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ARTICLE

Cloning and Expression Analysis of Carotenogenic Genes during Ripening of Autumn Olive Fruit (*Elaeagnus umbellata*)

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Autumn olive (*Elaeagnus umbellata* Thunb.) is an extremely lycopene-rich natural source, and lycopene concentration of ripe wild autumn olive fruit was 12 times higher than that of tomato. Lycopene formation was found to increase with significant reduction of other carotenoids and chlorophylls during fruit ripening. To elucidate the molecular basis of massive lycopene accumulation in autumn olive fruit, seven cDNA fragments were cloned encoding enzymes of the main steps of carotenoid biosynthetic pathway, which were geranylgeranyl pyrophosphate synthase (*EutGgps*), phytoene synthase (*EutPsy*), phytoene desaturase (*EutPds*), ζ-carotene desaturase (*EutZds*), lycopene β-cyclase (*EutLcy-b*), lycopene ε-cyclase (*EutLcy-e*), and β-carotene hydroxylase (*EutBch*). The accumulation of lycopene in the fruit was concomitant with the up-regulation of upstream genes of lycopene synthesis (*EutGgps*, *EutPsy*, *EutPds*, and *EutZds*) and down-regulation of downstream genes (*EutLcy-b* and *EutBch*) and in particular with the silence of *EutLcy-e* throughout fruit ripening. Thus, lycopene accumulation in autumn olive fruit was highly regulated by the coordination of the expression among carotenogenic genes and by fruit ripening.

KEYWORDS: Autumn olive; carotenoid biosynthesis; lycopene; gene expression; fruit ripening

INTRODUCTION

Lycopene, a red linear carotenoid, is the most potent antioxidant among the carotenoids of plant sources and exhibits the highest physical quenching rate constant with singlet oxygen due to its special molecular structure determined by conjugated double bonds (*1*). The tomato fruit is a major source of lycopene. A high intake of tomato and lycopene-rich diets could reduce the risk of chronic diseases such as cardiovascular diseases and different forms of cancer (*2*, *3*). Lycopene combined with eicosapentaenoic acid inhibited the growth of human colon cancer cells even at low concentration (*4*). Due to the important role of lycopene in human health, consumer demand for lycopene-rich food and nutraceutical products is growing. This increasing demand, in turn, has prompted research into identifying alternative lycopene-rich sources. By far the most promising is autumn olive (*Elaeagnus umbellata* Thunb.). Its fruits contain lycopene at 54 mg/100 g of fresh weight, which is about 17 times higher than that of tomato (*5*). This extremely rich source of lycopene may have a broad potential in healthcare food and clinical application.

All carotenoids are derived from isopentenyl diphosphate, and the first committed step is the head-to-head condensation of two geranylgeranyl diphosphate molecules to produce phytoene catalyzed by phytoene synthase (PSY) (**Figure 1**) (*6*). Phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) introduce four double bonds to yield lycopene (*7*). Then lycopene can undergo cyclization, which has been considered as a crucial branching point of carotenoid biosynthesis (*8*). Lycopene β-cyclase (LCY-b) and lycopene ε-cyclase (LCY-e) are responsible for the cyclization and convert lycopene to γ-carotene (one β-ring) and δ-carotene (one ε-ring), respectively. β-Carotene and α-carotene are subsequently synthesized, which are the precursors of the major leaf xanthophyll and lutein (*6*).

Carotenoid biosynthesis and its regulation have been studied in various plant species, such as *Arabidopsis* (*9*, *10*), tomato (*6*, *11*), *Citrus* (*7*), pepper (*12*), apricot (*13*), carrot (*14*), and *Crocus* (*10*). The accumulation of specific carotenoids in chromoplasts of fruits is developmentally regulated (*8*). During tomato fruit ripening, the expression of *Psy* and *Pds* increased, whereas both *Lcy-b* and *Lcy-e* disappeared, leading to massive lycopene accumultion (*6*, *7*, *11*). However, little is known about the molecular mechanism in autumn olive fruit. The objectives of the present study were therefore (i) to compare the carotenoid and chlorophyll compositions of fruits at the four selected ripening stages; (ii) to clone seven carotenogenic genes for *EutGgps*, *EutPsy*, *EutPds*, *EutZds*, *EutLcy-b*, *EutLcy-e*, and *EutBch*; (iii) to analyze the expression patterns of these genes; and (iv) to determine which gene or genes played major role in massive lycopene accumulation during fruit ripening.

