amersham Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare). Prehybridization, hybridization (overnight at 62 °C) with alkaline phosphatase-labeled probes, and subsequent washings of the membrane were carried out according to the AlkPhos Direct Labeling System protocol. Detection was performed using CDP-Star detection reagent following the manufacturer's directions (Amersham CDP-Star Detection reagent, GE Healthcare). The signal from the blot was accumulated on X-ray film (Retina XBE blue sensitive, Carestream Health Inc., New York) in film cassette at room temperature for 24 h. X-ray films were scanned on Amersham imager 600 (GE Healthcare Life Sciences, Japan) after development. The same blot was reprobed several times (ThauII, nptII, and RecR). The blot was striped in a 0.5% (w/v) sodium dodecyl sulfate (SDS) solution at 60 °C for 60 min, as described in the protocol for Amersham Gene Images AlkPhos Direct Labelling and Detection System.

Western blot analysis

Tomato plants were cultivated in the greenhouse and red, ripe fruit and leaves were used for the studies. To prepare total soluble protein, tomato fruit (0.5 g weight segment) or leaf (0.1 g) was ground in liquid nitrogen. The ground material was resuspended in four volumes of extraction buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10% (v/v) glycerol, 30 mM 2-mercaptoethanol, 4 µg/mL aprotinin, and 4 µg/mL leupeptin. Total proteins were extracted for 20 min at 4 °C, then centrifuged for 10 min at 16,000×*g* at 4 °C and the supernatant was taken for further analysis. Protein concentration was measured by DC™ protein assay (BioRad, Hercules, CA). Total proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (BioRad). Rabbit anti-thaumatin polyclonal antibodies (diluted 1:3000) served as the primary antibodies. Anti-rabbit IgG conjugated to alkaline phosphatase was used as the secondary antibody (1:6000) (Sigma). Blots were treated with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for visualization. Thaumatin from *T. daniellii* (Sigma) was used as a positive control.

Quantification of thaumatin accumulation

Recombinant thaumatin was quantified by enzyme-linked immunosorbent assay (ELISA). The protein samples were serially diluted in phosphate buffer saline (PBS), pH 7.4 (0.25, 0.5, 1, and 2 mg TSP (total soluble protein)/well), and thaumatin from *T. daniellii* (Sigma) was used as the reference standard. The plates were incubated for 2 h at room temperature and blocked with PBS containing 2% (w/v) bovine serum albumin and 0.05% (v/v) Tween-20 for 1 h

at 37 °C. The plates were then incubated with rabbit anti-thaumatin polyclonal antibody (diluted 1:6000) overnight at 4 °C, followed by anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1:8000, BioRad). Between each step, the plates were washed three times for 5 min with PBS plus 0.05% Tween-20. The plates were developed by addition of TMB Peroxidase EIA substrate (BioRad). One-way analysis of variance (ANOVA) was used to determine the significance of differences in thaumatin concentrations in leaves and fruit of transgenic lines with different promoters. ELISA was performed in triplicate for each analyzed line.

Results

Obtaining marker-free lines by the immediate selection strategy

After *Agrobacterium*-mediated transformation, two strategies were followed for the selection of marker-free transgenic tomato plants. In the early negative selection approach, a total of 65 and 90 green and tight kanamycin-resistant calluses were obtained by using pMF-ELI and pMF-E8 vectors, respectively (Table 1). The calluses were transferred to medium supplemented with dexamethasone to induce recombinase activity and then placed on the regeneration medium with 250 mg/L 5-FC. This concentration completely inhibited the regeneration of the hypocotyls and cotyledons of transgenic plants (Fig. 2). At the same time, this concentration did not have a negative effect on the regeneration of nontransgenic tissues, which remained at 100% for both types of explants. Only 40 of 155 calluses produced shoots that were able to pass selection on 5-FC. We obtained 116 lines and analyzed by PCR for the removal of undesired DNA and the presence of gene sequences of interest. Of the 65 sublines obtained using the pMF-ELI vector, 40 turned out to be nontransgenic escapes, 24 were transgenic in which the *nptII* gene is present, and only 1 plant was potentially marker-free. Among the 51 sublines produced using pMF-E8 vector, 43 plants were nontransgenic, 8 were transgenic, and no marker-free plants were obtained. Thus using the early selection strategy only one putative marker-free subline, named el-XI-14, with correctly excised DNA was produced from 155 independent kanamycin-resistant calluses.

