

Regulation of Carotenoid Content in Tomato by Silencing of Lycopene β/ϵ -Cyclase Genes

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Abstract To conduct RNAi interference of *Lyc- β* and *Lyc- ϵ* genes, two plant expression vectors were constructed by inserting the intron fragments of the *gusA* gene into the two target gene fragments, which were designed in anti-sense directions. After the *Agrobacterium tumefaciens*-mediated transformation, 13 transgenic tomato plants (seven and six for *Lyc- β* and *Lyc- ϵ* , respectively) were obtained, which was further validated by PCR. Real-time PCR revealed that the messenger RNA abundance of *Lyc- β* gene and *Lyc- ϵ* gene in transgenic tomato plants was significantly reduced to 8.95% and 13.16%, respectively, of the level of the wild-type plant. Subsequent high-performance liquid chromatography analysis found that transgenic tomato plant had significantly increased lycopene content, with the highest value of 13.8 $\mu\text{g/g}$ leaf dry weight, which was about 4.2-fold that of wild-type plant. Moreover, *Lyc- β* and *Lyc- ϵ* interference gene effects were observed on downstream products as well. β -Carotene and lutein contents decreased in *Lyc- β* RNAi lines, ranging from 40.7 to 117.3 $\mu\text{g/g}$ and 4.9 to 23.5 $\mu\text{g/g}$ leaf dry weight, respectively. In *Lyc- ϵ* RNAi lines, β -carotene content increased, ranging from 195.8 to 290.2 $\mu\text{g/g}$, while lutein content decreased, ranging from 3.7 to 11.3 $\mu\text{g/g}$. For total carotenoids, *Lyc- β* RNAi lines resulted in 2.9-fold decrease, while *Lyc- ϵ* RNAi lines yielded 1.7-fold increase in contents when compared to wild-type control. This study

demonstrated that RNAi gene technology is an effective method for enhancing lycopene content in plants.

Keywords β -Carotene · Lutein · Lycopene · Lycopene β -cyclase · Lycopene ϵ -cyclase · RNAi

Introduction

Carotenoids are groups of isoprenoid-derived molecules widely produced by photosynthetic organisms, bacteria, and fungi. The key step of carotenoid biosynthesis is a head-to-head coupling of two molecules of GGPP to yield phytoene (15-*cis*-phytoene, a colorless C40 compound) by phytoene synthase (PSY), with prephytoene diphosphate as an intermediate (Galpaz et al. 2006). Phytoene is converted to all-translycopene (a red pigment) by four desaturation reactions [mediated by phytoene desaturase (PDS) and ζ -carotene desaturase (ZDS)] and by an isomerization reaction (mediated by CRTISO), thus compound lycopene (Howitt and Pogson 2006). Finally, lycopene is metabolized by lycopene β -cyclases (LYC- β) and lycopene ϵ -cyclases (LYC- ϵ) producing α -carotene or β -carotene that are precursors of xanthophylls or abscisic acid (Baroli and Niyogi 2000; Howitt and Pogson 2006; Cunningham and Gantt 2001; Aluru et al. 2008) (Fig. 1).

Many genes of the carotenoid biosynthetic pathway have been cloned and manipulated in a large group of plants. In maize, overexpression of the bacterial genes *crtB* (for phytoene synthase) and *crtI* (function as PDS and ZDS in plants), significantly increased total carotenoids (34-fold) with the preferential accumulation of β -carotene in the maize endosperm (Aluru et al. 2008). In tomato, overexpression of the LYC- β and β -carotene hydroxylase remarkably increased β -carotene, β -cryptoxanthin, and

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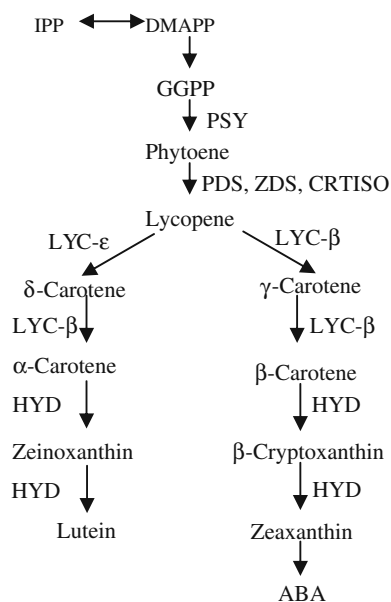


Fig. 1 Carotenoid biosynthetic pathway in plant. The pathway shows the primary steps found in many plant species. *Solid arrows* represent react steps. The following intermediate compounds and enzymes are represented. *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate, *ABA* abscissic acid, *PSY* phytoene synthase, *PDS* phytoene desaturase, *ZDS* ζ-carotene desaturase, *CRTISO* carotenoid isomerase, *LYC-β*, lycopene β-cyclase, *LYC-ε* lycopene ε-cyclase, *HYD* carotene hydroxylases

zeaxanthin (Dharmapuri et al. (2002). Also in tomato, suppression of *Def1* gene (an endogenous photo morphogenesis regulatory gene) increased notably both carotenoid and flavonoid contents (Davuluri et al. 2005), while suppression of the zeaxanthin epoxidase (*ZEP*) gene (which converts zeaxanthin to violaxanthin) resulted in the reduction of abscissic acid content in transgenic plants (Galpaz et al. 2008).

