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Isolation of functional RNA from different tissues of tomato suitable for developmental profiling by microarray analysis

Hsin-Mei WANG[1], Wan-Chun YIN[2], Chen-Kuen WANG[3], and Kin-Ying TO[3,*]

[1]*Institute of Plant Biology, National Taiwan University, Taipei 106, Taiwan*
[2]*Institute of Biotechnology, National Tsing Hua University, Hsinchu 300, Taiwan*
[3]*Agricultural Biotechnology Research Center, Academia Sinica, Taipei 115, Taiwan*

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ABSTRACT. An efficient and reproducible method is described for isolating high-quality RNA suitable for microarray analysis from vegetative and reproductive tissues of tomato plants at different stages of growth. This method was based on TRIzol method followed by lithium chloride (LiCl) precipitation and DNase treatment. Using this method, high yields of high-quality and undegraded RNA were obtained as confirmed by spectrophotometric method, gel electrophoresis and fluorescent quality control. The integrity and functionality of RNA isolated by this procedure have been further demonstrated as probe in tomato cDNA microarrays for identification of differentially expressed genes during fruit ripening, as template for cloning full-length cDNAs encoding phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene desaturase (ZDS) and lycopene β-cyclase (LCY) in the carotenoid biosynthesis pathway by the reverse transcription-polymerase chain reaction (RT-PCR), and as material for cDNA library construction.

Keywords: Carotenoid biosynthesis pathway; Fruit ripening; Microarray; RNA extraction; *Solanum lycopersicum*; Tomato; TRIzol reagent.

Abbreviations: cDNA, complementary DNA; **Cy3,** Cy3-dUTP fluorescent dye; **Cy5,** Cy5-dUTP fluorescent dye; **DEPC,** diethylpyrocarbonate; **RT-PCR,** reverse transcription-polymerase chain reaction.

Database Accession Nos: **EF650010** (tomato cv. CL5915 phytoene synthase mRNA), **EF650011** (tomato cv. CL5915 phytoene desaturase mRNA), **EF650012** (tomato cv. CL5915 ζ-carotene desaturase mRNA), and **EF650013** (tomato cv. CL5915 lycopene β-cyclase mRNA).

INTRODUCTION

Isolation of high-quality RNA is a critical step in many molecular biology experiments, such as cDNA synthesis, cDNA library construction, RT-PCR (reverse transcription -polymerase chain reaction), subtractive hybridization, SAGE (serial analysis of gene expression) technology, EST (expressed sequence tags) analysis, or DNA microarray analysis (To, 2000; To, 2004). Various methods have been developed to isolate high-quality RNA in reasonable amounts from plant tissues which may contain high levels of polyphenolic compounds, polysaccharides, pigments and RNase. High salt concentrations in the extraction buffer and an aqueous two-phase system coupled with conventional phenol/chloroform extraction and CsCl centrifugation have been used to isolate and purify RNA from different tissues of pine trees, which are especially rich in polyphenols (Schneiderbauer et al., 1991). By using higher buffering capacity, alkaline pH and polyvinylpyrrolidone (PVP), isolation of high-quality RNA and DNA from cotton plants containing high amounts of phenolic terpenoids and tannins has also been reported (John, 1992). A rapid and facile modification of a hexadecyltrimethyl ammonium bromide (CTAB) method which allows for the preparation of total RNA from recalcitrant materials such as pine needles without the use of toxic chemicals has also been reported (Chang et al., 1993). Based on the CTAB method with modifications, an easy and efficient protocol was developed to isolate high-quality total RNA from taxus and ginkgo (Liao et al., 2004). Another method, using soluble PVP and ethanol precipitation, has been reported and applied to several recalcitrant materials such as ripening grape berries, dry seeds of *Albizia procera* and radish, and leaf tissue of *A. procera* and *Griffonia simplicifolia* (Salzman et al., 1999). Extraction with phenol and polyvinyl polypyrolidone (PVPP), followed by two purifications with LiCl plus a

*Corresponding author: E-mail: kyto@gate.sinica.edu.tw; Tel: 886-2-26533161; Fax: 886-2-26515600.

2-butoxyethanol treatment between the LiCl steps was developed to isolate total RNA from pear plants which contain considerable amounts of plant polyphenolic compounds and polysaccharides (Malnoy et al., 2001). Total RNA and genomic DNA have also been isolated from an Australian native plant, *Hakea actities*, which is grown in low nutrient conditions (Mason and Schmidt, 2002). Hot borate buffer at alkaline pH supplemented with several adjuvants and followed by selective precipitations has been used to isolate functional RNA from small amounts of different grape and apple tissues (Moser et al., 2004). A method consisting of a two-step extraction with NaCl and trisodium citrate at high concentrations, followed by isopropanol and LiCl precipitations, was developed to isolate total RNA from siliques, dry seeds and other tissues of *Arabidopsis thaliana* (Suzuki et al., 2004).

In general, fruits are considered as one of recalcitrant tissues and different methods suitable for isolating fruit RNA have also been developed. A method consisting of a lysis buffer [50 μM aurintricarboxylic acid (an RNA inhibitor), 5 to 10% mercaptoethanol, 5% PVPP, MOPS buffer, EDTA, urea and Triton], followed by phenol/chloroform extraction and LiCl precipitation, was developed to isolate total RNA from tomato fruit (Rodrigues-Pousada et al., 1990). Another method, based on LiCl precipitation followed by CsCl precipitation, has been reported to isolate RNA from blackcurrant fruit, a tissue that contains high levels of phenolic compounds and polysaccharides and the high acidity (pH 2.5 to 2.9) (Woodhead et al., 1997). A protocol consisting of a hot lysis buffer containing 2% sodium dodecyl sulfate (SDS) and phenol with a high buffering capacity (300 mM Tris/Boric acid) and the subsequent use of a high concentration of PVPP (8.5%) to prevent polyphenol oxidation was developed to isolate RNA from the high acidity (pH 3.5 to 4.0) of raspberry fruit (Jones et al., 1997). An extraction buffer containing guanidine chloride and phenol and followed by a three-stage precipitation (NaCl, urea and LiCl, and potassium acetate) has been used to isolate total RNA from carotenoid-rich plant tissues of the Mexican marigold (Chi-Manzanero et al., 2000). A modified method of using CTAB, PVP and β-mercaptoethanol in an extraction buffer has also been reported to eliminate polysaccharides and prevent the oxidation of phenolic compounds in bilberry fruit (Jaakola et al., 2001). Isolation of functional RNA from cactus fruit, which is considered to contain high amounts of secondary metabolites and polysaccharides, using a lysis buffer of 150 mM Tris (pH 7.5) and 2% SDS, followed by phenol/chloroform extraction and LiCl precipitation (Valderrama-Cháirez et al., 2002).