Stable transgenic tomato plant production and polymerase chain reaction analysis

Another strategy with delayed selection of marker-free plants involves obtaining stable transformants and subsequent manipulations with them. For this we obtained a total of 114 transgenic tomato lines after two *Agrobacterium*-mediated transformations (Table 2). In event VI,

Table 1 Results of in vitro experiments for producing marker-free transgenic tomato plants via immediate selection

Vector	Total number of explants	Number of calluses					Number of sublines (shoots)			
		Kanamycin-resistant	Shoots produced (total)	*nptII*$^-$ shoots produced	*nptII*$^+$ shoots produced	Shoots of both types produced	Total number cut and analyzed	Nontransgenic (*nptII*$^-$ *thau*$^-$)	Transgenic (*nptII*$^+$ *thau*$^+$)	Marker-free (*nptII*$^-$ *thau*$^+$)
pMF-ELI	600	65	21	12	4	5	65	40	24	1
pMF-E8	600	90	19	15	3	1	51	43	8	0

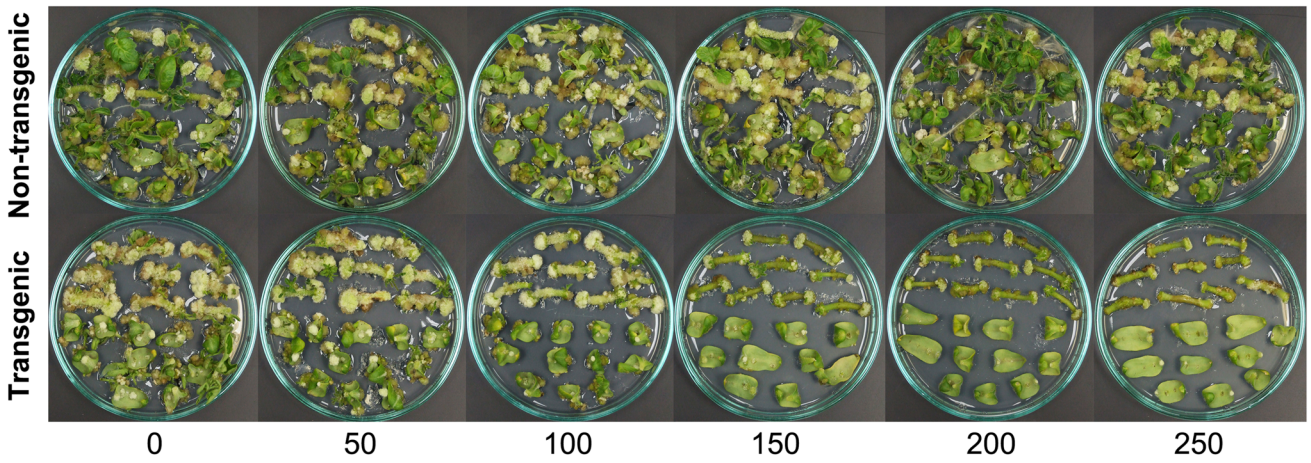

Fig. 2 Determination of an effective 5-fluorocytosine concentration for selection of marker-free tomato plants. Concentrations are presented as milligrams per liter

Table 2 Results of stable tomato transformation

Vector	Event	Explant type	Number of explants		Polymerase chain reaction positive (*nptII*$^+$)		Transformation efficiency, %	
			By type	Total	By type	Total	By type	Total
pMF1-E8	VI	Hypocotyl	124	124	33	33	26.6	
pMF1-ELI	VI	Hypocotyl	141	141	26	26	18.4	
pMF1-E8	VIII	Hypocotyl	32	96	15	27	46.9	28.1
		Cotyledon	47		7		14.9	
		Leaf	17		5		29.4	
pMF1-ELI	VIII	Hypocotyl	27	116	12	28	44.4	24.1
		Cotyledon	59		6		10.2	
		Leaf	30		10		33.3	

Table 3 Results of polymerase chain reaction analysis of stable primary tomato transformants

Vector	Event	Total number of transgenic lines	*thau*$^+$ *nptII*$^+$ RS$^+$		*thau*$^+$ *nptII*$^+$ RS$^-$		*thau*$^-$ *nptII*$^+$ RS$^-$	
			Number of lines	%	Number of lines	%	Number of lines	%
pMF1-E8	VI	33	22	66.7	10	30.3	1	3.0
pMF1-ELI	VI	26	6	23.1	17	65.4	3	11.5
pMF1-E8	VIII	27	15	55.6	11	40.7	1	3.7
pMF1-ELI	VIII	28	11	39.3	17	60.7	0	0
Total		114	54	47.4	55	48.2	5	4.4

only hypocotyls were used as explants. Transformation efficiency was 18% and 27% for pMF-ELI and pMF-E8 vectors, respectively. In event VIII three types of explants were used. Average transformation efficiency not considering the type of explant was 24 and 28% for pMF-ELI and pMF-E8 vectors, respectively. The lowest efficiency, of less than 20%, was observed when using cotyledons and the highest exceeded 40% in both cases when using hypocotyls. All observed putative transgenic lines were thoroughly analyzed by PCR for the presence of whole T-DNA and RS site sequences in their genomes (Table 3). Slightly less than half of them, 55 lines, contained a partial sequence of the T-DNA. There was no sequence of the recombination site at the left border in these plants. The proportion of such lines varied greatly, from 30 to 65% between the transformations. Fifty-four lines (47.4%) contained a complete T-DNA insert. Five plants contained only kanamycin-resistant gene sequences.