Tomato is a good source of carotenoids with especially high lycopene content. Therefore, regulating the biosynthetic enzyme in the carotenoid biosynthetic pathway through genetic engineering is a convenient and feasible manner to increase the content of lycopene in tomato fruits. The increase in lycopene content in tomato has been attempted by different strategies. Most of the studies carried so far mainly through overexpression methods to achieve an increase in plant carotenoids (Fraser et al. 2002; Diretto et al. 2007a). On the other hand, some studies started to manipulate the contents of carotenoids in plants using gene silencing techniques (Rosati et al. 2000; Wan et al. 2007). Carotenoid biosynthesis pathway is one of important biosynthetic pathway in plant. It is known that a certain number of secondary metabolites can accumulate in plants through the manipulation of related enzymes in carotenoid biosynthesis pathway. In the carotenoid biosynthesis pathway, lycopene can be cyclized by *LYC-β* and *LYC-ε* enzymes to produce β- and α-carotene. Current research

mainly focused on the overexpression and silencing of the upstream genes, such as *Psy* and *Pds*. Ronen et al. (2000) reported that increasing the *Lyc-β* gene expression may effectively increase β-carotene content in the tomato fruit and the lycopene content drops at the same time. By overexpressing an additional bacterial *crtB* in tomato, Fraser et al. (2002) increased phytoene, lycopene, carotenoids, and lutein levels in transgenic plant. Overexpressing of tobacco *Psy* gene increased the content of lycopene (Busch et al. 2002), but the transgenic plants showed abnormal morphology and growth, and lethal phenotypes. This problem was solved in potato by overexpressing them under tissue-specific promoters (Diretto et al. 2007a). Recently, RNA interference technology has been used to increase carotenoids in plants. By silencing *Lyc-β* gene expression in tomato, Wan et al. (2007) found that the transgenic plants had a maximum 3.2-fold lycopene as that of wild-type plants as measured by spectrophotometer. In potato, silencing *Lyc-ε* gene increased β-carotene and total carotenoids 14- and 2.5-fold, respectively (Diretto et al. 2006, 2007b). Interestingly, silencing β-carotene hydrogenase (*HYD*) gene in the background of *Lyc-ε* RNAi plants a significant increased β-carotene and total carotenoids content (38- and 4.5-fold, respectively).

Presently, most studies undertaken to increase carotenoid content in plants focused on overexpression of the upstream genes, such as *Psy* and *Pds*, in the carotenoid biosynthetic pathway. In this study, two RNAi expression vectors were constructed to silence *Lyc-β* and *Lyc-ε* genes, respectively, and then separately transformed into tomato. The relative messenger RNA (mRNA) abundance of *Lyc-β/ε* genes and the contents of lycopene, β-carotene, lutein, and total carotenoids were analyzed in different transgenic plants.

Materials and Methods

Construction of Vectors

The total RNA was isolated from tomato leaves with UNIQ-10 Column RNA Isolation kit (Sangon, SK1322). Reverse transcript assay was performed using the MMLV Reverse Transcript Kit (Sangon, K1622). PCR primers used for the amplification of *Lyc-β* (X-1S and X-1AS), *Lyc-ε* (Y-1S and Y-1AS) genes and *gusA* (*GusA-1S*, *GusA-1AS*, *GusA-2S* and *GusA-2AS*) gene intron fragments were listed in Table 1. The Pfu PCR assays, for the amplification of the *Lyc-β* and *Lyc-ε* gene, were carried out in 50 μL reactions containing a Super High PCR master [3.0 mM MgCl₂, 0.2 mM dNTP, and 2.5 U Super Pfu DNA polymerase (Sangon SK2084)], 0.4 μM of each primer, and 2.5 μL reverse transcript products. The PCRs were carried out in a MyCycle Thermal cycler System (Bio-Rad).

