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Controlling the Metabolic Flux through the Carotenoid Pathway Using Directed mRNA Processing and Stabilization

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A synthetic operon containing the *crtI* and *crtY* genes, encoding the phytoene desaturase and the lycopene cyclase, respectively, was placed under the control of the *araBAD* promoter. DNA cassettes encoding mRNA secondary structures were placed at the 5′ and 3′ ends of the genes and a putative RNase E site was placed between the genes. This construct was transformed into *Escherichia coli* cells harboring the genes for phytoene production. By varying the mRNA secondary structures, we were able to modulate the flux through the carotenoid pathway, resulting in a 300-fold variation in the production of β-carotene relative to lycopene. In addition, intermediates in the pathway from phytoene to β-carotene production that are not observed in cells expressing the recombinant operon were observed when the engineered operons were used, indicating that changes in levels of the enzymes affected the formation of intermediates. These results indicate that it is possible to coordinately regulate the genes encoding the enzymes of a metabolic pathway and balance the production of the intermediates. © 2001 Academic Press

INTRODUCTION

Many metabolic engineering applications necessitate the coordinated expression of several genes, encoding an entire metabolic pathway, in a heterologous host. In the native host, the genes for such a metabolic pathway might be expressed in an operon. In this case, the native host would have evolved mechanisms to coordinate expression of the genes in these operons at levels such that flux through a pathway is optimized or burden on the cell is minimized. Unfortunately, transferring the expression of this operon to a heterologous organism is not always trivial. Many times direct transfer of operons results in no expression or suboptimal expression within the new host. A better understanding of the elements involved in multicistronic gene expression could lead to better designs for the expression of heterologous genes.

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Many cells use mRNA processing and stability control to affect levels of transcripts from a multicistronic transcript and thus affect the amount of protein produced (Nilsson and Uhlin, 1991; Klug *et al.*, 1992; Klug, 1993; Alifano *et al.*, 1994; Bricker and Belasco, 1999). The cell uses many different types of elements simultaneously to achieve this coordinated decay, such as Shine–Delgarno sequences, sequences in the untranslated regions, codon usage, and mRNA stability control elements. While the effects of many of these elements on gene expression are well known, until recently little emphasis has been placed on altering mRNA stability of multicistronic transcripts to affect relative levels of gene expression (Smolke *et al.*, 2000).

An expression system was developed that allowed for the easy introduction of mRNA stability control elements, in the form of synthetic DNA cassettes, into various regions of a synthetic dual-gene operon consisting of two reporter genes. Control elements tested in this system included 5′ and 3′ RNA secondary structures, RNase cleavage sites, and gene order in the operon. Depending on the combination of control elements used, relative steady-state transcript and protein levels could be changed 1- to 500-fold and 1- to 1000-fold (Smolke *et al.*, 2000), respectively. This range of transcript and protein expression levels may be useful in both multi-subunit protein production and metabolic pathway engineering.

In this work, the technology previously designed using reporter genes was applied to the production of carotenoids. Carotenoids (C40 compounds) are a diverse group of pigments used in animal feeds, nutritional supplements, cosmetics, and pharmaceuticals (Sandmann *et al.*, 1999). Interest in these products has recently increased due to their antioxidant properties, which may play a role in tumor suppression and prevention of cancer and chronic disease (Edge *et al.*, 1997; Rock, 1997; Singh and Lippman, 1998; Bertram, 1999). Until recently, synthesis was limited to the few carotenoids that could be produced chemically, by extracting from native sources, or by microbial fermentation (Johnson and Schroeder, 1995). Due to anticipated

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TABLE 1

Strains and Plasmid Description

Strain or plasmid name	Phenotype/description	Reference/source
DH10B	F⁻ *mcr*A *Δ*(*mrr-hsd*RMS-*mcr*BC) *ϕ*80d*lacZΔ*M15*Δlac*X75 *deo*R *rec*A1 *ara*D139 *Δ*(*ara*, *leu*) 7697*gal*U *gal*K *λ*⁻ *rps*L *nup*G	Gibco BRL
p50gl	pBR origin, M13 intergenic region, *Amp*ʳ, *ara*C, P_{BAD}, *gfp*, *lacZ*; 8.5 kb	Smolke *et al.* (2000)
p70gl	p50gl with HP1 inserted 3′ of *gfp* and RNase E site (E2) inserted 5′ of *lacZ*	Smolke *et al.* (2000)
p70gHP41	p70gl with HP4 inserted 5′ of *lacZ*	Smolke *et al.* (2000)
p70gHP161	p70gl with HP16 inserted 5′ of *lacZ*	Smolke *et al.* (2000)
p70gHP171	p70gl with HP17 inserted 5′ of *lacZ*	Smolke *et al.* (2000)
p70yi	p70y1 with *crtI* and *crtY* in the *lacZ* and *gfp* locations, respectively	This study
p70yHP4i	p70yHP41 with *crtI* and *crtY* in the *lacZ* and *gfp* locations, respectively	This study
p70yHP16i	p70yHP16 with *crtI* and *crtY* in the *lacZ* and *gfp* locations, respectively	This study
p70yHP17i	p70yHP17 with *crtI* and *crtY* in the *lacZ* and *gfp* locations, respectively	This study
pAC-PHYT	pAC-LYC with *crtI* removed	This study
pAC-PHYT+	pAC-PHYT with *dxs* inserted between *crtE* and *crtB*	This study
pAC-LYC	p15A origin, *Cm*ʳ, *crtE*, *crtI*, *crtB*; 9.5 kb	Cunningham *et al.* (1994)
pAC-BETA	p15A origin, *Cm*ʳ, *crtE*, *crtI*, *crtB*, *crtY*; 10.6 kb	Cunningham *et al.* (1996)
pDdxs	pBR origin, M13 intergenic region, *Amp*ʳ, *ara*C, P_{BAD}, *dxs*; 6.1 kb	Kim and Keasling (2001)