Recombinase activation and obtaining marker-free plants

From 54 lines containing the complete T-DNA insertion, we selected 24 lines without morphological abnormalities and brought them to fruiting. Seeds were germinated on a medium with kanamycin and explants from resistant shoots were used to activate recombinase. Twenty lines demonstrated a segregation ratio of 3:1 between normal resistant and subdued seedlings (Fig. 3), indicating a single-locus insertion. Three lines showed non-Mendelian inheritance and one apparently had a two-locus (15:1) inserts (data not shown). After induction of recombinase activity in hypocotyl and cotyledon explants 11 of them did not produce any regenerants on negative selection medium with 5-FC (Table 4). Three lines with the *ELIP* gene promoter produced 11 sublines and 10 lines with the *E8* gene produced 91 sublines. Despite the fact that 77 of the 102 sublines obtained lost their ability to grow on kanamycin (being transferred after selection and multiplication), PCR analysis revealed the presence of the *nptII* gene sequence in 100 of them. These lines also were PCR negative for the *RecR* gene. Only two el-VIII-2-1 and e8-VI-22-6 potentially marker-free transgenic tomato lines were obtained. We then checked putative marker-free tomato lines for the presence of the CaMV35S promoter and the *nos* terminator sequences. As

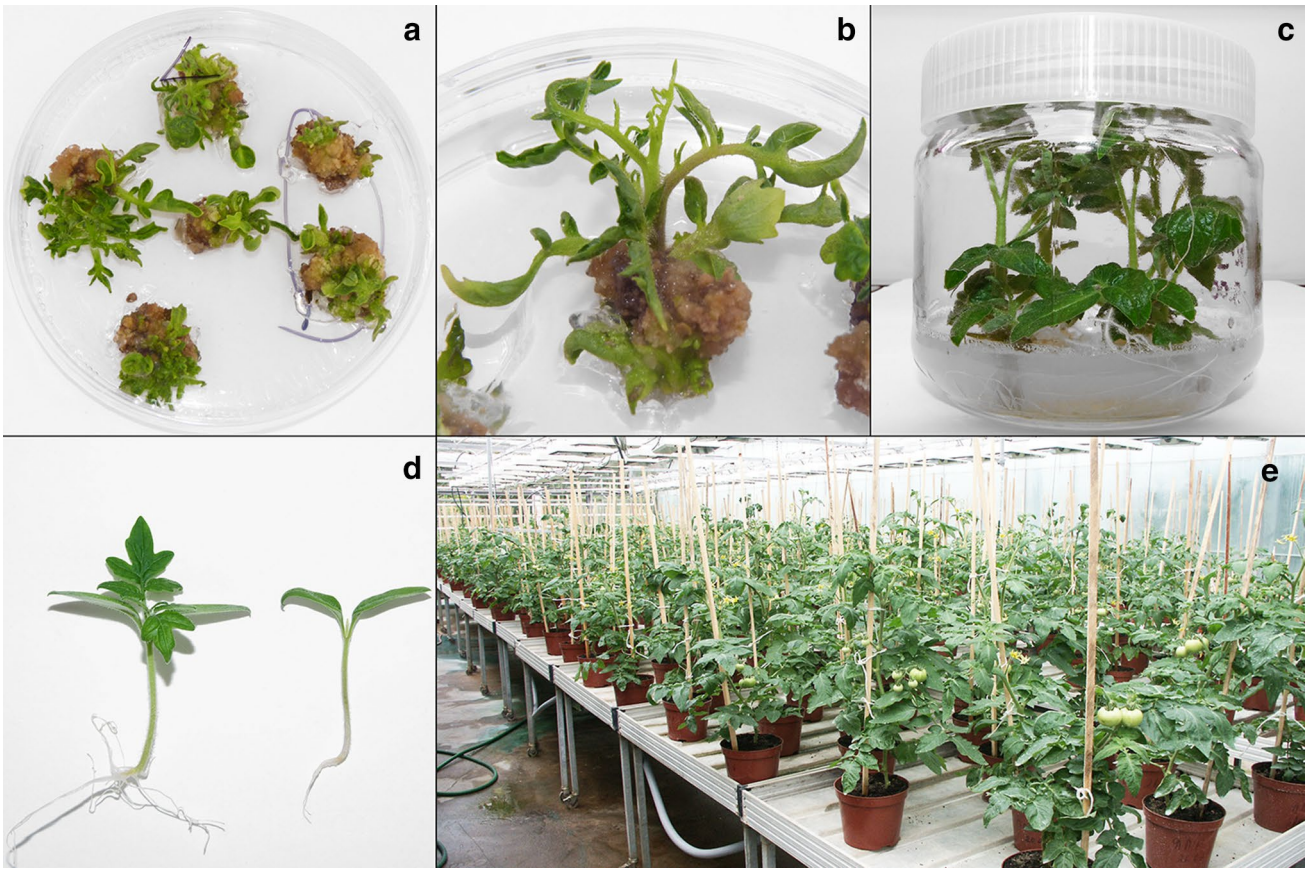

Fig. 3 Obtaining marker-free tomato plants. **a** Tomato regeneration on selective medium. **b** 5-Fluorocytosine-resistant shoot. **c** Rooting of tomato plants. **d** Segregation in the T1 tomato generation, kanamycin-resistant plant (left), and kanamycin-sensitive plant (right). **e** Transgenic and marker-free tomato plants in the greenhouse

Table 4 Results of in vitro experiments for producing marker-free transgenic tomato plants via delayed selection