Table 1 The primers of amplification for the *Lyc-β*, *Lyc-ε*, and *gusA* fragments

Primer name	Site	Primer sequence(5'–3')	Addition restrict site
X-1S	480–499 (X86452)	TTCTCGAGCTATGGTGTGGGTGGATG	<i>Xho</i> I
X-1AS	757–781 (X86452)	TAGAGAAGCCAGTTGCATCGAGCAC	\
Y-1S	347–364 (Y14387)	AACTCGAGTCCTGCTGGTCTTGCTCT	<i>Xho</i> I
Y-1AS	631–648 (Y14387)	CAATCCTATCCACTTTCG	\
GusA-1S	21–42 (AF234316)	CCGGATCCATTCTAGTTTTTCTCCTTCAT	<i>Bam</i> HI
GusA-1AS	167–188 (AF234316)	TTAGATCTATCATAGACACACGAAATAAAG	<i>Bam</i> HI
GusA-2S	11605–11622 (AF234316)	ATTTGGAGAGAACACGGG	\
GusA-2AS	190–207 (AF234316)	TCTGTAACATATCATCATC	\
Ubi-S	92–111 (EU294350)	CAGATCTTCGTCAAAACCCT	\
Ubi-AS	263–282 (EU294350)	GACTCCTTCTGGATGTTGTA	\

The PCRs condition were as follows: one cycle denaturalization step at 94°C for 3 min, 30 cycles of denaturalization step at 94°C for 45 s, primers annealing at 59°C (X-1S and X-1AS)/52°C (Y-1S and Y-1AS) for 45 s and extension at 72°C for 30 s, with the final extension step of 72°C for 7 min. Amplification products were run in a 1% (w/v) TAE agarose gel and visualized by staining with 0.5 μg ethidium bromide per milliliter. The amplification products were cloned into pUCm-T vector (Sangon), and its identification was verified by DNA sequencing (Sangon Company). The plasmids were referred to as pPUCM-X-1 and pPUCM-Y-1 separately. For the amplification of the fragment of *gusA* gene intron, the PCR primers GusA-1S/GusA-1AS and GusA-2S/GusA-2AS were used with the annealing temperature at 57°C and 50°C separately. The PCR products were amplified using the same PCR conditions. The plasmids of pGusA-1 and pGusA-2 were then obtained after the cloning and sequencing verification.

The pPUCM-X-1 was digested with *Xho*I and *Bam*HI, and then the small *Xho*I–*Bam*HI fragments were inserted into pPUCM-X-1 at the *Sal*I–*Bam*HI sites. Following the digestion with *Bam*HI and its dephosphorylation, this constructor was inserted into a *Bam*HI–*Bam*HI intron fragment of pGusA-1 to generate pPUCM-X1-RNAi. To that end, a series containing a *gusA* gene intron flanked by *Lyc-β* fragments were obtained. Final assembly of plant expressive vector was performed as follows. The pPUCM-X1-RNAi was digested with *Hind*III following the blunting with Klenow enzyme, and then it was digested with *Sac*I. The small fragments were inserted into the p2300-121 expressive vector (Ma et al. 2009) at *Sac*I–*Sma*I sites. The resulting plasmid containing the expression cassettes of the P35s-*Lyc-β*-Tnos in series was designated as p2300-121-X1-RNAi (Fig. 2a). Following the comprehensive digestion analyze, the vector p2300-121-X1-RNAi was verified by sequencing and used for the interference of *Lyc-β* gene. For construction of the *Lyc-ε* gene interference vector, pPUCM-Y-1 and pGusA-2 were used to construct

p2300-121-Y1-RNAi (Fig. 2b) using the same strategy as described above.

Genetic Transformation

Agrobacterium tumefaciens-mediated transformation of tomato (*Solanum lycopersicum* Mill. cv. Leeger 87-5) was performed following the protocol described by Zhang et al. (2006). Kanamycin-resistant plantlets were grown under controlled greenhouse conditions at 25°C and 50% relative humidity. Plants were grown under 16/8 h (light/dark) photoperiod with supplementary light provided by 400 W fluorescent tubes (200 μmol m⁻² s⁻¹).

Screening of Transgenic Plants

The genomic DNA was extracted from the transgenic and wild-type tomato leaves with UNIQ-10 Column DNA Isolation kit (Sangon, SK1204). The fragments of P35s promoter, used for screening of transgenic plants, was amplified with the primers 5'-AGGCTTACGCAGCAGGTC-3' and 5'-GCAGAGGCATCTTCAACG-3'. The

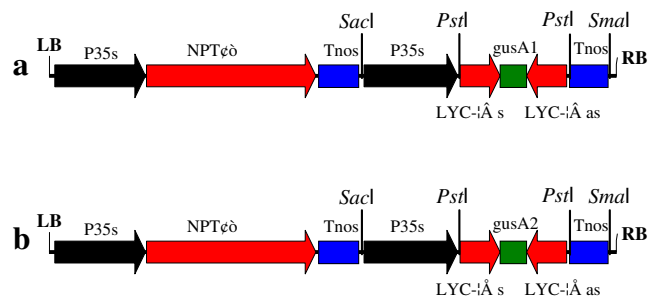


Fig. 2 The diagram of *Lyc-β* (a) and *Lyc-ε* (b) genes' RNAi vectors. P35s-NPTII-Tnos represent the antibiotic resistance gene used as selectable marker. The *Lyc-β/ε* S-gusA1-*Lyc-β/ε* AS represents the *Lyc-β/ε* genes RNAi sequences; it is promoted by P35s and terminated by Tnos. LB and RB represents left and right borders respectively