increased demand for natural and novel carotenoids, focus has shifted to heterologous production of carotenoids and the design of new pathways for novel carotenoid production (Albrecht *et al.*, 2000; Schmidt-Dannert, 2000; Schmidt-Dannert *et al.*, 2000; Sandmann, 2001). While more of the genes responsible for carotenoid synthesis are being cloned and expressed in heterologous hosts, an understanding of this type of "rational operon design" is necessary to optimize, balance, and control flux through these pathways.

Two genes responsible for *β*-carotene and lycopene synthesis (*crtI,* encoding a phytoene desaturase; *crtY,* encoding a lycopene cyclase) from phytoene were inserted into the novel dual-gene constructs containing a variety of mRNA stability control elements. Genes necessary to produce phytoene in *Escherichia coli* [*crtE,* geranylgeranyldiphosphate (GGPP) synthase; *crtB,* phytoene synthase; *dxs,* 1-deoxy-D-xylulose-5-phosphate (DXP) synthase] were expressed on a second plasmid. The flux through the carotenoid pathway was controlled by varying the relative half-life of the *crtI* coding region to the *crtY* coding region, thus affecting the relative amount of protein produced from the individual coding regions. Depending on the mRNA control elements used, the relative ratio of *β*-carotene to lycopene could be affected 300-fold.

METHODS AND MATERIALS

Bacterial Strains, Media, Chemicals, and Enzymes

E. coli DH10B (Gibco BRL) was used for all cloning steps and in all carotenoid assays (Table 1).

Luria–Bertani (LB) medium and 2 × YT medium were made as described by Maniatis *et al.* (Sambrook *et al.*, 1989). Ampicillin, used at a concentration of 100 *μ*g/ml, chloramphenicol, used at a concentration of 35 *μ*g/ml, and arabinose were purchased from Fisher Scientific.

Restriction enzymes were purchased from Roche and New England Biolabs. T4 DNA ligase was purchased from New England Biolabs. High-Fidelity PCR enzyme mix was obtained from Roche.

Reagents and Standards

Lycopene, α-carotene, and *β*-carotene were obtained from Sigma to be used as standards. Acetone, acetonitrile, and methanol were of high-performance liquid chromatographic (HPLC) grade from Fisher Scientific. Unless otherwise stated, all carotenoids were dissolved in acetone.

Metabolic Engineering **3**, 313–321 (2001)
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Plasmid Construction

All plasmids used in this study are listed in Table 1. The dual-gene plasmid systems containing the reporter genes *gfp* and *lacZ* were previously described in Smolke *et al.* (2000). Primers used in each PCR step were synthesized by Genemed Synthesis, Inc. (South San Francisco, CA). DNA amplification was performed with High-Fidelity PCR enzyme mix. All PCRs were performed with 20 mM Tris–HCl (pH 8.3), 30 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs), 300 μM primers, 0.5 μg of template, and 2.6 U of enzyme. Cycle times and temperatures followed those suggested by the enzyme manufacturer.

The base carotenoid plasmid pAC-PHYT, enabling *E. coli* to synthesize phytoene, was generated in one cloning step. Plasmid pAC-LYC (Cunningham *et al.*, 1994) was digested with *Bgl*II and *Sbf*I to remove the *crtI* coding region so that lycopene could no longer be synthesized by cells harboring this plasmid. The cohesive ends were filled in using the Klenow enzyme. The reaction was performed with 1 U of enzyme in a 50-μl reaction containing 0.5 mM dNTPs, 50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM dithioerythritol (DTE) at 37° C for 2 h. The blunt-end fragment was circularized using T4 DNA ligase, transformed into *E. coli* DH10B, and the cells were plated on LB agar containing chloramphenicol. Screening for the correct plasmid was performed by isolating plasmid from several white colonies (Sambrook *et al.*, 1989), digesting with *Afl*II and *Hin*dIII, and analyzing by gel electrophoresis.

The base carotenoid plasmid pAC-PHYT+ was also generated in one cloning step. PCR was performed on pDdxs (Kim and Keasling, 2001) using primer 1 (5′-atacctgcaggtgataacgaggcgcaaaaaatgagttttgatattgccaaatacccgac-3′) and primer 2 (5′-ataagatctcatgccagccaggccttgatt-3′) to amplify the *dxs* gene and place a strong RBS in front of the coding region to enhance expression. The 1900-bp PCR product and plasmid pAC-LYC were digested with *Bgl*II and *Sbf*I and ligated together to form pAC-PHYT+. Screening for the correct plasmid was performed by digesting with *Sal*I.