Tomato is an important crop worldwide. It provides abundant sources of antioxidant micronutrients such as β-carotene, lutein, phytoene, phytofluene, γ-carotene, vitamins C and E, and phenolic compounds; some of which may contribute to the health-giving properties of tomato (Dumas et al., 2003). We are interested in the tissue-specific profiling of differentially expressed genes by using microarray approach and engineering of carotenoid biosynthesis pathway in tomato plants. Therefore, we initially need to set up a method for RNA isolation from different developmental tissues of tomato plants, especially in ripening fruits. Although several methods as mentioned above have been described to isolate high-quality RNA from various plant species as well as plant tissues, only one method used in *Arabidopsis* (Suzuki et al., 2004) has been examined at the microarray-based level, which is extremely sensitive to the quality of the RNA (Duggan et al., 1999; Burgess, 2004; To, 2004). Our method is originally based on the use of a reagent containing an acid guanidine thiocyanate-phenol-chloroform mixture (Chomczynski and Sacchi, 1987); this mixture is well-known as TRIzol reagent (Invitrogen; Chomczynski, 1993). Here we report an efficient and reproducible protocol, which is based on the TRIzol method followed by LiCl precipitation and DNase treatment, to isolate high-quality total RNA from various tissues and developmental stages of tomato plants. The quality of the total RNA obtained was then evaluated at the molecular level.

MATERIALS AND METHODS

Plant material

Seeds of tomato (*Solanum lycopersicum* var. CL5915) were kindly provided by the AVRDC-The World Vegetable Center in Taiwan. To obtain synchronized growth, tomato seeds were shaked gently (50 rpm) in a 50-ml tube containing 10 ml water at room temperature for 2 days. Those germinating seeds with visible roots were sown in a mixture of peat and vermiculite, and grown in a walk-in growth chamber under a cycle of 14-h (6:00 a.m. to 8:00 p.m., 25°C) illumination (250 μmol/m^2/s) and 10-h (8:00 p.m. to 6:00 a.m., 20°C) darkness. Different tissues including cotyledons, stems, leaves, roots, flowers and fruits were harvested during different developmental stages (young seedlings, 1-month-old; vegetative growth, 2-month-old; flowering stage, 3-month-old; fruiting stage, 4-month-old) and stored at -80°C until used.

Isolation of total RNA and mRNA from different tissues in tomato plants

For total RNA isolation, the protocol was based on the TRIzol reagent user manual provided by the manufacturer (Invitrogen), followed by LiCl precipitation and DNase treatment. Plant tissues were grinded to a fine powder in liquid nitrogen with a pre-cooled pestle and mortar, and then put separately into a 50-ml plastic screw-cap centrifuge tube. To one gram samples, 10 ml extraction buffer [TRIzol reagent: 38% phenol (USB Cooperation, Cleveland, Ohio, USA) was equilibrated to pH 4.0 with Tris-HCl buffer; 0.8 M guanidine thiocyanate; 0.4 M ammonium thiocyanate; 0.1 M sodium acetate (pH 5.0); 5% glycerol] was added and mixed well. Samples were

incubated for 5 min at room temperature. After incubation, 0.2 ml chloroform was added for each one ml extraction buffer and tubes were shaked vigorously with votex for 15 sec. Tubes were incubated at room temperature for 3 min and then centrifuged at 10,000 *g* at 4°C for 15 min. Aqueous phase was carefully transferred into a clean screw-cap centrifuge tube and then added 0.5 ml of isopropanol for each one ml extraction buffer. Tubes were covered and mixed by gentle inversion, and then sit at room temperature for 10 min. After incubation, tubes were centrifuged (10,000 *g*; 4°C; 10 min) and the supernatant was discarded. For each gram of tissue, 50 µl DEPC-treated H_2O was added to dissolve the pellet, 1/3 volume of 8 M LiCl was added to each tube, and tubes were placed at 4°C overnight. After centrifugation (10,000 *g*; 4°C; 10 min), supernatant was discarded and pellet was dissolved in DEPC-treated H_2O. For each gram of tissue, 0.5 µl RNase-free DNase (10 U/µl; Roche Diagnostics GmbH, Germany) was added and incubated at 37°C for 15 min. Two volumes of isopropanol were then added and mixed. Tubes were centrifuged at 10,000 *g* at 4°C for 10 min. After centrifugation (10,000 *g*; 4°C; 10 min), supernatant was discarded and pellet was washed with 75% ice-cold ethanol, and then re-centrifuged (10,000 *g*; 4°C; 10 min). Supernatant was discarded and RNA pellet was dissolved in RNase-free water. Total RNA concentration was determined by measuring absorbance at 260 and 280 nm. Samples were stored at -80°C until further use.

The Oligotex mRNA spin-column protocol for isolation of poly(A) mRNA from total RNA was based on the manufacturer (Qiagen, Valencia, CA, USA).

Labeling 1st strand cDNA with Cy3-dye for mRNA quality control

In an Eppendorf tube, 2 µl RNA sample containing 80 ng mRNA was mixed with 0.5 µl oligo $d(T)_{23}N$ (2 µg/µl; MD Bio Inc., Taiwan). The tube was incubated at 70°C for 10 min and quickly chilled on ice. After incubation, 1 µl 5X Superscript II buffer (Invitrogen), 0.5 µl 0.1 M DTT, 0.1 µl 50X dNTPs (25 mM dNTPs except dTTP at 10 mM), 0.5 µL Cy3-dUTP (Amersham) and 0.4 µl Superscript II reverse transcriptase (200 U/µl; Invitrogen) were added into the tube and then incubated at 42°C for 2 h. The reaction was cleaned up by a Qiagen PCR clean up kit, and the eluted DNA was dried and the pellet was dissolved in 3 µl TE buffer (pH 8.0). Three µl of 2X loading buffer was mixed with 3 µl of labeled 1st strand cDNA, and run on a 1.2% agarose gel in 1X TAE buffer at 100 V for 30 min. A labeled DNA molecular weight marker was used for comparison.