MATERIALS AND METHODS

Plant Materials. Autumn olive (*E. umbellata* Thunb.) plants were grown in our orchard, and fruits were sampled at four ripening stages: A, green fruit (approximately 19 weeks after fertilization); B, yellow fruit (approximately 22 weeks after fertilization); C, dark yellow fruit (approximately 24 weeks after fertilization); and D, full-ripe fruit (approximately 27 weeks after fertilization) (**Figure 2**). Stem, leaf, and flower as well as fruit samples of autumn olive were randomly collected periodically, rapidly frozen in liquid nitrogen, and stored at −80 °C until processed for RNA and DNA extraction and pigment analysis.

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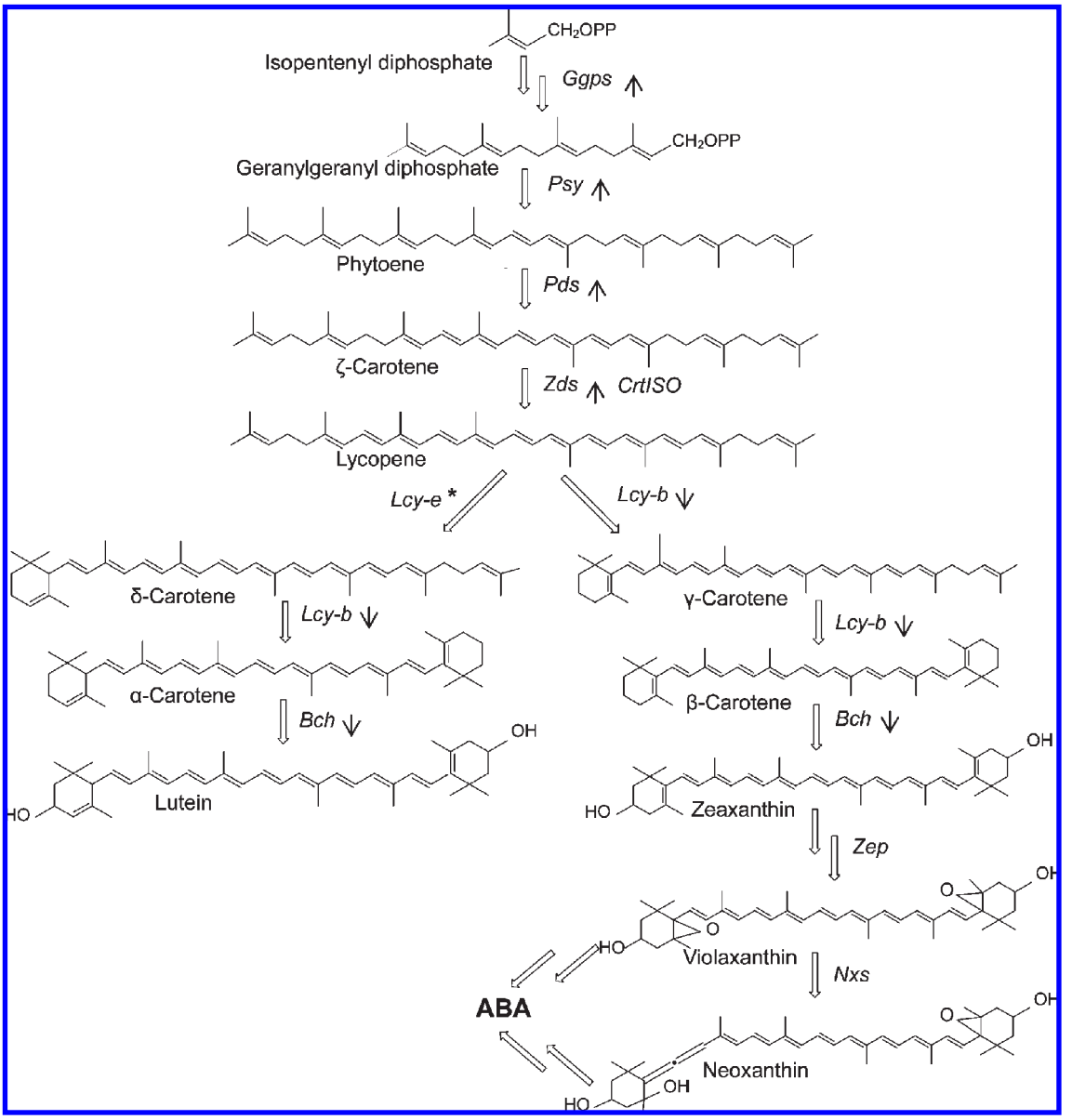