The dual-gene carotenoid plasmids were constructed in several cloning steps. PCR was performed on pAC-BETA (Cunningham *et al.*, 1996) using primer 3 (5′-ccggctagcgtattttggatgataacgaggcgcaaaaaatgagggatctgattttagtcggcgg-cgg-3′) and primer 4 (5′-ccgagatctcatcctttatctcgtctgtcagga-aaatggaag-3′) to amplify the *crtY* gene and place a 5′ untranslated region in front of the coding region. The 1200-bp PCR product and plasmids p70gl, p70gHP4l, p70gHP16l, and p70gHP17l (described in Smolke *et al.*, 2000) were digested with *Nhe*I and *Bgl*II (to remove *gfp* from the plasmids) and ligated together to form p70yl, p70yHP4l, p70yHP16l, and p70yHP17l, respectively. Transformed cells were plated on LB agar containing arabinose, ampicillin, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Gibco BRL). Screening for the correct plasmid was performed by isolating plasmid from blue colonies which did not fluoresce green (due to loss of *gfp*) and digesting it with *Bgl*II and *Nhe*I.

PCR was performed on pAC-LYC (Cunningham *et al.*, 1994) using primer 5 (5′-c ggggtaccgtattttggatgataacgaggc-gcaaaaaatgaaaaaaaaccgttgtgattggcgcagg-3′) and primer 6 (5′-cgggaattcattgcagatcctcaatcatcaggctggc-3′) to amplify the *crtI* gene and to place a 5′ untranslated region in front of the coding region. The 1500-bp PCR product and plasmids p70yl, p70yHP4l, p70yHP16l, and p70yHP17l were digested with *Asp*718 and *Eco*RI (thereby removing *lacZ* from the plasmids) and ligated together to form p70yi, p70yHP4i, p70yHP16i, and p70yHP17i, respectively. Screening for the correct plasmid was performed by isolating plasmid from white colonies (due to the loss of *lacZ*) and digesting it with *Bgl*II.

The two plasmids were combined into one cell by transforming plasmids p70yi, p70yHP4i, p70yHP16i, and p70yHP17i into electrocompetent cells containing pAC-PHYT and pAC-PHYT+. Electrocompetent cells were made according to Maniatis *et al.* (Sambrook *et al.*, 1989). Screening for the correct plasmids was done by isolating plasmid from yellow colonies and digesting with *Eco*RI and *Sal*I. All plasmids were sequenced to confirm the integrity of the coding regions and the control elements used.

HPLC UV–vis and MS Analysis of Carotenoids

To determine relative carotenoid levels, cells with the appropriate plasmids were plated from freezer stocks onto LB agar with ampicillin and chloramphenicol. Cells were grown at 30° C for 2 days. Colonies from the plates were inoculated into 2×YT. When the cells reached exponential phase, this culture was used as a stock to inoculate to an optical density at 600 nm (OD_{600}) of 0.1 fresh 2×YT and 8×10^{-5}%, 1×10^{-3}%, or 2×10^{-2}% arabinose. Cells were grown at 30° C for 24–48 h prior to carotenoid analysis.

A volume of cells between 1.0 and 5.0 ml was centrifuged at 18,000 rcf in a microfuge tube and the supernatant was removed from cell pellets. Carotenoids were extracted by suspending the cells in 0.2–1.0 ml of acetone, immediately vortexing for 1 min, and incubating for 15 min in the dark in a 55° C water bath, with intermittent vortexing. Cell debris was centrifuged at 18,000 rcf for 5 min. Acetone extractions, containing the carotenoids, were