PCR amplifications were carried out in 20 μ L reactions containing a PCR buffer (500 mM KCL, 100 mM Tris/HCL, pH 9), 1.5 mM $MgCl_2$, 0.4 mM dNTP, 0.2 mM each of primer, 1 U of *Taq* DNA polymerase, and 100 ng DNA template. The condition of PCR reactions were as follows: one cycle denaturalization step at 94°C for 5 min, 30 cycles of denaturalization step at 94°C for 45 s, primers annealing at 55°C for 45 s, and extension at 72°C for 1 min, with the final extension step of 72°C for 7 min.

Real-Time PCR Analysis

Total RNA was extracted from transgenic and wild-type tomato leaves, and its concentration and purity were calculated by measuring A260 and A280 value with Nano Drop Meter (Gene Cop). Two micrograms of total RNA was reverse transcribed into complementary DNAs (cDNAs). Real-time PCRs were performed in an optical 96-well plate with the Mx3000sp QPCR system (Stratagene). The primer X-1S/X-1AS and Y-1S/Y-1AS were used to determine the abundance of *Lyc- β* and *Lyc- ϵ* genes. The ubiquitin (*ubi*) gene, amplified by primer UBI-s/UBI-as, was used as the reference to normalize data. Twenty-microlitre reactions contained 10 μ L SYBR® Green Real time PCR Master Mix, 0.4 μ L of each primer (20 mM), and 1 μ L previously synthesized cDNA. The following standard thermal profile was used for PCRs: one cycle denaturalization step at 94°C for 30 s, 40 cycles of denaturalization step at 94°C for 5 s, primers annealing at 59°C (X-1S and X-1AS)/52°C (Y-1S and Y-1AS)/55°C (UBI-S and UBI-AS) for 5 s and extension at 72 for 20 s. A dissociation step was performed after amplification to confirm the presence of a single amplification. Three technical replicates were carried out at the same time. The standard curve was made by the 10-fold dilutions of pPUCM-X-1, pPUCM-Y-1, and pUBI vectors ranging from 10 to 10⁶ copy number, respectively. The standard curves were as follows: pPUCM-X-1, $Y = -3.464 \times \log(X) + 47.70$ (Eff.=94.4%; RSq, 0.996); pPUCM-Y-1, $Y = -2.273 \times \log(X) + 42.38$ (eff.=175.4%, RSq, 0.994); pUBI, $Y = -3.348 \times \log(X) + 45.28$ (Eff.=98.9%; RSq, 0.999). The gene copies were calculated through the standard curves, and then converted into gene concentration by the following formulae: (gene concentration)=(gene copies) \times (plasmid length) \times 660 \times 6.02 \times 10²³. At last, the mRNA relative abundance of target gene equaled the ratio of target gene concentration and reference gene concentration (Schmidt and Parrott 2001).

Carotenoids Extraction and HPLC Analysis

Carotenoids were extracted from 0.5 g leaves of transgenic and wild-type tomato separately. A solvent extraction system assisted by ultrasonication was run at room temperature. For the extraction of lycopene, the methanol was firstly added into

samples and filtered until all the color was removed from the plant materials. The toluene of 5 mL was used as the solvent, and the extraction was performed for 30 min at 20°C under a 200-W ultrasonic power (Kunshan). The insoluble materials were removed by centrifugation (4,000 \times g for 4 min). The final extracts were filtered through nylon filters, 0.45 μ m pore size, and stored at -70°C. All of the above steps were done in dark. Chromatography was carried out with an Agilent liquid chromatography system (Agilent 1100). Lycopene in tomato leaves was determined by reversed phase high-performance liquid chromatography (HPLC) using an Agilent C18 column (150 \times 4.6 mm and 5 μ m particle diameter) at 20°C, at with methanol/acetonitrile/dichloromethane (7:7:2 v/v) at 1 ml/min. The eluate was monitored at 472 nm. Peak was identified by its retention time, and absorption spectra was compared to the known standards (Sigma-Aldrich, L9879). Amounts were quantified using the standards curve in concentrations of 20, 10, 2, 1, and 0.2 μ g/mL. Three replicates were performed to decrease the experiment error.

For the extraction of β -carotene and lutein, the petroleum was used as the solvent. The ultrasonic power was exchanged at 140 W, and the acetonitrile/dichloromethane/isopropanol (8:1:1 v/v) was used as mobile phase in differently. The peak assignment of β -carotene and lutein, monitored at 450 nm, was based on the retention time of their commercial standards (Sigma-Aldrich, C9750, X6250).