Labeling λ/HindIII DNA with Cy3 fluorescent dye as molecular size marker

In an Eppendorf tube containing 4 µl Klenow 10X buffer (Amersham), 4 µl 10X dNTPs (0.25 mM each except dTTP at 0.09 mM), 4 µl λ/*Hin*dIII DNA, 2 µl Cy3-dUTP, 2 µl Klenow enzyme (5 U/µl; Amersham) and 24 µl H_2O was incubated at 37°C for 1 h. After incubation, reaction mixture was clean up by a Qiagen PCR clean up kit. The eluted DNA was dried, resuspended in 10 µl TE (pH 8.0) and mixed with 10 µl of 2X loading buffer. Ten µl of mixture was loaded into each well and electrophoresed together with labeled samples on a 1.2% agarose gel in 1X TAE buffer at 100 mA for 30 min.

Quality control gel analysis

After electrophoresis, the gel was trimmed to no more than 25 mm × 75 mm, put on a glass slide (25 mm × 75 mm), and then placed in a 70°C baking oven until completely dry. The dried gel was scanned by a scanner (GenePix 4000B; Axon Instruments, Foster City, CA, USA) under 350 nm and analyzed the data with GenePix version 5.1 software.

cDNA synthesis and labeling for microarray analysis

For the first strand cDNA synthesis, two 0.2-ml Eppendorf tubes were labeled with "1" and "2", and 1 µg poly(A) mRNA of sample 1 and sample 2 were added respectively. To each tube, 1 µg oligo $d(T)_{23}N$ (MD Bio Inc., Taiwan) was added and the final volume was adjusted to 24.5 µl by adding DEPC-treated H_2O. The tubes were incubated at 70°C for 10 min and then immediately transferred to ice. To each tube, 8 µl 5X Superscript buffer (Invitrogen), 4 µl 0.1 mM DTT, 2 µl 10 mM dNTP and 1.5 µl Superscript II reverse transcriptase (200 U/µl; Invitrogen) were added and then incubated at 42°C for 1 h. After incubation, 0.25 µl RNase H (5 U/µl; Amersham) was added and then further incubated at 37°C for 30 min. The reaction product was clean up by a Qiagen column according to the manufacturer. The final volume was adjusted to 28 µl by H_2O.

For the second strand cDNA synthesis and labeling, 28 µl first-strand cDNA product from sample 1 and sample 2 were added to two 0.2-ml tubes labeled with “1” and “2”, respectively. To each tube, 4 µl Klenow buffer and 1 µl random primer (3 µg/µl; Invitrogen) were added and incubated at 100°C for 2 min. Tubes were put at room temperature for 5 min. To the tubes labeled with “1” and “2”, 1 µl Cy3-dUTP (25 nmol; Amersham) and 1 µl Cy5-dUTP (25 nmol; Amersham) were added, respectively. And then to each tube, 4 µl 10X dNTPs (0.25 mM each except for dTTP at 0.09 mM; Amersham) and 2 µl Klenow DNA polymerase (5 U/µl; Amersham) were added and incubated at 37°C for 3 h. The reaction product was clean up according to the user manual of MinElute PCR purification kit (Qiagen). DNA sample was dissolved in 10 µl H_2O.

Microarray analysis

To set up cDNA microarray analysis, approximately 12,000 tomato cDNA clones including approximately 6,000 cDNA clones from four tomato root libraries (mineral deficiency, pre-anthesis stage, post-anthesis stage and

germination seedlings) purchased from Clemson University Genomics Institute, USA, and approximately 6,000 subtractive clones or normal cDNA clones from individual laboratories of Integrative Plant Stress Biology (iPSB) group at the Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan (To, 2004), were amplified by PCR and purified, and then printed onto a slide (25 mm × 75 mm) by an arrayer (Cartesian SynQUAD, USA). The microarray slide was put in an 80°C oven for 5 h and incubated in 250 ml pre-hybridization buffer (25% formamide; 5X SSC; 0.1% SDS; 0.1 mg/ml BSA) for 1 h at 42°C. The slide was washed by immersing in 250 ml extra-pure H_2O twice, rinsed in isopropanol, and then dried by centrifugation (110 *g*, 10 min; Megafuge 2.0R, Heraeus Instruments, Germany). For probe preparation, 12.75 µl hybridization buffer (25% formaide; 5X SSC; 0.1% SDS), 8.25 µl labeled cDNA samples, 2 µl poly(dA)$_{10}$ (10 µg/µl) and 2 µg human Cot-1 DNA (10 µg/µl; Invitrogen) were added into an Eppendorf tube and mixed thoroughly. For hybridization, the probe was denatured at 94°C for 5 min and then incubated at room temperature for 20 min. Slide was placed in the hybridization chamber (HybChamber, GeneMachines), and the probe solution (25 µl) was added onto the center of the array. A cover glass (24 mm × 60 mm) (TaKaRa Space Cover Glass TX705, Takara Bio Inc., Japan) was carefully placed onto the slide, and 4 drops of 10 µl 3X SSC were added on the each lower edge of the slide. After assembling the hybridization chamber, the chamber was gently placed into 42°C water bath for 16 h without shaking.

After hybridization, the slide was placed in a jar containing the pre-warm (42°C) wash I solution (2X SSC; 0.1% SDS) until the cover glass moves freely away. Then the jar was shaked (50 rpm) at room temperature for 5 min. The slide was immediately transferred to wash II solution (0.1X SSC; 0.1% SDS) and shaked (50 rpm) at room temperature for 5 min. The slide was repeatedly washed 2 more times in wash II solution. Afterwards, the slide was placed in wash III solution (0.1X SSC) up and down for 1 min, and repeatedly washed 4 more times in wash III solution. After washing, the slide was rinsed with H_2O and then dried by centrifugation for 5 min at 110 *g*. Slides were scanned (GenePix 4000B scanner; Axon Instruments, Foster City, CA, USA) as soon as possible. Otherwise, slides may be stored at -80°C for 1 to 2 weeks.