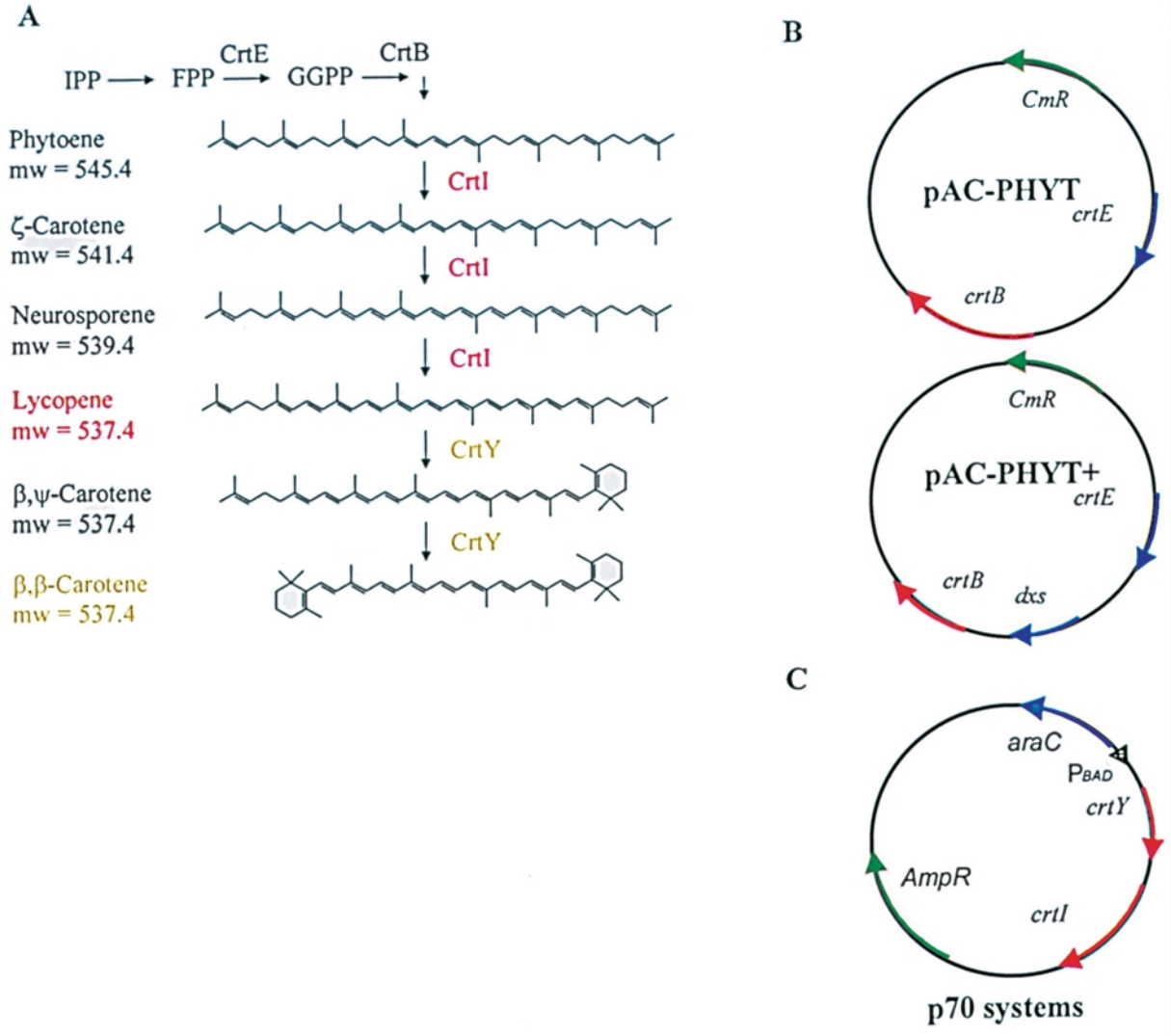

FIG. 1. Pathway and plasmids. (A) Detailed carotenoid biosynthesis pathway, including structures and molecular weights of compounds. CrtE, GGPP synthase; CrtB, phytoene synthase; CrtI, phytoene desaturase; CrtY, lycopene cyclase. (B) Plasmid backbones for pAC-PHYT and pAC-PHYT+. (C) Plasmid backbone for p70 constructs.

transferred to microfuge tubes and stored in the dark at −20° C until HPLC–MS analysis.

Lycopene, α-carotene, and β-carotene standards were dissolved in acetone to final concentrations of 11.6 and 11.9 μg/ml (2:1 ratio of α-carotene to β-carotene), respectively. These standards were kept in the dark at −20° C until ready to use. Since standards for phytoene could not be obtained, phytoene levels were estimated by assuming that the amounts of total carotenoid in pAC-BETA and pAC-PHYT were identical. From the calibration curve for β-carotene, a calibration curve for phytoene was extrapolated by comparing relative signals for these two carotenoids in pAC-BETA and pAC-PHYT. Corrections were made for differences in responses using extinction coefficients (Ebenezer and Pattenden, 1993; Hagiwara *et al.*, 1998).

The procedure used for HPLC–MS analysis was based upon that described in Hagiwara *et al.* (1998). Modifications from that procedure are described here. The chromatographic analysis of carotenoids was performed on a Hewlett–Packard Series 1100 high-performance liquid chromatographic system equipped with a diode array detector (DAD) and a mass selective detector (MSD). The chromatographic system was computer controlled using HP Chem Station software. Chromatography was carried out on a Spherisorb S5 ODSI C18 column (Waters Corp.; 4.6 × 250 mm). The MSD was used in the positive-ionization mode of the atmospheric pressure chemical ionization (APCI) module.

Metabolic Engineering **3**, 313–321 (2001)
doi:10.1006/mben.2001.0194

Columns and lines were washed sequentially with 100% methanol and 100% acetonitrile to equilibrate the column. The isocratic mobile phase consisted of 70% methanol and 30% acetonitrile. HPLC separations were performed in 9 min, with a 2-min postrun wash and a flow rate of 1.5 ml/min. When the MSD was used, separations were 20 min in length, with a 5-min postrun wash and a solvent flow rate of 1.0 ml/min. The injection volume was set to 15 μl and the HPLC column was heated to 35° C. Blanks of acetone were run on the column before each sample series.