Vector	Number of lines in experiment	Total number of explants	Number of lines did not produce resistant shoots	Number of lines produced resistant shoots	Number of obtained and analyzed sublines	Sublines that have lost resistance to kanamycin	Number of marker-free sublines
pMF1-ELI	8	1311	5	3	11	9	0
pMF1-E8	16	2820	6	10	91	68	1

confirmation of the presence of the target expression cassette in obtained sublines, the primers for the *rbcs3A* gene terminator were also taken. The results of the analysis are shown in Fig. 4. Regulatory elements of selective genes were not found in selected tomato sublines, whereas in the original lines there was a complete insert of T-DNA.

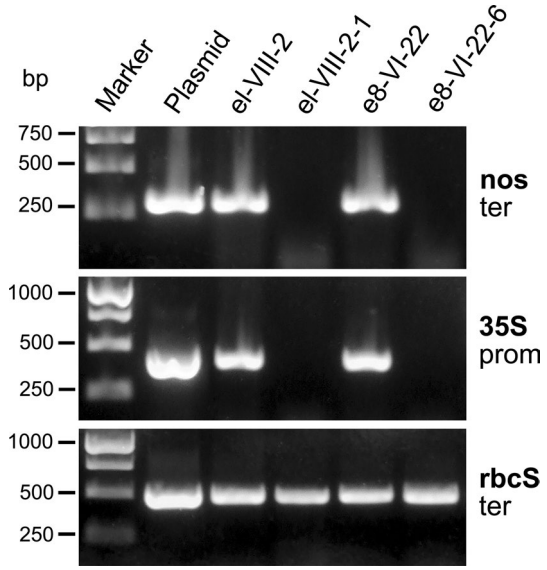

Fig. 4 Polymerase chain reaction analysis for the presence of genetic regulatory elements in parental transgenic plants (el-VIII-2, e8-VI-22) and putative marker-free plants (el-VIII-2-1, e8-VI-22-6). M, molecular weight marker; Plasmid, pMF-ELI vector; nos ter, nopaline synthase gene terminator; 35S prom, cauliflower mosaic virus 35S gene promoter; rbcS ter, ribulose-1,5-bisphosphate carboxylase/oxygenase gene terminator

Molecular analysis of the transgenic and marker-free tomato lines

Southern blot analysis was performed to confirm the excision of DNA flanked by RS sites. For this, the genomic DNA of the parental transgenic lines and potentially marker-free lines were hybridized with probes on the *thau*, *recR,* and *nptII* genes (Fig. 5). Three T-DNA copies were found in the genome of e8-VI-22 line. After DNA removal, only the thaumatin II gene remained in the e8-VI-22-6 line. The inheritance pattern (3:1) of the *nptII* gene and these data appear to indicate a tandem arrangement of inserts in the parental line. Unfortunately, the *nptII* gene sequence was detected in the second selected el-VIII-2-1 line. At the same time, the probe for the *recR* gene gave a negative result. Apparently, incomplete multiple inserts occurred in the el-VIII-2 line. Only one el-XI-14 line obtained by early selection was confirmed as marker-free. Only two copies of the thaumatin II gene were detected.