RT-PCR analysis

Total RNA samples from different developmental stages of tomato fruits were treated with RNase-free DNase I to eliminate genomic DNA contamination. After enzyme removal by phenol/chloroform, approximately 200 ng of RNA samples were used to perform reverse transcription-polymerase chain reaction (RT-PCR) analysis, using a one-step RT-PCR kit (Qiagen). For gene-specific amplification, primers PSY-F1 (5′-ATGTCTGTTGCCTTGTTATGG-3′) and PSY-R1 (5′-TTATCTTTGAAGAGAGGCAGTTT-3′) specific for tomato phytoene synthase (PSY), primers ZDS-F1 (5′-ATGGCTACTTCTTCAGCT-3′) and ZDS-R1 (5′-TCAGACAAGACTCAACTC-3′) specific for tomato ζ-carotene desaturase (ZDS), primers LCY-F1 (5′-ATGGATACTTTGTTGAAA-3′) and LCY-R1 (5′-TCATTCTTTATCCTGTAA-3′) specific for tomato lycopene β-cyclase (LCY), and primers PDS-F1 (5′-ATGCCTCAAATTGGACTT-5′) and PDS-R1 (5′-CTAAACTACGCTTGCAAC-3′) specific for tomato phytoene desaturase (PDS) were synthesized to amplify full-length reading frames of PSY (1239 bp), ZDS (1767 bp), LCY (1503 bp) and PDS (1752 bp), respectively. Reverse transcription was carried out at 50°C for 30 min. The PCR mixtures (20 µl) were initially denatured at 94°C for 5 min, and then subjected to 35 cycles (30 sec at 94°C, 30 sec at 55°C, 1.5 min at 72°C) with a final extension at 72 °C for 10 min. PCR products (2 µl) were analyzed on 1% TAE-agarose gel stained with ethidium bromide.

RESULTS AND DISCUSSION

RNA isolation from different tissues of tomato plants

To analyze differentially expressed genes in different tissues of tomato during development, we divided plant development into 4 stages, namely the young seedling stage (1-month-old plant), vegetative growth stage (2-month-old plant), flowering stage (3-month-old plant) and fruiting stage (4-month-old plant), as shown in Figure 1A. In addition, the typical 8 stages of fruit development in tomato were also observed (Figure 1B). To simplify the difficulty in collecting each stage of tomato fruits, we roughly divided fruit into 3 classes during tomato fruit ripening: green fruit represented immature and mature green stages, orange fruit represented breaker, turning and pink stages, and red fruit represented light-red, red and over-ripen stages.

Using the TRIzol method coupled with LiCl precipitation and DNase treatment as described, the yield of total RNA ranged from 30 to 857 µg/g of developing vegetative tissues including leaf, stem, and root, as well as cotyledon, and 1184 to 1313 µg total RNA/g of flower tissue (Table 1). For tomato fruits, the yield of total RNA ranged from 118 to 183 µg total RNA/g of green fruit, 15 to 34 µg total RNA/g of orange fruit, and 20 to 32 µg total RNA/g of red fruit (Table 1). Our results were found similar or a little higher than the reported yields of between 20 to 50 µg total RNA per gram fresh weight using protocol developed for tomato fruit (Rodrigues-Pousada et al., 1990), and those reported yields of between 12 and 100 µg total RNA per gram fresh weight using protocols developed for other fruit tissues (Woodhead et al., 1997; Jaakola et al., 2001; Valderrama-Cháirez et al., 2002). The ratio of A_{260}/A_{280} was 1.67 to 2.05 in all samples. In general, the average yield of total RNA in vegetative tissues was higher than in fruit tissues. Agarose gel electrophoresis revealed that two major bands of 28S and 18S rRNA were observed in root, stem,

flower and ripening fruit tissues, and 5 major RNA bands were detected in cotyledon and developing leaf tissues, indicating that the RNA was not degraded (Figure 2).

RNA samples from ripening fruits (Figure 2) were selected to examine the quality of total RNA isolated. The obtained mRNA were further labeled with Cy3 dye and then checked for quality by gel electrophorsis analysis (Figure 3). Abundant messages with high molecular sizes (up to 4 kb) were detected, suggestive of high-quality mRNA.

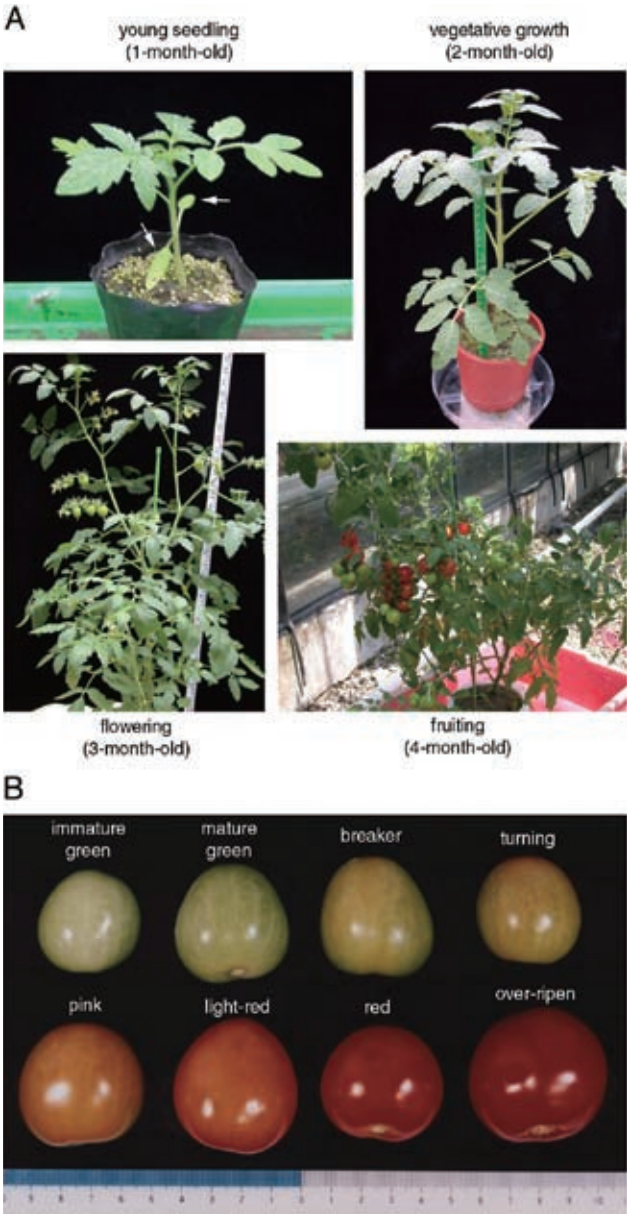

Figure 1. Different developmental stages in plants and fruits of tomato (*Solanum lycopersicum* cultivar CL5915). (A) Growth stages in tomato plants were divided into four stages: young seedling (1-month-old), vegetative growth (2-month-old), flowering (3-month-old) and fruiting (4-month-old). White arrows indicate two cotyledons in a young seedling plant. (B) Typical eight growth stages in tomato fruits.