Concentrations of lycopene, β-carotene, and phytoene were tracked by monitoring the output with the diode array detector at wavelengths of 472, 452, and 295 nm, respectively. Carotenoids were identified on the HPLC–MSD by examining the UV–vis and mass spectra of individual peaks.

RESULTS AND DISCUSSION

Design of Base Plasmid for Phytoene Production

E. coli naturally produces a precursor in the isoprenoid pathway, isopentyl-pyrophosphate (IPP) (Fig. 1A). By expressing *crtE* and *crtB* in *E. coli,* the cells can produce phytoene, the first carotenoid in the pathway, from IPP. Plasmids pAC-PHYT and pAC-PHYT+ were designed to enable the cells to produce phytoene at a constant level. The design of these plasmids was based on pAC-LYC, which carries the *crtEBI* genes from *Erwinia herbicola* and confers on cells the ability to produce lycopene (Cunningham *et al.*, 1994). *crtI* was removed from pAC-LYC to make pAC-PHYT, and *dxs* was inserted into the partially deleted *crtI* to make pAC-PHYT+ (Fig. 1B). The plasmid was maintained using the selective pressure of a chloramphenicol resistance marker. We introduced *dxs* into pAC-PHYT because previous studies have shown increases in *dxs* expression to increase carotenoid production (Albrecht *et al.*, 1999; Matthews and Wurtzel, 2000; Wang *et al.*, 2000; Kim and Keasling, 2001). However, *dxs* expression did not affect carotenoid production with this system (data not shown), and thus we will report only those results obtained from using pAC-PHYT in the two-plasmid system.

It is not clear why *dxs* expression did not affect carotenoid production in our system. It is possible that removing some of the genes from the original *crt* operon and placing them in the new operons created bottlenecks in carotenoid production downstream of *dxs,* such that overexpression of *dxs* had no effect on product titers. Regardless of the lack of an effect by *dxs,* cells containing the various constructs produced equivalent amounts of total carotenoids because the changes in expression of *crtI* and *crtY* did not affect farnesyl-pyrophosphate (FPP) production. However, the relative amounts of β-carotene, phytoene, lycopene, and other intermediates changed with the relative expression levels of *crtI* and *crtY*.

Design of Dual-Gene Systems

The design of the dual-gene construct has been described previously in Smolke *et al.* (2000). Briefly, a synthetic operon containing two reporter genes, *gfp* and *lacZ,* under the control of the *araBAD* promoter was designed to allow introduction of secondary structures and RNase cleavage sites in the form of DNA cassettes at various locations in the operon. A DNA cassette encoding a 3′ hairpin for *gfp* and an intercistronic region with a putative RNase E site immediately 5′ of *lacZ* was inserted into the dual-gene plasmid p50gl, making p70gl. Three DNA cassettes encoding previously designed hairpin structures (HP4, HP16, and HP17) (Carrier and Keasling, 1999) were inserted immediately downstream of the putative RNase E site, making plasmids p70gHP4l, p70gHP16l, and p70gHP17l. The system was designed to ensure that inserted secondary structures would not interfere with translation and that there would be no differences in translation initiation.

The decay intermediates of the p70 systems used in this paper have been described previously (Smolke *et al.*, 2000). Briefly, the engineered RNase cleavage site located between the two genes directs processing of the primary transcript in this area. Two stable secondary transcripts result, each containing a single coding region. The secondary transcripts are protected from immediate decay at both their 5′ and 3′ ends through engineered secondary structures. Depending on the combination of secondary structures and RNase sites used, different secondary transcript stabilities resulted (Smolke *et al.*, 2000). The *gfp* coding region was replaced by *crtY* and the *lacZ* gene was replaced by *crtI* in the systems described here (Fig. 2).

Identification of Carotenoids by Visual Inspection

Carotenoid production in cells was determined initially by visual inspection of colonies harboring the two plasmids grown on solid medium or cell pellets harvested from liquid culture. Cells carrying p70yi, p70yHP4i, and p70yHP17i appeared yellow on plates. Cells harboring p70yHP4i had the darkest yellow color, followed by cells harboring p70yi. Cells harboring p70yHP17i had the lightest yellow color, whereas cells harboring p70yHP16i appeared pink. When induced cells were grown in liquid

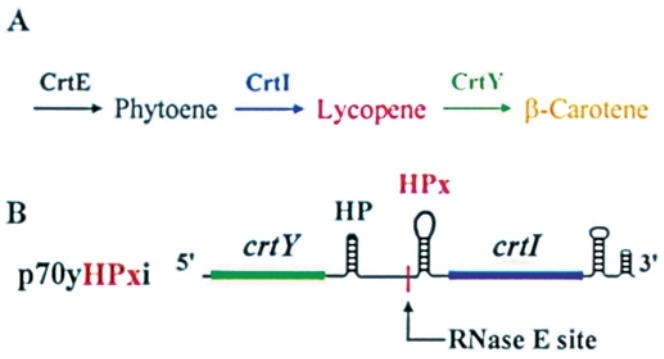

FIG. 2. Simplified pathway and RNA constructs. (A) Simplified form of the section of the carotenoid biosynthesis pathway through which the flux was modified and monitored. (B) General mRNA representation of the constructs used to modify flux in the p70 systems. p70yHP4i, p70yHP16i, and p70yHP17i differ in the hairpin located 5′ of the *crtI* coding region. p70yi lacks this hairpin 5′ of *crtI*. All other features of these constructs are identical.

culture, the harvested pellets had the same pigment patterns (Fig. 3). The intensity of colors varied with induction levels, indicating that higher gene expression could increase the flux through the metabolic pathway (data not shown). Cells harboring pAC-PHYT, pAC-PHYT+, or only the p70 plasmids had no pigment associated with them.