To confirm the expression of the target gene by RNA level, RT-PCR was performed. For this, total RNA was isolated from the leaves of young sterile plants and red, ripe fruit, and then used as a matrix for reverse transcription. In addition to the target gene, we analyzed the expression of selective genes of neomycin phosphotransferase (*nptII*) and recombinase (*recR*) in parental transgenic plants and some of the sublines obtained. As confirmation of normalization of cDNA produced, a product of the housekeeping gene *rpl2*

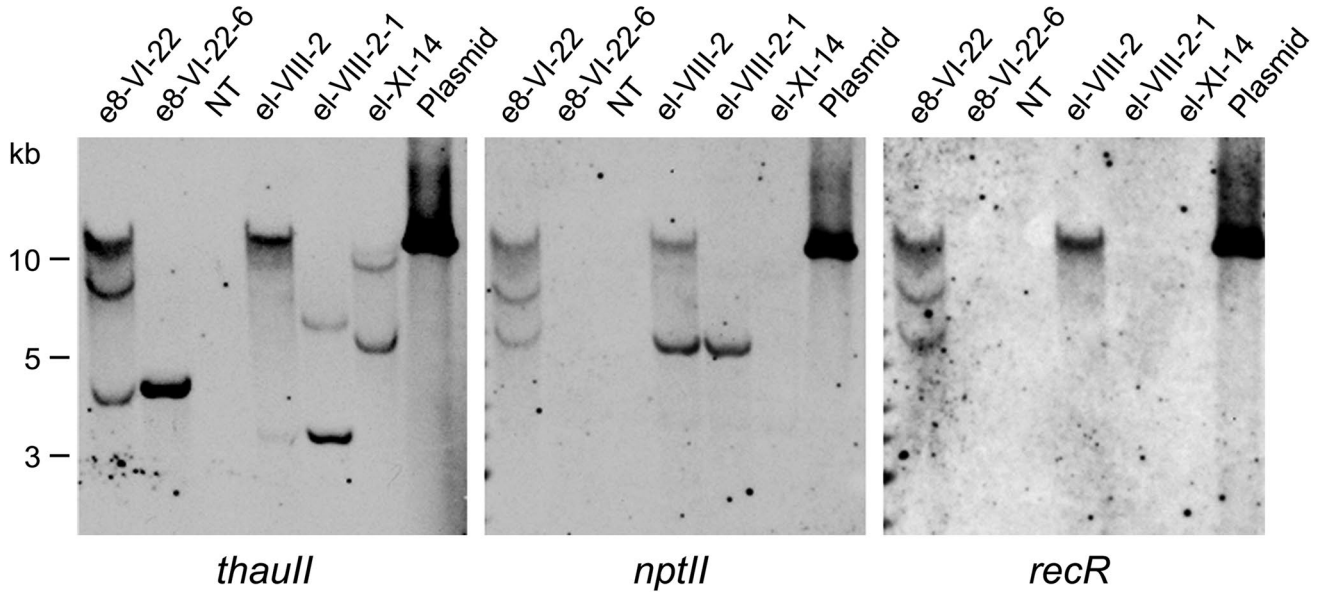

Fig. 5 Southern blot analysis of tomato plants before (el-VIII-2, e8-VI-22) and after DNA excision (el-VIII-2-1, e8-VI-22-6), and the line obtained with early selection (el-XI-14). NT, nontransgenic control; Plasmid, pMF-ELI vector

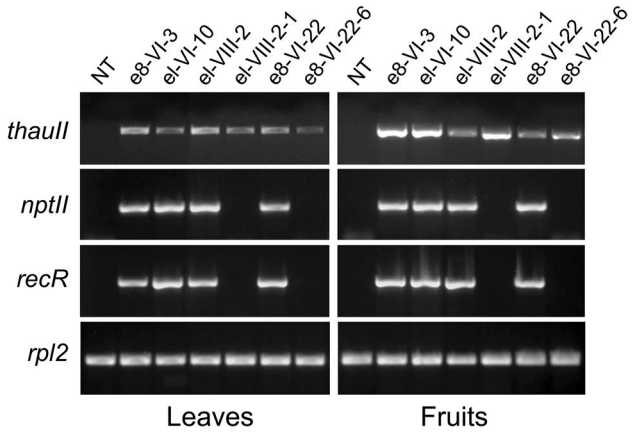

Fig. 6 Reverse transcriptase-polymerase chain reaction analysis of four genes in leaves and fruit of tomato plants. NT, nontransgenic tomato plant; el-VIII-2-1 and e8-VI-22-6, sublines obtained after negative selection step; other numbers, primary transgenic tomato lines