Table 1. Quantity and quality of total RNA isolated from different tissues in tomato plants.

Sample	Experiment 1				Experiment 2				Experiment 3			
	Fresh weight (g)	Total RNA isolated (μg)	Yield (μg RNA/g FW)	A_{260}/A_{280}	Fresh weight (g)	Total RNA isolated (μg)	Yield (μg RNA/g FW)	A_{260}/A_{280}	Fresh weight (g)	Total RNA isolated (μg)	Yield (μg RNA/g FW)	A_{260}/A_{280}
Coty (1MO)	4	462	116	1.69								
Leaf (1MO)	4	798	120	1.85	7	6000	857	1.83	15	7528	502	2.05
Stem (1MO)	4	270	68	1.79	15	705	47	2.02				
Root (1MO)	7	749	107	1.82	16	2037	127	1.94	24	2460	103	1.82
Leaf (2MO)	12	4100	342	2.00	12	3750	313	1.99	80	26558	332	2.03
Stem (2MO)	11	1925	175	1.88	100	6440	64	1.96	100	9655	97	1.86
Root (2MO)	16	2001	125	1.79	21	3150	150	1.85	60	6161	103	1.84
Leaf (3MO)	12	3528	294	1.88	12	2780	232	1.89	12	2858	238	1.93
Stem (3MO)	15	1463	98	1.94								
Root (3MO)	12	594	50	1.77	24	1138	47	1.84	26	1858	71	1.85
Leaf (4MO)	4	1542	386	2.03	16	3440	215	1.65				
Stem (4MO)	20	600	30	1.78								
Root (4MO)	24	1250	50	1.83	24	1600	67	1.81	24	1840	77	1.78
Flower	8	9470	1184	1.84	10	10500	1313	1.88				
Green fruit	7.5	1374	183	1.68	16	1908	119	1.67	16	1883	118	1.67
Orange fruit	9	306	34	1.79	30	522	17	1.92	80	1162	15	1.76
Red fruit	24	624	26	1.76	100	1967	20	1.99	140	4379	32	1.80

Abbreviations: Coty, Cotyledon; 1MO, 1-month-old; 2MO, 2-month-old; 3MO, 3-month-old; 4MO, 4-month-old.

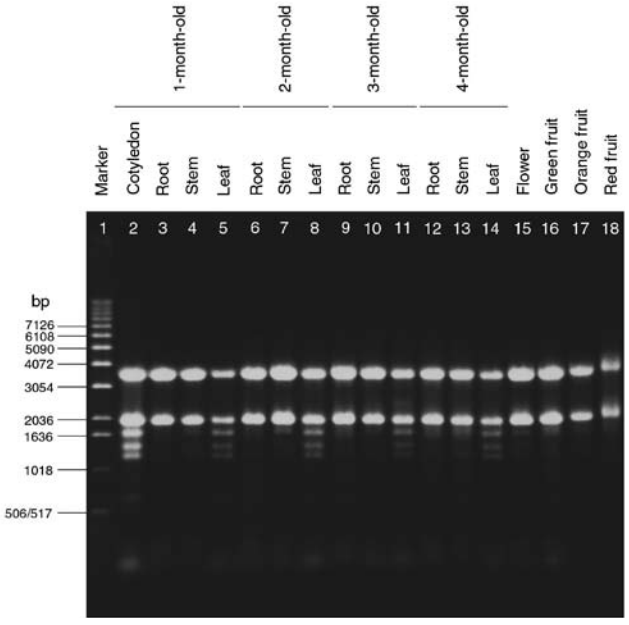

Figure 2. RNA gel electrophoresis analysis of total RNA from tomato. Total RNA from different tissues, as indicated, were isolated. Equal amounts of 20 µg RNA from each sample were separated at a 1.2% agarose gel.

Microarray analysis

To genome-wide identify differentially expressed genes during tomato fruit ripening, the obtained mRNA from green fruit and orange fruit (Figure 1) were labeled with different fluorescent dyes, as indicated in Figure 4A and Figure 4B, and hybridized to our home-made microarray chip containing approximately 12,000 PCR-amplified fragments. After hybridization, signals were detected by a highly sensitive laser scanner and analyzed by GeneSpring software (see the legend in Figure 4). We believe our microarray data are convincing based on dye swap analysis. For example, green spots in Figure 4A are considered as up-regulated genes in green fruit as comparison with orange fruit, since mRNA isolated from green fruit was labeled with a green fluorescent dye Cy3 and mRNA isolated from orange fruit was labeled with a red fluorescent dye Cy5. Therefore, the gene labeled as "1" (green color) in Figure 4A is believed to be more highly expressed in green fruit than in orange fruit. However in Figure 4B, those up-regulated genes in green fruit should appear as red, since the method for probe labeling has been swapped (mRNA isolated from green fruit and orange fruit were labeled with a red fluorescent dye Cy5 and a green fluorescent dye Cy3, respectively). Thus for a particular gene "1" which appears as red Figure in 4B is equivalent to an up-regulated gene in green fruit in Figure 4A. In addition, genes having similar expression pattern between green and red fruits appear as yellow (a third pseudo color representing similar expression levels between two samples was generated by the GeneSpring software), no matter which labeling method was employed (for example, 8 yellow spots labeled with "a" to "h" in Figure 4A and Figure 4B). Furthermore, our microarray data proved reproducible upon comparison with the consistent expression pattern of the repetitious α-tubulin 3 (*tubA3*) sequence among different blocks in our microarray slide (e.g., those spots labeled with "★" in Figure 4A appeared as green, those spots labeled with "★" in Figure 4B appeared as red). Interestingly, we found from this study that the expression of the α-tubulin 3 gene was also affected by development (i.e., higher expression level of this gene in green fruit than in orange fruit). Previously, a gene list including DnaJ-like protein, translationally controlled tumor protein, two α-tubulins (*tubA1* and *tubA3*), cyclophilin and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) had been compiled as so-called "housekeeping" gene candidates in