Identification of Carotenoids and Intermediates

Samples were analyzed by HPLC–MS to identify intermediates of the carotenoid pathway. In the positive chemical ionization mode, the protonated quasi-molecular ion $(M+H)^+$ is usually observed. Due to the gentler nature of this mode of ionization, the baseline ion and the molecular ion (carotenoid without protonation or breakage) are identical. Therefore, only the molecular ion was recorded as verification of the carotenoid identity (Table 2). Based on the elution order, the molecular ion, and the UV–vis spectrum (Fig. 4) (Ebenezer and Pattenden, 1993; Schmidt-Dannert *et al.*, 2000), we identified ζ-carotene, neurosporene, and *β*, *ψ*-carotene, which are intermediates of the *β*-carotene pathway (Fig. 1). These intermediates were seen in all samples at different relative levels.

Quantification of Carotenoids

The levels of carotenoids produced at various inducer concentrations were determined by UV–vis absorbance of peaks eluted from the HPLC column (Fig. 3). Since relative carotenoid levels for a particular dual-plasmid system were identical at all inducer concentrations, only the low inducer concentration studies are shown for each system. Retention times and absorbance maxima are listed for each compound (Table 2).

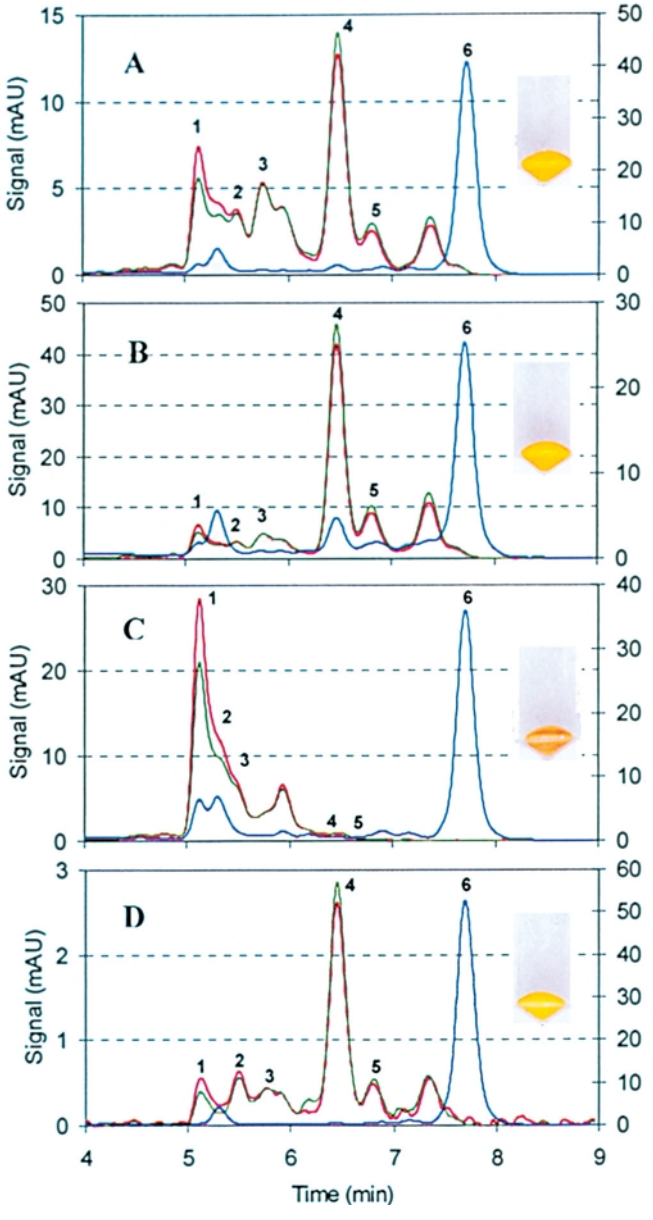

FIG. 3. HPLC analysis of carotenoid extracts of cells harboring various plasmid constructs. Red line, 472 nm (left axis); green line, 452 nm (left axis); blue line, 295 nm (right axis). The peaks are lycopene (1), neurosporene (2), *β*, *ψ*-carotene (3), *β*-carotene (4), ζ-carotene (5), and phytoene (6). Retention times are listed in Table 2. Inserts in the plots are pictures of corresponding cells concentrated in microfuge tubes prior to carotenoid extraction. (A) p70yi. (B) p70yHP4i. (C) p70yHP16i. (D) p70yHP17i.