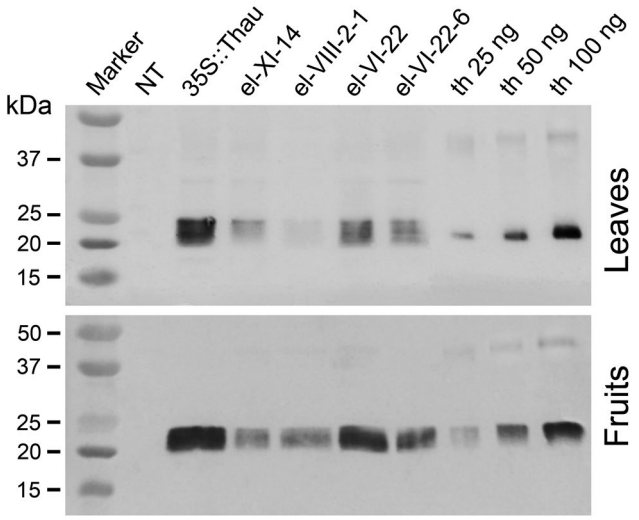

Fig. 7 Western blot analyses of transgenic tomato plants. Marker, molecular weight marker; NT, nontransgenic tomato plant; 35S::Thau, transgenic tomato plant expressing thaumatin II gene driven by CaMV35S promoter; e8-VI-22-6 and el-XI-14, marker-free lines; e8-VI-22, parental transgenic line; el-VIII-2-1, line after recombinase activation; transgenic tomato lines; th 25 ng, 50 ng, and 100 ng, commercial thaumatin II protein

was used. The results of the analysis are shown in Fig. 6. Expression of analyzed genes from T-DNA was detected in all primary transformants. In both sublines only mRNA of the thaumatin II gene was detected after undesired DNA excision. In the subline el-VIII-2-1, the *nptII* gene mRNA, previously detected by Southern blotting on a selective gene fragment, was absent. This result, together with the fact that plants lost the ability to grow on kanamycin, indicated that there is a nonfunctional disrupted *nptII* gene in the genome of the el-VIII-2-1 subline.

We have shown accumulation of the target protein in some transgenic and marker-free tomato plants using the western blotting method (Fig. 7). About 22-kDa bands corresponding to the mature thaumatin were detected. The thaumatin II gene expression was observed both in ripe fruit and in leaves of the analyzed lines under the control of *ELIP* and *E8* promoters. In addition, a line with high expression level containing the CaMV35S promoter was used as a positive control in this experiment. Although it is a quality assessment method, it can be seen that in plants with *ELIP* and *E8* promoters, protein accumulated in larger amounts in fruit than in leaves. In plants with a constitutive promoter, as expected, the amount of protein was about the same in both tissues.

A quantitative estimation of the thaumatin II content in leaves and ripe tomato fruit was performed by ELISA (Fig. 8). For analysis, we used transgenic lines selected to obtain marker-free tomato plants. All lines showed an accumulation of protein in leaves and fruit except for one line and nontransgenic plants. The amount of protein was four to six times higher in ripe fruit than in leaves for both promoters. The maximum level of thaumatin accumulation, 3.7%, and 2.9% of TSP were observed in the lines el-VIII-19 and e8-VI-22, respectively. Interestingly, in two lines obtained after DNA excision, the protein level was reliably lower than in their parental lines. For the marker-free e8-VI-22-6 subline, the amount of protein decreased from 2.9 to 2.1%, and for the el-VIII-2-1 subline protein decreased from 1.12 to 0.75%. In the tissues of tomato lines with the *ELIP* promoter, the concentration of thaumatin was on average 1.5 times higher than in the lines obtained using the pMF-E8 vector. The fruit of the most transgenic tomato plants used in experiments on marker-free plant selection and ELISA analysis demonstrated a well-defined sweet taste with a licorice aftertaste typical for thaumatin II. This indirectly indicates correct folding of thaumatin into the tomato plants obtained.

Discussion

To obtain the marker-free transgenic tomato plants we used the vector pMF1 containing recombinase R from the yeast (*Z. rouxii)* fused to the ligand-binding domain of the glucocorticoid receptor and the bifunctional *CodA-nptII* gene, allowing the selection of plants on 5-FC after removal of the undesirable region of DNA from the genome. As the gene of interest we used the supersweet thaumatin II protein gene from the tropical plant katemfe (*T. daniellii).* In addition to obtaining marker-free plants, another important task in our study was to ensure a high level of protein accumulation in tomato fruit. Therefore, to control the expression of the thaumatin II gene, we chose the recently characterized strong promoter of the tomato *ELIP* gene (Timerbaev and