Figure 1. Carotenoid biosynthesis pathway in higher plants. The up-regulation, down-regulation, and silence of carotenogenic gene expression during fruit ripening are indicated by the symbols "↑", "↓", and " * ", respectively.

Figure 2. Four stages during autumn olive fruit ripening: (**A**) green fruit; (**B**) yellow fruit; (**C**) dark yellow fruit; (**D**) full-ripe fruit.

Quantification of Carotenoids and Chlorophylls. The frozen samples were pulverized in liquid nitrogen and then subjected to carotenoid and chlorophyll extraction. High-performance liquid chromatography (HPLC) using a ternary gradient elution was carried out on a Waters system (Waters Corp., Milford, MA) as previously described (*15*). Carotenoids were identified on the basis of the same retention times and same spectral characteristics with standards. Peak areas were recorded at 286, 400, 470, and 450 nm for phytoene, chlorophyll *a*, chlorophyll *b*, lycopene, and the others, respectively. The reference standards of α-carotene, β-carotene, lutein, and lycopene were purchased from Sigma-Aldrich (St. Louis, MO). The other carotenoid standards were kindly provided by Prof. Changjie Xu (*15*). The total carotenoid content was the sum of identified carotenoids including phytoene, lycopene, α-carotene, lutein, β-carotene, zeaxanthin, violaxanthin, and neoxanthin. All tests were replicated at least three times. SPSS version 12.0 statistical analysis software was used to analyze data.

RNA Extraction and cDNAs Synthesis. Total RNA extraction was performed as described by Hunter and Reid (*16*) and digested with DNaseI (40 U/mL) at 37 °C for 30 min. RNA was quantified spectrophotometrically and checked out by 1.0% agarose−formaldehyde gel electrophoresis. About 500 ng−1 μg of total RNA isolated from stem, leaf, flower, and fruit tissues harvested at four ripening stages was used for the reverse transcription reaction. First-strand cDNA was synthesized using an oligo$(dT)_{18}$ primer and Superscript II kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

cDNA Cloning of Seven Carotenogenic Gene Fragments. The degenerate primers were designed on the basis of conserved amino acid sequences of each carotenogenic enzymes from the National Center for Biotechnology Information (NCBI) database (**Table 1**). cDNAs were amplified in 25 μL of PCR mixture using rTaq polymerase kit (Takara, Dalian, China). The PCR conditions were 30 cycles of 30 s at 94 °C, 30 s at 45 to 60 °C depending on the primers, and 90 s at 72 °C. Purified amplified products of appropriate length were ligated to the pUCm-T vector for