Cells harboring p70yi had all carotenoids present at intermediate levels. *β*-Carotene was present (peak 4, Fig. 3A) in cells harboring p70yi at levels lower than in cells harboring p70yHP4i, but higher than in cells harboring the other two constructs. Phytoene (peak 6, Fig. 3A) was present in cells harboring p70yi at levels similar to cells

Metabolic Engineering **3**, 313–321 (2001)
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TABLE 2

Properties of Carotenoids from HPLC and LC–MS Analysis

Carotenoid	Retention time (min)	absorbance maxima (nm)[a]	Molecular ion (m/z)
Phytoene	7.7	276, 286, 295	545.4
ζ-Carotene	6.8	376, 396, 422	541.4
Neurosporene	5.5	420, 440, 468	539.4
Lycopene	5.1	446, 472, 504	537.4
β, ψ-Carotene	5.9	438, 462, 490	537.4
β, β-Carotene	6.5	424, 452, 478	537.4

[a] In 70% methanol, 30% acetonitrile.

harboring p70yHP16i. ζ-Carotene (peak 5, Fig. 3A) was present in cells harboring p70yi at levels lower than cells harboring p70yHP4i, but higher than cells harboring the other two constructs. Lycopene, neurosporene, and β, ψ-carotene (peaks 1, 2, and 3, respectively, Fig. 3A) were present at intermediate levels in cells harboring p70yi, similar to cells harboring p70yHP4i (Fig. 3B).

Cells harboring p70yHP4i accumulated more β-carotene than the other three constructs (Fig. 3B). ζ-Carotene and phytoene were present at levels higher and lower, respectively, than cells harboring the other three constructs.

Cells harboring p70yHP16i accumulated more lycopene than the other three constructs (Fig. 3C). ζ-Carotene and β-carotene were present at low levels, similar to cells harboring p70yHP17i. Neurosporene and β, ψ-carotene were present at intermediate levels.

Cells harboring p70yHP17i accumulated more phytoene than the other three constructs (Fig. 3D). All other carotenoids were present at very low levels.

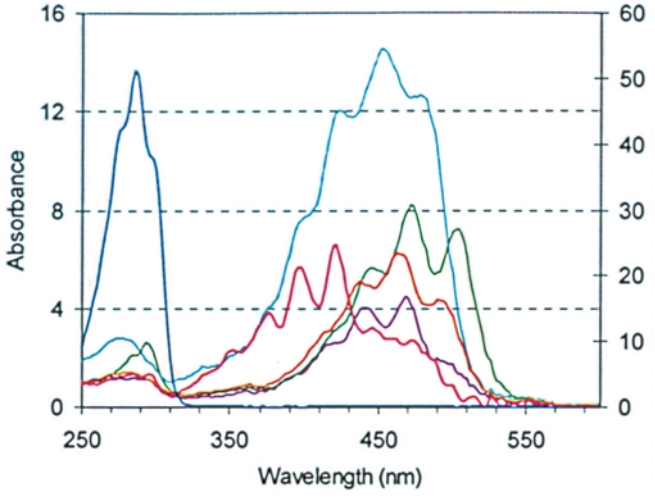

FIG. 4. Spectra of carotenoid extracts. Corresponding peak maxima are given in Table 2. Dark blue, phytoene; light blue, β-carotene; red, ζ-carotene; orange, β, ψ-carotene; green, lycopene; purple, neurosporene. All spectra magnitudes are read on the left axis except dark blue (phytoene) which is read on the right axis.

Expression Analysis

The constructs used in this study are examples of how one can use mRNA stability control elements to alter the accumulation of intermediates in a metabolic pathway of interest. Plasmid p70yi, which contains a secondary structure 3′ of *crtY* and a putative RNase E site between *crtY* and *crtI* but no secondary structure 5′ of *crtI,* serves as the base case. Cells harboring this plasmid produced intermediate levels of β-carotene and lycopene (Fig. 5A).

The addition of a hairpin 5′ of *crtI* in plasmid p70yHP4i directs the flux through the pathway to high levels of β-carotene (relative to lycopene) (Fig. 5A). In the *gfp–lacZ* reporter system, the hairpin 5′ of the second gene stabilized the transcript containing that coding region. While the transcript stabilities and enzyme levels were not determined for the *crtYI* system, it appears that the hairpin 5′ of *crtI* had a similar effect, resulting in a larger amount of CrtI. As such, more phytoene is converted to lycopene, which can then be converted to β-carotene by CrtY. In this construct, the relative expressions of *crtI* and *crtY* were balanced such that a higher flux is obtained through the pathway.

The replacement of HP4 with HP16 drastically changed the relative levels of lycopene and β-carotene (Fig. 5A). In the *lacZ–gfp* reporter system (p70gHP16l), HP16 stabilized the *lacZ* secondary transcript, resulting in higher production of β-galactosidase (relative to GFP) than from p70gHP4l. A similar phenomenon appears to be occurring with p70yHP16i; the hairpin 5′ of *crtI* appears to stabilize the transcript containing that coding region. A very large amount of CrtI is produced from this stabilized transcript resulting in very high lycopene production. The flux from lycopene to β-carotene is now limited by *crtY* expression. Therefore, very low amounts of β-carotene were produced. It would appear that the expression level of *crtY* in this construct is being changed, potentially due to some secondary effect.