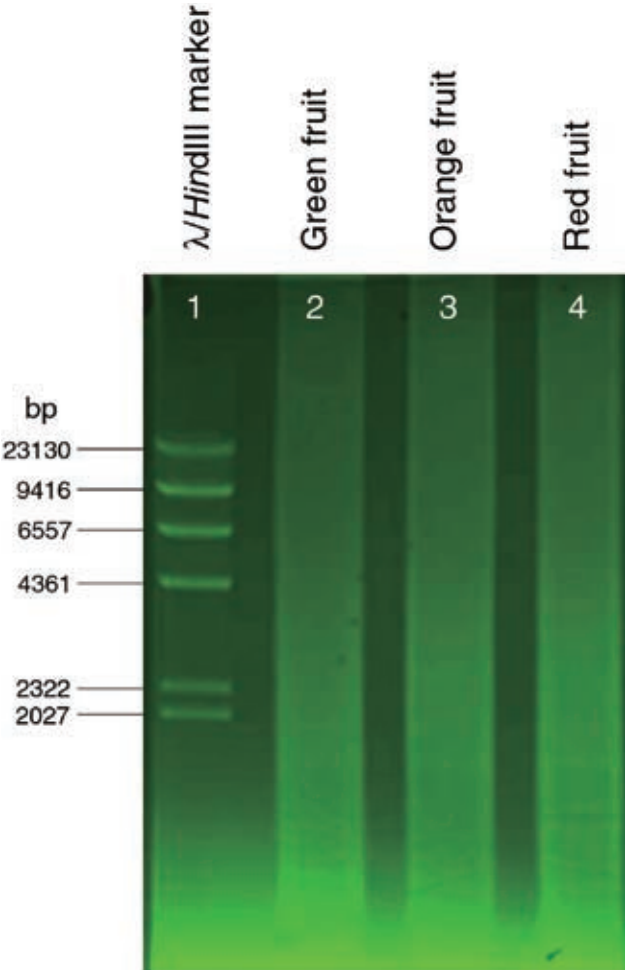

Figure 3. Quality control of poly(A) mRNA. Poly(A) mRNA from green fruit, orange fruit and red fruit of tomato were isolated, equal amounts of 80 ng mRNA were used to label the 1st strand cDNA with a fluorescent dye Cy3, and the labeled 1st strand cDNA products were separated on 1.2% agarose gel in 1X TAE buffer. After electrophoresis, the gel was trimmed, transferred onto a glass slide, and then completely dried in a 70°C oven. Images were obtained by scanning the dried gel with a GenePix 4000B Scanner.

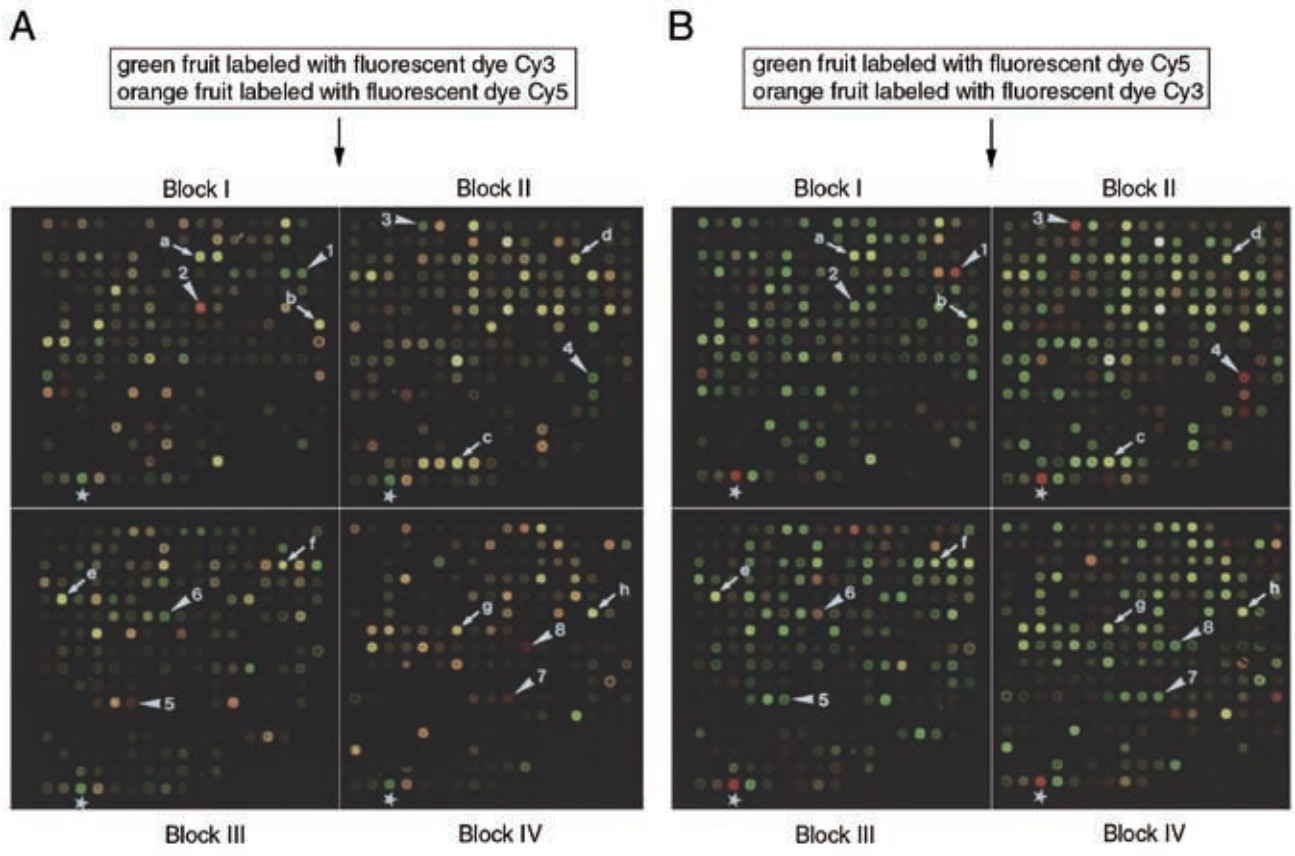

Figure 4. The dye swap method showing reliable reproduction and accuracy in tomato microarray analysis. Poly(A) mRNA from two samples of green fruit and orange fruit to be compared were labeled with fluorescent dyes Cy3 (green color) and Cy5 (red color), as indicated in panels A and B, by reverse transcription. The fluorescent probes were then pooled and allowed to hybridize under stringent conditions to the tomato array containing approximately 12,000 PCR-amplified gene fragments and printed on a coated glass microscope slide by a computer-controlled, high-speed arrayer. After hybridization, highly sensitive laser scanner was used to detect any expression signal in each spot on the slide. Monochrome images from the scanner were imported into GeneSpring software in which the images pseudo-colored and merged. This image represents the relative expression in the two samples we examined (for example, spots in panel A with green color represent relatively high expression in green fruit, while spots in the same panel with red color represent relatively high expression in orange fruit). A third color yellow was introduced to represent a similar expression level between two samples. The selected 8 spots labeled "1" to "8" represent up-regulated or down-regulated genes in one method of labeling probes, as shown in panel A; if the labeling method was effective, the same spots should show up-regulation or down-regulation while another labeling method is employed, as shown in panel B. This procedure is called the "dye swap" method. In parallel, the selected 8 spots labeled "a" to "h" represent similar expression pattern between two samples, as confirmed by dye swap method. Spots labeled with an asterisk (★) in each block represent the same DNA sequence of α-tubulin 3 gene from *Hordeum vulgare*, and were used as an internal control for microarray analysis.