Results

RNAi Vectors

According to the sequences of *LYC- β* (X86452) and *LYC- ϵ* (Y14387) genes, two segments of 302 bp were amplified using PCR from tomato leaf total RNA. At the same time, the partial fragments (168 bp) and the full length fragments (238 bp) of the *gusA* first intron were obtained from the plasmid pCAMBIA-2301 by PCR. All the cloned fragments were subcloned and sequenced, with the 100% homology to the corresponding gene fragments.

Using pCAMBIA-2300, two RNAi expressive vectors were constructed to silence *LYC- β* and *LYC- ϵ* genes separately. The recombinant vector constructs is shown in Fig. 2. After the confirmation by sequencing and enzyme digestion analysis, the resultant plant expressive vectors were then used for the transformation into tomato plants.

PCR Detection on Transgenic Tomato

The transformed plants were selected firstly based on their resistances to the kanamycin. Total 15 resistance plants were obtained to perform PCRs detection by the amplification of

35s promoter. The predicted 35s fragments of 550 bp were amplified in plasmid pCAMBIA-2300 (positive control) and transgenic tomatoes but not in the wild-type tomatoes (negative control). Finally, 13 transgenic plants were obtained (Fig. 3). These transgenic tomatoes were planted in the glasshouse; they did not show any visible abnormal phenotype, and all exhibited the same growth as wild-type plants, indicating that the RNAi of the *Lyc-β* or *Lyc-ε* gene does not affect the plant growth.

Lyc-β and *Lyc-ε* Genes mRNA Abundance

To examine the effect of RNAi on the expression of the *Lyc-β* and *Lyc-ε* in transgenic plants, real-time PCR was performed on six transgenic plants for each gene. The target gene and the reference gene were detected at the same time. The value of ΔC_t [(target gene)–(reference gene)] of the transgenic lines were higher than that of the wild type (Fig. 4c). The dissolution curves showed that the reaction was independent, and all reactions had only one PCR production (Fig. 4d). The endogenous *Lyc-β* or *Lyc-ε* genes relative abundance was analyzed separately, while the *ubi* gene was used as reference gene to eliminate the error of reverse transcription reactions. As shown in Fig. 5, reverse transcription PCRs revealed that mRNA abundance of *Lyc-β* and *Lyc-ε* were significantly reduced in all tested 12 RNAi plants. Transgenic plant line X1-2 exhibited the lowest mRNA abundance of *Lyc-β* gene, which was only 1.44% of that of the wild-type plants. Silencing *Lyc-β* gene did not change the mRNA abundance of *Lyc-ε* gene. Transgenic plant line Y1-3 exhibited the lowest mRNA abundance of *Lyc-ε* gene, which was 2.98% of that of the wild-type plants. Interestingly, silencing *Lyc-ε* gene increased slightly the mRNA abundance of *Lyc-β* gene, with the line Y1-3 rising to 116% of that of the wild-type plants. All in all, the average of the reduction of the mRNA abundance of *Lyc-β* gene by the transformation of p2300-121-X1-RNAi was 91.05%, while the average of the reduction of the mRNA abundance of *Lyc-ε* gene by the transformation of p2300-121-Y1-RNAi was 86.84%. Compared with the p2300-121-Y1-RNAi, the p2300-121-X1-RNAi vector showed a better silencing effect.

Lycopene, β -carotene, Lutein, and Total Carotenoids Content

The lycopene, β -carotene, and lutein of tomato leaves from individual transgenic lines were analyzed through HPLC. Lycopene contents in the all six *Lyc-β* RNAi transgenic plants were increased significantly, with the transgenic line X1-2 showing the highest (13.8 $\mu\text{g/g}$). The average content of lycopene of *Lyc-β* RNAi transgenic plants was 7.1 $\mu\text{g/g}$, which was 2.2-fold of that of the wild-type plants (3.2 $\mu\text{g/g}$). The silencing of *Lyc-β* gene also impacted the production of downstream substances in carotenoids biosynthesis pathway. The contents of β -carotene and lutein in transgenic plants were both decreased, ranging from 40.7 to 117.3 $\mu\text{g/g}$ and from 4.9 to 23.5 $\mu\text{g/g}$, respectively. The transgenic line X1-2 had the lowest β -carotene content of 40.7 $\mu\text{g/g}$ (29% of the wild-type plant), while the transgenic line X1-1 had the lowest lutein content of 4.9 $\mu\text{g/g}$ (11.8% of the wild-type plant). Accompanying with the reduction of β -carotene content, the total carotenoids contents of *Lyc-β* RNAi transgenic plants decreased 29.7–70.3%; The transgenic line X1-2 has the lowest total carotenoids content of 64.4 $\mu\text{g/g}$. This means that the total carotenoids content decreased 2.9-fold compared with the 185.1 $\mu\text{g/g}$ from the control plant (Fig. 6a).