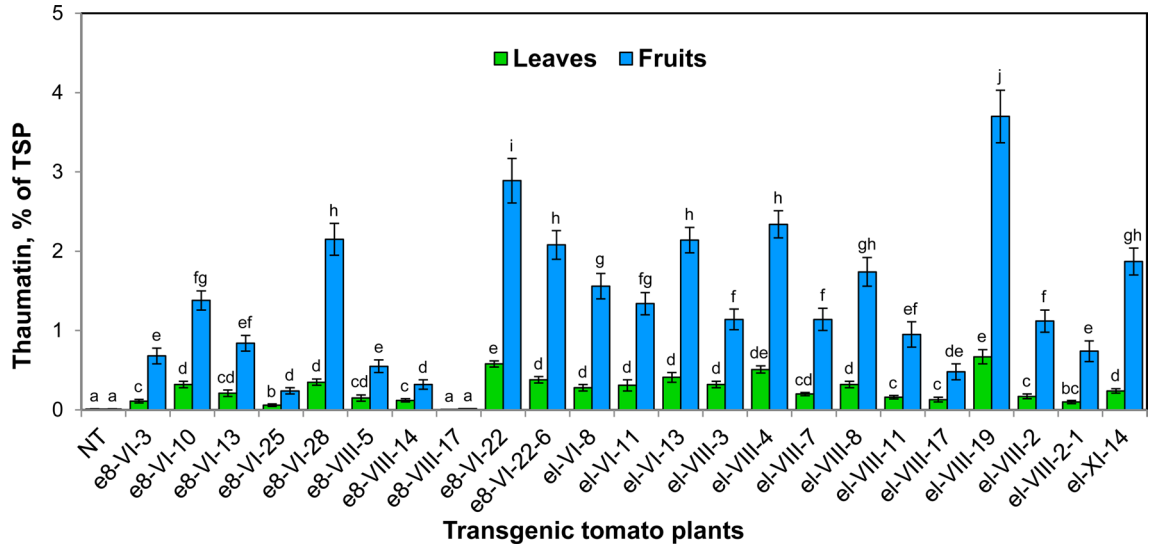

Fig. 8 Quantitative enzyme-linked immunosorbent assay for thaumatin II in tomato lines. NT, nontransgenic control; el-VIII-2-1 and e8-VI-22-6, sublines obtained using delayed selection; el-XI-14, the line obtained with early selection; other numbers, primary transgenic tomato lines. Statistical analysis was performed using one-way analysis of variance. Values represent the mean ± standard deviation from three replicates per transgenic line. Different letters above the bars indicate significant differences at $p < 0.05$

Dolgov 2019). Since this is the first report of using the *ELIP* promoter for directed heterologous expression, we also used the well-proven classical tomato *E8* promoter as a positive control, produced on the basis of the pMF1 vector we used for *Agrobacterium*-mediated transformation of tomato.

Vector system manufacturers offer two variants of marker-free plant selection, immediate and delayed. In our study we used both. The low efficiency of rapid selection without bringing to transgenic plants, in which out of 155 putative transgenic calluses only one marker-free plant was obtained, is due primarily to the heterogeneity of the calluses used in the experiments. Despite careful selection and visual control, we failed to obtain calluses that did not contain nontransgenic cells. This led to the fact that 72% of plants turned out to be nontransformed shoots. Twenty-seven percent of the remaining shoots that overcame selection contained genes from an undesirable region of T-DNA, which indicates the weak selective pressure of the chosen concentration of 5-FC. Despite the fact that in preliminary experiments at 250 mg/L, regeneration was completely absent (Fig. 2), for the early selection strategy this concentration was insufficient. In addition, the achieved results indicate the low efficiency of recombinase-mediated excision. To understand why this is happening as well as compare the approaches, we obtained stable tomato transformants. Thorough analysis of transgenic plants revealed that almost half of them did not contain the complete T-DNA sequence, with mainly the RS site missing near the left border. This result corresponds to the data that most T-DNA integrations in tomato require sequence homology between the left T-DNA border (LB) and plant target DNA (Thomas and Jones 2007). Similar DNA structures in the proposed integration mechanism were also found in *Nicotiana tabacum*, which suggests a common mechanism for Solanaceous species. The principal requirement of the presence of both recombination sites for successful DNA excision makes it especially critical to obtain plants with full T-DNA inserts. Therefore, only lines with complete T-DNA inserts were chosen for recombinase activation and marker-free plant selection. Most of them (13 of 24) produced 5-FC-resistant shoots, among which only one was marker-free. Despite the fact that most of the remaining sublines lost the ability to grow on kanamycin, they contained nonfunctional fragments of the *nptII* gene. We suppose that an incomplete excision and chromosomal rearrangements owing to the presence of multiple and aberrant or partial T-DNA insertions occurred in these cases. Possible variants of chromosomal rearrangements after recombinase activation in events with multiple inserts were described by Krens et al. (2010). The requirement of sequence homology between the LB and tomato DNA for complete T-DNA integration, as well as the likelihood of multiple inserts with high transformation efficiency (up to 47%), makes tomato a rather difficult object to use for specific removal of selective genes. Nevertheless, using two strategies, we managed to obtain two fully marker-free tomato lines, demonstrating the accumulation of thaumatin protein in fruit under the control of both promoters. Here it was shown also that there were no analyzed regulatory elements of viral and bacterial origin in the subline genomes as well as the mRNA of selective genes. Despite the more than 20-year history