tomato plants using EST and bioinformatic approaches (Coker and Davies, 2003); however, our microarray data clearly showed that α-tubulin 3 gene is not a housekeeping gene in tomato plants, at least it is not suitable for studying tomato fruit ripening.

By analyzing microarray data, we have identified 98 up-regulated genes and 37 down-regulated genes showing at least 5-fold difference in orange fruit as compared to green fruit. Top 10 putative differentially expressed genes are listed in Table 2. Among them, a heat-inducible gene encoding class II small heat shock protein 17.6 kDa from tomato (*LeHSP17.6*) has been characterized and no *LeHSP17.6* mRNA could be detected in mature green tomato fruit (Kadyrzhanova et al., 1998). More recently, a novel tomato small heat shock protein gene (*vis1*) was isolated and showed strong similarity with members of the small heat shock proteins including *LeHSP17.6*, and *vis1* transcripts were barely detected at early stages (e.g., mature green) of fruit development but rapidly accumulated in fruit after onset of ripening with maximum accumulation at the turning stage fruit (Ramakrisha et al., 2003). Taken together, it is reasonable to expect that higher level of *LeSHP17.6* expression was detected in orange fruit than in green fruit by our microarray analysis (Table 2). Currently, we are picking up several clones including *LeSHP17.6* from the gene list for further characterization.

Gene cloning, sequence analysis and construction of cDNA library from the RNA samples

RT-PCR is a sensitive amplification procedure that has been used to detect the presence of a gene in a plant genome. If RNA is a poor template for reverse transcription, it is very difficult or no chance to amplify a longer DNA fragment such as full-length cDNA, and thus, a good cDNA library cannot be prepared. To further prove the integrity of the RNA we isolated, full-length cDNA sequences encoding phytoene synthase (PSY), ζ-carotene desaturase (ZDS), lycopene β-cyclase (LCY) and phytoene desaturase (PDS) in the carotenoid biosynthesis pathway (To and Wang, 2006) were amplified by RT-PCR from total RNA of tomato ripening fruits (Figure 5). All PCR fragments with the expected sizes were detected in RNA samples amplified by gene-specific primers (Figure 5). Furthermore, these PCR products were purified, cloned into pGEM-T Easy cloning vector (Promega), and then sequenced. DNA sequence analysis revealed clearly that our PSY clone is 1239 bp in length and contains a reading frame of 412 amino acids. In comparison with the published tomato *psy1* mRNA (1239 bp; Bartley et al., 1992), a base change at nucleotide position 583 in the coding region of our PSY clone is detected, where an adenine is replaced by a guanine residue. As a result, the corresponding amino acid at position 195 changes from methionine (ATG) to valine (GTG). Different cultivars (CL5915 *versus* MicroTom) may be the possible explanation for this observation. Thus, we concluded that our PSY clone is encoded an enzyme catalyzing 2 molecules of geranylgeranyl diphosphate (GGDP) into phytoene. Nucleotide sequence of our tomato cDNA clone encoding phytoene synthase has been submitted into NCBI database with an accession number EF650010. The second clone PDS (accession number EF650011) is 1752 bp in length and contains a reading frame of 583 amino acids. In comparison with the published tomato *pds* mRNA (1752 bp; Giuliano et al., 1993), a base change at nucleotide position 705 in the coding region of our PDS clone is detected, where an adenosine is replaced by a guanosine residue. However, the corresponding amino acid residue at 235 (proline) is not altered. Thus, we conclude that our clone PDS is encoded an enzyme catalyzing phytoene into ζ-carotene. The third clone ZDS (accession number EF650012) is 1767 bp in length and contains a reading frame of 588 amino acids. In comparison with the published tomato *zds* mRNA (1767 bp; Bartley and Ishida, 1999), alterations at nucleotide positions 709, 1133 and 1639 in the coding region of our PSY clone are detected. A different codon 709 from thymidine is replaced by a cytidine residue and the corresponding amino acid residue is also altered from serine (TTC) to proline (TCC); a different codon 1133 from thymidine is replaced by a cytidine residue and the

Table 2. Top putative up-regulated or down-regulated genes in tomato orange fruits in comparison to tomato green fruits.

Up- or down-regulation	Systematic name on array	Description	Fold change in replicate
Up	LE023I12	Class II small heat shock protein Le-HSP17.6 (tomato)	27, 32
Up	LE022E08	FIN21.18 protein (*Arabidopsis thaliana*)	22, 32
Up	LE017D11	Metallothionein-like protein	20, 22
Up	LE023G18	Early light-induced protein-like protein (*Retama raetam*)	20, 47
Up	LE022G10	Heat shock protein 18p (tobacco)	17, 28
Up	LE017B21	Ribosomal protein S20 (*Arabidopsis thaliana*)	15, 28
Up	LE022H20	Type I small heat shock protein 17.6 kDa isoform (tomato)	15, 13
Up	LE009P11	Similar to AT4g27450 (*Arabidopsis thaliana*)	15, 13
Up	LE017H05	Zinc-finger protein (*Petunia hybrida*)	14, 24
Up	LE022K12	Mitochondrial small heat shock protein	14, 15
Down	CLEX1M5_1	Alpha-tubulin 3 (*Hordeum vulgare*)	16, 15
Down	CLEX1M5_12	Alpha-tubulin 3 (*Hordeum vulgare*)	15, 15
Down	CLEX1M5_10	Alpha-tubulin 3 (*Hordeum vulgare*)	14, 12
Down	LE001D09	Argonaute (AGO1)-like protein (*Arabidopsis thaliana*)	7, 11
Down	LE032C13	Unknown	6, 8
Down	LE030C01	Heat shock protein	6, 8
Down	LE011I11	Unknown protein F19K16.21 (*Arabidopsis thaliana*)	6, 5
Down	LE013M11	Glutathione peroxidase	6, 5
Down	LE004E24	Chlorophyll synthetase G4	6, 6
Down	LE001B10	Ethylene-responsive methionine synthase	5, 8