The lycopene contents were also increased in *Lyc-ε* transgenic plants. The transgenic line Y1-3 had the highest lycopene content of 8.5 $\mu\text{g/g}$ (2.66-fold of that of the wild-type plants). The average content of lycopene of *Lyc-ε* transgenic plants was 6.2 $\mu\text{g/g}$ (1.94-fold of that of the wild-type plants). It is noteworthy that the silencing of *Lyc-ε* gene exerted different impacts on the downstream substances. The content of lutein decreased 72.3–90.9%. The transgenic line Y1-3 has the lowest lutein content of 3.7 $\mu\text{g/g}$ (9.1% of that of the wild-type plants). Conversely, the β -carotene and total carotenoids increased 38.9–105.8% and 13.2%–65.0%, respectively. The transgenic line Y1-6 had the highest β -carotene contents of 290.2 $\mu\text{g/g}$ (2.06-fold of that of the wild-type plants) and the highest total carotenoids contents of 305.4 $\mu\text{g/g}$ (1.7-fold of that of the wild-type plants; Fig. 6b).

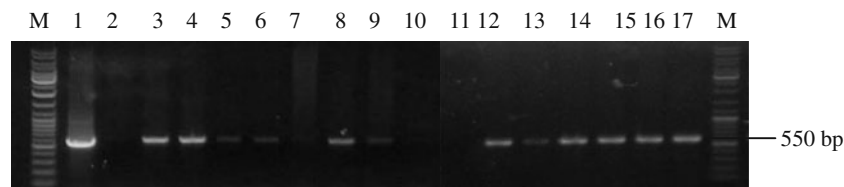


Fig. 3 Photographs of ethidium bromide-stained 1% (w/v) gels carrying PCR products obtained from 35s promoter. The lane labeled M contain the 1 kb DNA ladder (GeneRuler™ DNA Ladder Mix) as size marker; the lane labeled 1 shows the result from plasmid

pCAMBIA-2300; the lane labeled 2 shows the result from wild-type tomato plant; the lanes labeled 3–10 show the results from *Lyc-β* gene RNAi plants; the lanes labeled 11–17 show the results from *Lyc-ε* gene RNAi plants

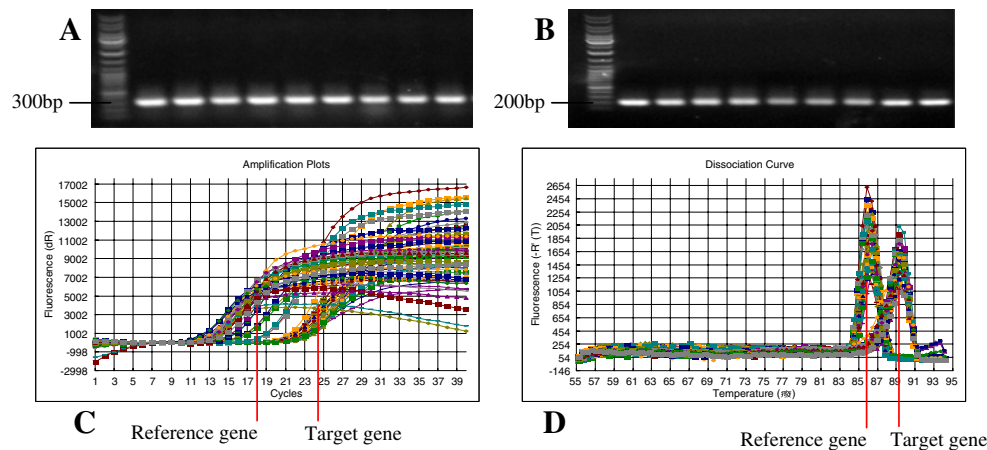


Fig. 4 The real time PCR results. **a** Photographs of ethidium bromide-stained 1% (*w/v*) gels carrying PCR products obtained from the target gene real time PCR products; **b** photographs of ethidium bromide-stained 1% (*w/v*) gels carrying PCR products obtained from the reference gene real time PCR products; **c** the real-time PCR amplification curves. The *X*-axis showed cycle number, and the *Y*-axis show the fluorescence in the amplification curves. The reference

gene abundance is higher than the target gene, so the *Ct* value is lower than the target gene; **d** the real-time PCR dissolution curves. The *X*-axis showed temperature, and the *Y*-axis shows the fluorescence in the dissolution curves. Both of the reference gene and target gene had a single peak in dissolution curves. It means that the product of the amplification was unique; no other products were amplified