Table 1. Primers Used in Carotenogenic Gene Cloning and Expression Analysis[a]

gene	forward primer 5′→3′	reverse primer 5′→3′	application
EutGgps	ATGGAYAAYGMCGAYCTCC	CRAGWATRTCATCMACYACYTG	cloning
	AGAAGGGCTCGTTGCTGGTC	ACCCACCTCCCAAAATTGC	real-time
EutPsy	GWACHWTGCTHATGACWCCBGA	CYAADGCAGCRTTRTABACRC	cloning
	CGCTTTCTGTTGTGGCTTGTG	CTGCTATTATGTTGCTGGGACTG	real-time
EutPds	AAGGTDGCTGCWTGGAA	GTGTARTCACCAGCTAAATA	cloning
	GCTACGATGAAAGAACTGGCTAA	AGGGACGGCAAGGTTCACAA	real-time
EutZds	CTHMGMTACAATGGHTGGGT	GAVARAGTTGCTCCTTCCAT	cloning
	TTTCCATCGTCCCAAGGTTT	TTGCTCCTTCCATGCTGTCT	real-time
EutLcy-b	GATGARCRWTGTGTDATYCCAATGGG	AGYTTWAGCARWATMTCCAT	cloning
	TATGGTCCACCCTTCAACTGG	TGTCTCCTCCGCTCTATGGG	real-time
EutLcy-e	GTGGTDATTGGTTGTGGYCC	GGAYTRTTTTCCACCTCAACCTC	cloning
	CGAGGGTCGAAAGGATTATAG	GACCACCGACCTCATACTGC	real-time and semi-Q
EutBch	TACAGATTYTCDTGGCAAATGGA	CCATCGTGRACRAACATGTA	cloning
	CATAATAAACGCCGTTCCAG	GCACCAAAGCATAATCCAGG	real-time
EutGapdh	GGGTGGTGCTAAGAAGGTTG	TGGAATAATGTTGAAGGAGGC	cloning
	TAGGTTTGGAATTGTTGAGGG	TGGAATAATGTTGAAGGAGGC	real-time and semi-Q

[a] Y = C or T; M = A or C; W = A or T; H = A, C, or T; B = C, G, or T; D = A, G, or T; R = A or G; V = A, C, or G.

Table 2. Carotenoid and Chlorophyll Content (Micrograms per Gram of Dry Weight) during Autumn Olive Fruit Ripening

	ripening stage[a]			
	A	B	C	D
phytoene	3.33 ± 0.28[b]	0	0	0
lycopene	0	188.37 ± 61.02	472.39 ± 110.97	1822.28 ± 522.45
α-carotene	5.10 ± 0.61	1.87 ± 0.16	0	0
lutein	50.77 ± 3.76	18.21 ± 1.37	5.72 ± 0.68	5.55 ± 0.51
β-carotene	31.76 ± 2.67	17.48 ± 3.20	11.38 ± 1.66	22.79 ± 5.24
zeaxanthin	1.11 ± 0.16	0	0	0
violaxanthin	45.41 ± 2.51	13.29 ± 1.53	6.30 ± 0.97	0
neoxanthin	5.47 ± 0.53	0.80 ± 0.23	0	0
total carotenoids	142.94 ± 9.26	240.02 ± 66.73	495.78 ± 113.07	1850.63 ± 527.96
chlorophyll *b*	198.53 ± 11.76	26.46 ± 2.63	0	0
chlorophyll *a*	97.73 ± 4.00	13.19 ± 0.53	4.32 ± 0.45	0

[a] A, green fruit; B, yellow fruit; C, dark yellow fruit; D, full-ripe fruit. [b] Values represent means ± SE.

sequencing. Fruit cDNA was used as a template for all of the genes except *EutLcy-e*, which was cloned from autumn olive flower. Protein sequences for putative homologues from a number of plant taxa were obtained from GenBank and subjected to ClustalW analysis (http://align.genome.jp/) (*17*).