of the development and use of site-based specific recombinase systems, not much research has been done with tomato. Among them are reports of successful use of the Cre/loxP DNA excision system (Zhang et al. 2006, 2009; Ma et al. 2008) and R/RS site-specific recombination based on the multi-auto-transformation vector system (Khan et al. 2011).

To our knowledge this is the second report on the absence of strict fruit-specificity of the *E8* promoter. It was previously shown that the absence of 1.1 kb*E8* promoter activity was observed not only in the leaves, but even in unripe fruit (Deikman et al. 1992; Good et al. 1994). Here, gene expression under its control was detected in the leaves at the mRNA and protein levels. Timerbaev et al. (2019) also showed an accumulation of thaumatin mRNA in apple leaves under the control of a tomato *E8* promoter. For apple, this promoter is heterologous, which may partly explain gene expression under its control in the leaves. There is an assumption that certain terminators can not only enhance the transcription and translation of a gene of interest but also change the tissue specificity of a promoter. For example, the use of a combination of the *E8* promoter and the heat shock protein (HSP) terminator not only increased the expression level of miraculin (a glycoprotein sweetener) to values higher than for the 35S promoter, but also led to the appearance of expression in mature green fruit (Kurokawa et al. 2013). It was concluded that HSP can disrupt the tissue-specificity of expression of *E8* gene promoter. It is also possible in our case that the combination of the *E8* promoter and the terminator of Rubisco small subunit gene (*rbcS3A)* provides a similar effect. There are also reports of the successful use of the Rubisco terminator in combination with its promoter. For example, using the promoter and terminator of the *rbcS1* gene of chrysanthemum, expression levels increased eightfold compared to the constitutive CaMV35S promoter (Outchkourov et al. 2003). Similar combination for apple resulted in the same expression level as provided by the 35S promoter (Schaart et al. 2011). The most abundantly expressed *rbcS* gene contributes 5 to 6% of total soluble protein (Outchkourov et al. 2003). The values we achieved were slightly less and represent 3.7% the *ELIP* promoter, which indicates almost the maximum efficiency of the created expression cassette. In most works devoted to the expression of foreign protein genes in plants with stable nuclear transformation, the accumulation does not usually exceed 1 to 2% of TSP (Floss et al. 2007). We have previously obtained transgenic tomato plants with the thaumatin gene under the control of the 35S promoter (Firsov et al. 2012). The amount of protein accumulation in ripe fruit ranged from 1.8 to 4.6% of TSP. High concentrations of thaumatin under the control of the *ELIP* promoter obtained in the present study correspond to data where the activity level of the reporter gene *GUS* driven by full-version (2165 bp) of the *ELIP* promoter was comparable to that provided by the CaMV35 promoter in red, ripe tomato fruit (Timerbaev and Dolgov 2019).

Conclusions

In about half of transgenic tomato plants obtained using vectors based on the pMF1 system there is incomplete integration of the T-DNA region. Despite the loss of resistance to kanamycin after activation of recombinase, the fragment of the *nptII* gene was detected in most of the selected tomato sublines, which indicates that chromosomal rearrangements owing to the presence of multiple and aberrant or partial T-DNA insertions occur in most cases in transgenic tomatoes.

This is the first report of using the *ELIP* promoter for targeted protein production in tomato fruit. The protocol, developed based on the use of the pMF1 system, is applicable to create marker-free tomato plants, but objective features of T-DNA integration inherent in Solanaceae reduce the efficiency of both approaches to marker-free plant selection. For both approaches increased efficiencies may be obtained by optimizing the conditions for obtaining plants with one full copy of T-DNA.

Thus, using site-specific recombinase and two methodological approaches, we obtained marker-free transgenic tomato lines. Plants did not contain nonplant genetic regulatory elements and accumulated up to 3.6% of the total soluble protein in fruit owing to the activity of the strong *ELIP* promoter. Plants can potentially be producers of supersweet thaumatin II protein to meet the needs of the food industry.

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Author contributions VT designed the study, constructed the transformation vectors, obtained transgenic and marker-free tomato plants, performed the PCR and RT-PCR, and wrote the manuscript. AP performed the Southern blot and ELISA. SD supervised the project and provided financial support. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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