Discussion

RNAi technology has become a powerful tool for the study of the functions of individual genes in a range of organisms, including plants, such as *Arabidopsis*, soybean, rice, maize, tomato, potato, and so on (Padmanaban et al. 2004; Subramanian et al. 2005; Miki et al. 2005; McGinnis et al. 2007; Liu et al. 2004; Kim et al. 2008). It becomes a common method for gene silencing. In the siRNA pathway, any of the 21–23 nucleotides' change may disturb the work of the siRNA-targeted mRNA sequence. For vector-mediated RNAi, it has been shown that the RNAi fragment should include complete sequence of intron, and the intron

boundary sequences play an important role in the formation of hairpin RNA structures (Li et al. 2006). The RNAi fragments designed in this experiment was 302 bp, which obtained from tomato through Pfu PCR with a 100% homological rates compared with the target mRNA. Two intron sequences used in the RNAi vectors for silencing *Lyc-β* and *Lyc-ε* gene were different; the one used for *Lyc-ε* RNAi was whole length of the intron sequence, and the one used for *Lyc-β* RNAi did not include its boundary sequence. The results showed that both of them had obvious silencing effects on their corresponding target mRNAs. Overexpressing the RNAi vector with the partial intron sequence resulted in an average of 91.05% deregulation of the expression of the target mRNA, while overexpressing the RNAi vector with the full intron sequence caused an average of 86.84% downregulation of the corresponding target genes. Therefore, the results suggested that the intron boundary sequence may be not the necessary component of the RNAi silence machinery.

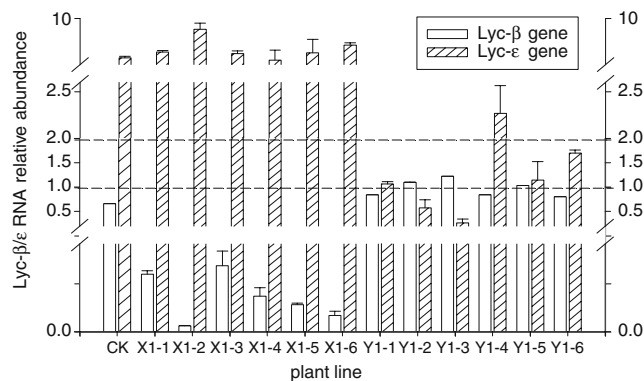


Fig. 5 *Lyc-β/ε* genes mRNA abundance analysis in transgenic tomatoes. The lane labeled CK shows the *Lyc-β/ε* genes relative abundance in wild-type plant; the lanes labeled X1-1 to X1-6 showed the *Lyc-β/ε* genes relative abundance in p2300-121-X1-RNAi vector transgenic tomatoes; the lanes labeled Y1-1 to Y1-6 showed the *Lyc-β/ε* gene mRNA relative abundance in p2300-121-Y1-RNAi vector transgenic tomatoes

Earlier studies revealed that overexpression of genes in the carotenoid biosynthetic pathway enhanced carotenoid content in plants. Moreover, increased downstream components were also observed, but only to limited levels. In this study, using reverse genetics to control *Lyc-β* or *Lyc-ε* gene expression enhanced lycopene content in tomato. Earlier, conserved sequences of interfering segments of *Lyc-β* and *Lyc-ε* genes were used in tobacco first, and subsequently, these were used in tomato plants. In tomato, both *Lyc-β* gene (X86452) and the chromoplast *Lyc-β* gene (AF254793) regulated lycopene degradation. The chromoplasts *Lyc-β* gene was expressed during flower and fruit color development, but the *Lyc-β* gene was expressed at all