Expression Profiles of Seven Carotenogenic Genes during Fruit Ripening. Primers for quantitative real-time PCR were designed on the basis of the sequence of partial cDNAs of *EutGgps*, *EutPsy*, *EutPds*, *EutZds*, *EutLcy-b*, and *EutBch* (**Table 1**). Real-time PCR was carried out in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (catalog no. 4367659, Applied Biosystems, Warrington, U.K.) according to the supplied protocol. Transcript level of *EutLcy-e* was further confirmed by semiquantitative (semi-Q) PCR using the primers listed in **Table 1**. PCR was run with an initial denaturation of 3 min at 94 °C followed by 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. The PCR products were applied to 1.2% agarose gel and analyzed using Quantity One software (Bio-Rad, Hercules, CA). All transcript levels were normalized to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*EutGapdh*) (*18*).

RESULTS

Changes in the Carotenoid and Chlorophyll Composition during Autumn Olive Fruit Ripening. The color of the autumn olive fruit changed from green to red during fruit ripening (**Figure 2**). The carotenoid and chlorophyll composition and concentration of the fruit flesh at four ripening stages were analyzed by HPLC (**Table 2**). At stage A, lutein, β-carotene, and violaxanthin were predominant in the carotenoid components. Lycopene was barely detected, and other carotenoids including phytoene, α-carotene, zeaxanthin, and neoxanthin were considerably low. After stage A, the concentration of lutein and β-carotene gradually decreased and then remained stable or increased at stage D. Violaxanthin content decreased dramatically during fruit ripening. The other carotenoids including phytoene, α-carotene, zeaxanthin, and neoxanthin were not detected after stage B. As expected, lycopene content increased with fruit ripening and reached its highest levels at stage D. When autumn olive fruit fully ripened, lycopene was the most abundant carotenoid followed by β-carotene. The lycopene content reached 1822 μg/g of dry weight or 341 μg/g of fresh weight, which accounted for about 98% of the total identified carotenoids. In contrast, the chlorophyll content decreased with fruit ripening (**Table 2**). It is apparent that the massive lycopene accumulation during autumn olive fruit ripening

Table 3. Seven Genes and Their Deduced Amino Acid Sequence Identities

gene	accession no.	identities[a] (%)		
EutGgps	FJ643540	89 (AAM21638)$^{C.i.}$	88 (ABA64563)$^{C.p.}$	86 (AAG10424)$^{E.u.}$
EutPsy	FJ643541	92 (BAF49052)$^{P.m.}$	92 (ABB72444)$^{C.s.}$	92 (ABB52068)$^{D.c.}$
EutPds	FJ958187	91 (AAR81605)$^{M.c.}$	90 (AAX33347)$^{P.a.}$	89 (ABB72445)$^{C.s.}$
EutZds	FJ643542	97 (CAD55814)$^{H.a.}$	96 (AAG10425)$^{T.e.}$	96 (ABD67160)$^{L.e.}$
EutLcy-b	FJ643543	91 (BAF49055)$^{P.m.}$	91 (AAK07430)$^{A.p.}$	90 (CAA67331)$^{N.p.}$
EutLcy-e	FJ643544	87 (EEF48090)$^{R.c.}$	84 (AAK07431)$^{A.p.}$	84 (BAE93359)$^{C.u.}$
EutBch	FJ643545	92 (BAE92729)$^{G.l.}$	92 (ABB49053)$^{C.s.}$	92 (ABA43903) $^{C.a.}$

[a] *C.i.*, *Cistus incanus* subsp. *creticus*; *C.p.*, *Carica papaya*; *E.u.*, *Eucommia ulmoides*; *P.m.*, *Prunus mume*; *C.s.*, *Citrus sinensis*; *D.c.*, *Daucus carota* subsp. *sativus*; *M.c.*, *Momordica charantia* var. *abbreviata*; *P.a.*, *Prunus armeniaca*; *H.a.*, *Helianthus annuus*; *T.e.*, *Tagetes erecta*; *L.e.*, *Lycopersicon esculentum*; *A.p.*, *Adonis palaestina*; *N.p.*, *Narcissus pseudonarcissus*; *R.c.*, *Ricinus communis*; *C.u.*, *Citrus unshiu*; *G.l.*, *Gentiana lutea*; *C.a.*, *Coffea arabica*.

occurred concomitantly with significant reduction of other carotenoids and chlorophylls.