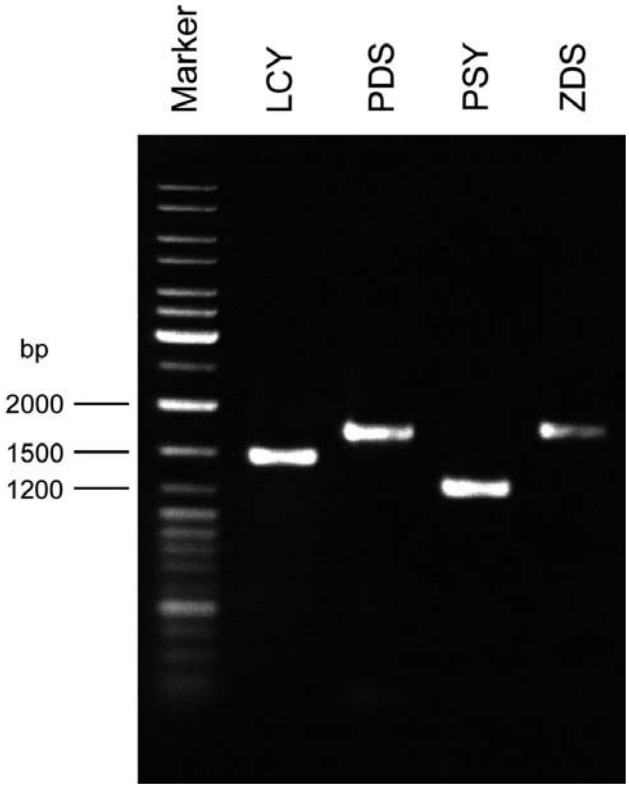

Figure 5. Gel electrophoresis of RT-PCR products. Approximately 200 ng of DNA-free RNA samples from different developmental stages of tomato fruits were amplified by gene-specific RT-PCR. Forward and reverse primers were synthesized to amplify full-length reading frames of 1503 bp, 1752 bp, 1239 bp and 1767 bp encoding lycopene β-cyclase (LCY; lane 2), phytoene desaturase (PDS; lane 3), phytoene synthase (PSY, lane 4) and ζ-carotene desaturase (ZDS; lane 5), respectively. PCR products (2 µl out of 20 µl) were analyzed on 1% TAE-agarose gel stained with ethidium bromide.

corresponding amino acid residue is altered from phenylalanine (ATT) to serine (ATC); and a different codon 1639 from adenosine is replaced by a guanosine residue and the corresponding amino acid residue is altered from alanine (AGG) to glycine (GGG). To exclude the possibility of 3 alternations in our ZDS clone is due to the random occurrence of sequencing error in the *Taq* DNA polymerase during PCR amplification, two more ZDS clones were picked up from the plate and then sequenced. These two clones have identical sequence, and is consistent with our previous ZDS clone we mentioned above. Thus, we believe that our clone ZDS is encoded an enzyme catalyzing ζ-carotene into lycopene. The fourth clone LCY (accession number EF650013) is 1503 bp in length and contains a reading frame of 500 amino acids. In comparison with the published tomato *lcy* mRNA (1503 bp; Cunningham et al., 1996), a base change at nucleotide position 919 in the coding region of our LCY clone is detected, where a thymidine was replaced by a cytidine residue. However, the corresponding amino acid at position 307 (lysine) is not altered. Thus, we conclude that our LCY clone is encoded an enzyme catalyzing lycopene into β-carotene. In brief, DNA sequence analysis demonstrated clearly that our cDNA clones are the same sizes and almost identical to the corresponding sequences from tomato, suggesting that the RNA samples we isolated are intact and functional.

In addition, we have also constructed a cDNA library representing 8 developmental stages of tomato fruits (Figure 1B). Our tomato fruit cDNA library contains approximately 1.0 x 10^5 independent clones with inserts ranging from 0.6 kb to 4 kb (data not shown). In conclusion, the protocol for RNA isolation presented here allows for the recovery of high-quality and functional RNA from different tissues of tomato suitable for use in tissue-specific or developmental-specific expression and regulation assays such as Northern blot, RT-PCR, microarray analysis and for the construction of cDNA libraries. Furthermore, tomato is considered as one of the model species in vegetative crops and its transformation has been routinely carried out for both basic and applied researches (Pfitzner, 1998; Bhatia et al., 2004; Cortina and Culiáñez-Macià, 2004), our method should also be applied to isolate functional RNA from different tissues in transgenic tomato plants and then monitor transgene expression by molecular tools.

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從番茄不同組織分離功能性 RNA 並以微矩陣方法進行發育圖譜分析

王幸美[1] 銀琬春[2] 王貞觀[3] 陶建英[3]

[1] 國立臺灣大學 植物科學研究所

[2] 國立清華大學 生物科技研究所

[3] 中央研究院 農業生物科技研究中心

我們詳述一個從番茄不同發育時期之營養及生殖組織中分離高品質 RNA 的方法，所獲得之 RNA 可應用在微矩陣分析上。這個方法基本上是依照 TRIzol 方法並加入氯化鋰 (LiCl) 沉澱及 DNase 處理。使用這個方法，可以獲得產量較多之高品質及完整的 RNA，其品質以分光光度儀、膠體電泳及螢光儀驗證之。我們進一步以下述方法闡明所獲得的完整 RNA 具有功能 ：(1) 這些 RNA 以螢光試劑標記後用作探針，與番茄 cDNA 微矩陣雜交，找到了果實成熟時期不同表現之基因 ； (2) 利用反轉錄聚合酶鏈反應 (RT-PCR) 並以這些 RNA 作為模板，我們成功地選殖了番茄類胡蘿蔔素生合成路徑的數個關鍵基因，包括 phytoene synthase (PSY)、phytoene desaturase (PDS)、ζ-carotene desaturae (ZDS) 及 lycopene β-cyclase (LCY) 等 ； 及 (3) 這些 RNA 可以供作構築 cDNA 基因庫。

關鍵詞 ： 類胡蘿蔔素生合成路徑 ； 果實成熟時期 ； 微矩陣 ；RNA 萃取方法 ；*Solanum lycopersicum*；番茄 ；TRIzol 試劑。