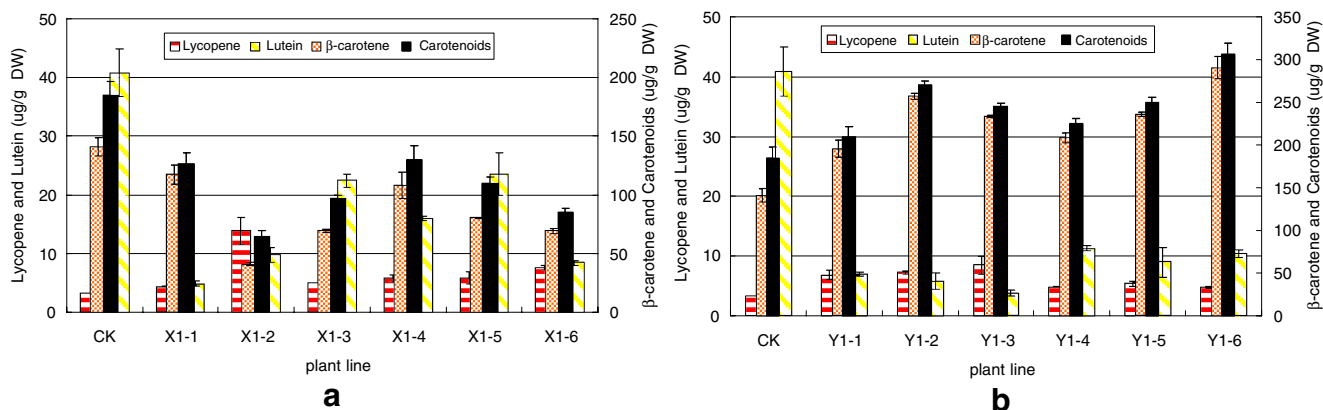


Fig. 6 The alterations in pigment contents between the transgenic plants and the wild-type plants: **a** HPLC analyses of the lycopene, β -carotene, and lutein contents in the p2300-121-X1-RNAi vector transgenic plants; **b** HPLC analyses of the lycopene, β -carotene, and lutein contents in the p2300-121-Y1-RNAi vector transgenic plant. The CK showed the content of three pigments and total carotenoids

stages of plant growth. When the tomato *Lyc- β* gene and chromoplast *Lyc- β* gene sequence were aligned, before amplification of interfering segments, it was revealed that these two genes had 57% homology and without a 21 nucleotide sequence identity. Based on the RNAi protocol, the hairpine structure would be cut by Dicer 4, yielding 21–22nt double strands of small RNA in the cytoplasm. These would bind to Ago1, resulting in degradation of target mRNA in the cytoplasm. Therefore, the *Lyc- β* gene silencing would not have an effect on the function of the chromoplast *Lyc- β* gene. In this study, the *Lyc- β* and *Lyc- ϵ* genes were separately blocked through RNA interference and resulting in high accumulation of lycopene. Both vectors for *Lyc- β* and *Lyc- ϵ* genes used in this study had high degenerating effects on mRNA of target genes, average of 8.95% and 13.16% compared to those of control plants, respectively. Moreover, lycopene content in these transgenic tomato plants, average of 7.07 and 6.2 μ g/g, respectively, did not increase significantly. This might be due to the presence of two branches downstream of the lycopene metabolic pathway. While one branch was blocked in this study, the other proceeded with production of downstream substances. Another likely reason might be attributed to the fact that the chromoplast *Lyc- β* and chromoplast *Lyc- ϵ* genes (that has not yet been reported) could regulate lycopene biosynthesis in tomato, and the *Lyc- β/ϵ* gene interference did not influence the chromoplast *Lyc- β/ϵ* gene, thus resulting in no significant change in lycopene content.

In the carotenoid biosynthesis pathway, the lycopene can be cyclized by lycopene β -cyclase and lycopene ϵ -cyclase. The lycopene β -cyclase exists in both of the two branches to yield the downstream substance β -carotene and lutein, but the lycopene ϵ -cyclase only exists in the lutein branch.

in wild-type tomato leaves. X1-1 to X1-6 and Y1-1 to Y1-6 showed the content of three pigments and total carotenoids in different individual transgenic tomato plant lines. The lycopene and lutein contents were measured with the left y-axes; the β -carotene and the total carotenoids contents were measured with the right y-axes. DW, Dry weight

Thus, when the *Lyc- β* gene was silenced, both of the two branch downstream substance was decreased, which is in agreement with the changes in the expression of the corresponding genes (Figs. 5 and 6a). In *Lyc- ϵ* RNAi plants, the changes in the mRNA abundance were consistent with the changes in the downstream substance contents. The changes in total carotenoids depend mainly on the changes in β -carotene in transgenic plants. Silencing *Lyc- ϵ* gene (blocking one way of lycopene catabolism) induced the accumulation of β -carotene, which contributed to the increase in the total carotenoids. On the other hand, silenced *Lyc- β* gene (blocking both ways of lycopene catalism) induced the increase of lycopene, but decreased total carotenoids. Our results supported the efficiency of RNAi in silencing the endogenous gene and confirmed that the silencing either *Lyc- β* or *Lyc- ϵ* gene impacted not only the lycopene content but also the downstream substances. Further study will focus on the transform of both *Lyc- β* RNAi and *Lyc- ϵ* RNAi into tomato plants and will examine their effects on the regulation of carotenoids.

Conclusion

The manipulation of tomato carotenoids can be done through the silencing of either *Lyc- β* or *Lyc- ϵ* gene using vector mediated RNAi technology. The strongest silencing of the target genes was obtained by use intron fragment without the boundary sequence, and the efficiency of the downregulation of the corresponding target genes by RNAi in transgenic plants was significant, which caused obvious changes in the contents of β -carotene, lycopene, total carotenoids, and downstream lutein. All those metabolic changes are consistent with the changes of corresponding

genes resultings from the transformation of the RNAi vectors. Therefore, RNAi is an effective method for enhancing lycopene content in tomato plants.

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