Isolation of the cDNAs Fragments of Carotenogenic Genes. On the basis of the conserved amino acid sequences among plant species in carotenogenic genes, seven sets of degenerated primers were designed for *EutGgps*, *EutPsy*, *EutPds*, *EutZds*, *EutLcy-b*, *EutLcy-e*, and *EutBch* (**Table 1**). Seven cDNA fragments of about 300−1200 bp from autumn olive for carotenogenic genes were obtained by RT-PCR, which were submitted to the GenBank database as *EutGgps* (294 bp), *EutPsy* (408 bp), *EutPds* (1175 bp), *EutZds* (452 bp), *EutLcy-b* (286 bp), *EutLcy-e* (489 bp), and *EutBch* (341 bp), respectively (**Table 3**). Alignments of the deduced amino acid sequences of seven autumn olive cDNAs with those from other plant species showed high identities, from 84 to 97%. The phylogenetic tree representing the multiple sequence alignment for the deduced amino acid sequences showed that a clear dichotomy was apparent between monocotyledonous and dicotyledonous plants for these seven genes (data not shown).

Expression of Seven Carotenogenic Genes during Fruit Ripening. The expression profiles of seven carotenogenic genes in autumn olive fruit were analyzed by quantitative real-time PCR at four ripening stages (**Figure 3a**). The expression of all upstream genes of lycopene synthesis (*EutGgps*, *EutPsy*, *EutPds*, and *EutZds*) increased clearly during ripening although their expression patterns were different. Whereas *EutGgps* expression increased about 40% at stage D compared to stage A, the expression of *EutPsy* significantly increased about 3.0-fold. The level of *EutPds* transcript showed a maximum at stage B, and *EutZds* transcript reached its maximum at stage C. The expression of two genes to produce zeaxanthin (*EutLcy-b* and *EutBch*) decreased with fruit ripening (**Figure 3a**). Interestingly, the *EutLcy-e* transcript encoding another cyclase that utilizes lycopene for further biosynthetic steps was below the level of real-time PCR detection in fruit at any ripening stage (**Figure 3a**). To ensure this result, tissue expression of *EutLcy-e* was analyzed by semiquantitative PCR. However, still no transcript was observed in fruit (**Figure 3b**).

Concurrently, the expressions of seven carotenogenic genes were detected in stem, leaf, and flower. *EutLcy-e*, in contrast to silence in fruit, was expressed in stem, flower, and strongly high in leaf (**Figure 4**). Whereas the expressions of *EutGgps*, *EutPsy*, and *EutPds* were comparatively lower in stem, the expressions of *EutLcy-b* and *EutBch* were quite low in ripe fruit, but the expressions of both *EutGgps* and *EutZds* were highest in ripe fruit. The transcript levels of *EutPsy* and *EutBch* were highest in flower, whereas those of *EutPds* and *EutLcy-b* were highest in autumn olive leaf (**Figure 4**).

DISCUSSION

Ripe fruit size from sampled plants ranged from 6 to 9 mm in diameter, and its lycopene content averaged 1822 μg/g of dry weight or 341 μg/g of fresh weight. The U.S. Department of Agriculture reported in the USDA National Nutrient Database for Standard Reference that the value for fresh tomato was 2573 μg/100 g (*19*). Thus, the lycopene concentration of wild autumn olive fruit is about 12 times higher than that of ordinary tomato. A similar result was also obtained by Fordham et al. (*5*).

Lycopene Accumulation and Carotenogenic Gene Expression during Autumn Olive Fruit Ripening. In tomato fruits, the induction of lycopene accumulation coincided with increased expression of upstream carotenogenic genes and reduced expression of genes downstream of lycopene synthesis (*20*). In red-flesh citrus mutant Cara Cara, lycopene accumulation was highly regulated by the coordinated expression of the different carotenoid biosynthetic genes (*21*). Whereas the transcript level of *Psy* was upregulated in the juice and peel sacs during fruit ripening (*22*), *Pds* increased during maturation of the juice sacs and remained constant once fruits were fully developed (*23*). An orange mutant, "Hong Anliu", accumulated lycopene in the pulp, and an upregulation of most carotenogenic genes was recently proposed as the mechanism responsible for its particular carotenoid composition (*24*).

Altogether, the expression profiles of carotenogenic genes in autumn olive fruit were different from those in tomato observed previously during fruit ripening (*6*, *11*) (**Figure 3**). The low expression of *EutGgps*, *EutPsy*, *EutPds*, and *EutZds* in green autumn olive fruit (stage A), which produces linear carotenoids, was responsible for the low concentration of carotenoids. When autumn olive fruit changed color from green to yellow (from stage A to stage B), a significant increase in the gene expression of *EutPds* led to an undetectable level of phytoene. Simultaneous increases in the expression of *EutGgps*, *EutPsy*, *EutPds*, and *EutZds* induced by fruit ripening, which make up a set of genes to produce lycopene, led to the accumulation of lycopene at this stage. The high expression of *EutLcy-b*, in contrast to the silence of *EutLcy-e*, suggested that cyclization to the β-ring was more active than that to the ε-ring, resulting in some accumulation of β-carotene. Thus, it was thought that the pathway changing from other carotenoid synthesis to lycopene synthesis occurred in autumn olive fruit at the yellow stage. After the dark yellow stage, it seems that a lasting increase in the expression of *EutPsy* played an important role in the continuous increase of lycopene concentration because the increase of *EutPds* and *EutZds* expression began to fall and the *EutGgps* transcript remained stable, which was consistent with PSY catalyzing the first committed step in the carotenoid biosynthetic pathway (*6*). Recovery of β-carotene content at full-ripe stage could be explained by remarkably decreased expression of its downstream genes *EutLcy-b* and *EutBch*. The results above were useful for biotechnological regulation of carotenoid biosynthetic pathway. The corresponding enzyme activities may increase by overexpression of four genes mediating lycopene synthesis from autumn olive fruit, respectively or concomitantly, under the control of a constitutive or inducible strong promoter, thus enhancing lycopene production of other fruits.

Cyclization of lycopene by either LCY-e or LCY-b has been demonstrated to play a key role in the regulation of carotenoid composition in fruits of different plant species (*21*). A loss or silence of lycopene cyclase function could dramatically affect carotenoid composition. In tomato, both *Lcy-e* and *Lcy-b* expressions were detected at a significant level at the green stage and disappeared at the subsequent ripening stages, which led

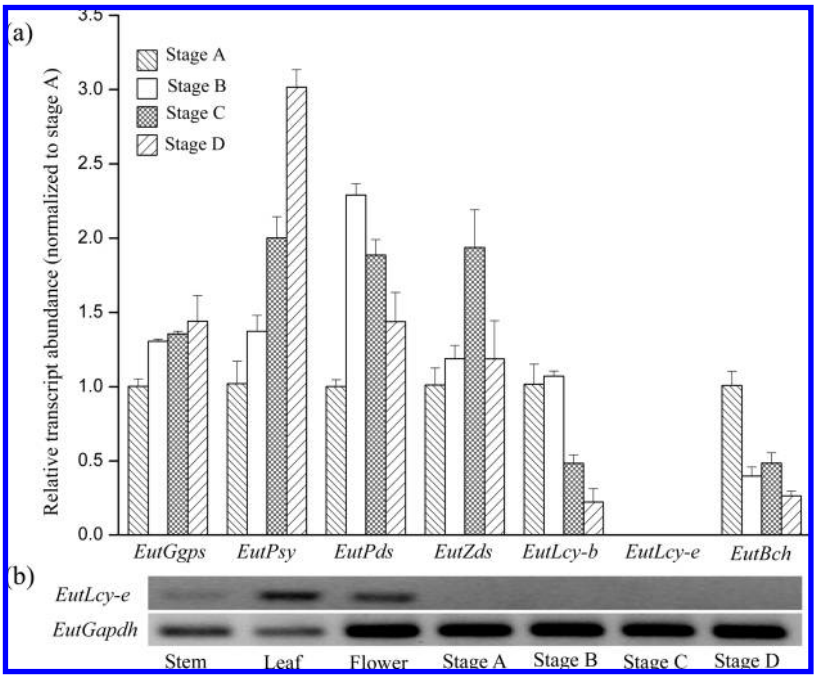

Figure 3. (**a**) Relative accumulation of carotenogenic gene transcripts through autumn olive fruit ripening. Transcript levels were measured by quantitative real-time PCR and were first normalized to a housekeeping gene, *EutGapdh*, and then to that in green fruit (stage A). Each gene expression was analyzed at least three times for samples from at least three different plants. (**b**) Semiquantitative PCR analysis of *EutLcy-e*. A housekeeping gene, *EutGapdh*, was used as an internal amplification control.

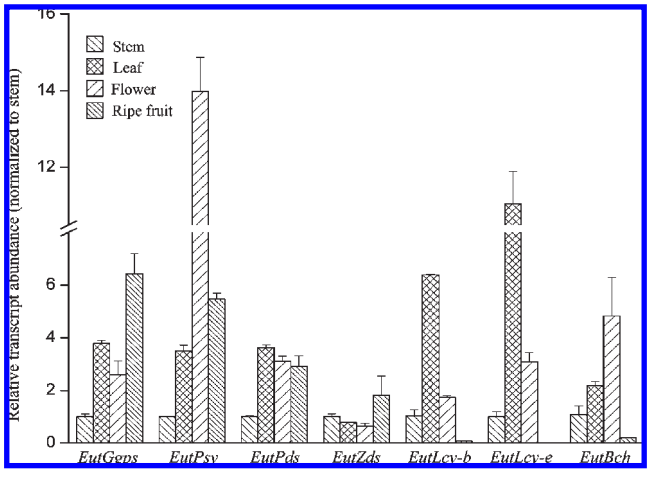

Figure 4. Relative accumulation of carotenogenic gene transcripts in autumn olive stem, leaf, flower, and ripe fruit. Transcript levels were measured by quantitative real-time PCR and were first normalized to a housekeeping gene, *EutGapdh*, and then to that in stem.

to the accumulation of lycopene (*11*). In autumn olive fruit, *EutLcy-e* was silent throughout fruit ripening and the expression of *EutLcy-b* decreased significantly, which helped to accumulate lycopene due to a pulp-specific blockage in the conversion of lycopene to α-carotene or *β*-carotene. Therefore, antisense and RNA interference techniques can be used to silence *Lcy-b* and *Lcy-e* expression to enhance lycopene accumulation in some fruits.

***EutLcy-e* May Contribute to Lutein Synthesis in the Early Stage of Autumn Olive Fruit Development.** Although *EutLcy-e* was silent throughout autumn olive fruit ripening, there still was some accumulation of lutein in the fruit (**Table 2**). In general, lutein biosynthesis and expression of *Lcy-e* were quite strong in the early stage of fruit development, and the synthesized lutein could be present for a long time (*25*). Here, semiquantitative PCR analysis showed that *EutLcy-e* expressed in flower (**Figure 3b**), thus the formation of lutein was feasible before stage A. The level of lutein decreased progressively from stage A to stage D (**Table 2**), probably due to a dilution effect caused by the enlargement of the fruit (**Figure 2**).

Carotenoid biosynthesis enzymes aggregated into multienzyme complexes specific for accumulating each of the carotenoids (*9*). There is a channeling of intermediates through the multienzyme complex, and multiple enzymes interact (*26*). During the process, the enzymes could arrange in a coordinated fashion to produce a specific carotenoid. These seven genes from autumn olive fruit are available for genetic modification of lycopene-producing fruits.

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