Substitution of HP17 for HP4 or HP16 significantly affected the production of both lycopene and β-carotene (Fig. 5A). From studies with this hairpin in the dual-gene reporter system (Smolke *et al.*, 2000), it was shown that this structure in the 5′ position leads to very low expression of the second gene in the operon, directly following the hairpin. Although this hairpin stabilizes the

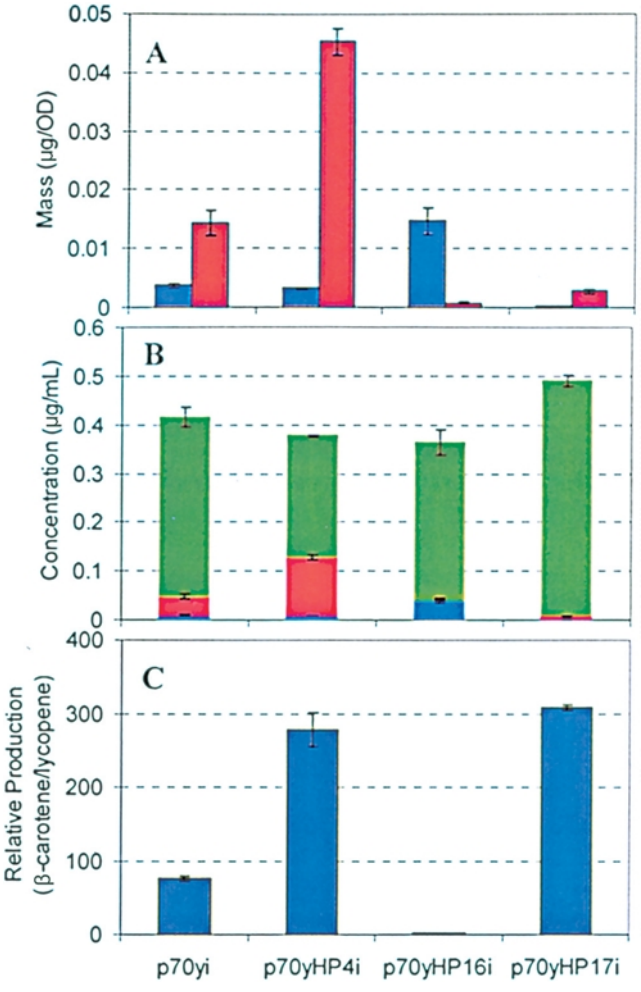

FIG. 5. Carotenoid analysis. (A) Amounts of β-carotene (red bars) and lycopene (blue bars) from various constructs. (B) Amounts of β-carotene (red), lycopene (blue), and phytoene (green) from various constructs. (C) Ratios of β-carotene to lycopene in cells harboring the various constructs (relative to p70yHP16i). Values represent means from three replicates and error bars represent standard deviations from the mean.

transcript, it appears that its structure somehow interferes with translation of the gene. Therefore, we presume that a small amount of CrtI was produced resulting in low levels of lycopene. Since the levels of β-carotene are dependent on how much lycopene is synthesized, low levels of β-carotene were present as well. Interestingly, the ratios of β-carotene to lycopene were nearly the same using HP4 and HP17, indicating that production of CrtY was unaffected by the hairpin 5′ of *crtI*.

The presence of intermediates in these samples can be compared to the carotenoids present in cells harboring pAC-BETA, which carries the genes for β-carotene production. With this plasmid, HPLC–MS analysis indicated that only β-carotene and phytoene were present in the cells (data not shown). The only difference between the two plasmid systems described in this paper and pAC-BETA is the transfer of the *crtI* and *crtY* coding regions from pAC-BETA (a pACYC184-based plasmid) to the pBAD24-based plasmid (under P_{BAD} control) and the presence of the engineered mRNA control elements to direct the processing and stabilities of transcripts containing the individual coding regions. This indicates that rational design of metabolic pathways can control the flux through the pathway and affect the levels of intermediates that accumulate. Previously, it was reported that cells harboring a mutant *crtI* allowed accumulation of intermediates (that were subsequently converted to novel products using mutated cyclases) that were not present in cells harboring wild-type versions of *crtI* (Schmidt-Dannert *et al.*, 2000). The work presented here demonstrates that one need not have a mutation in *crtI* to accumulate these intermediates; changes in the relative levels of the two enzymes in the pathway can lead to the same intermediates. Thus, it appears that an enzyme's ability to catalyze two or more subsequent reactions (phytoene to ζ-carotene to neurosporene to lycopene by CrtI and lycopene to β, ψ-carotene to β, β-carotene by CrtY) is affected by its production level or by the levels of precursors that accumulate inside cells.

As expected, the total levels of carotenoids (the sum amount of β-carotene, lycopene, and phytoene) within the cells were similar for all constructs studied, while the distribution of individual carotenoids varied (Fig. 5B). This result was expected as we did not manipulate the production of enzymes upstream of phytoene.

The relative production of β-carotene to lycopene for these systems varied from 1- to 300-fold (Fig. 5C). This range is larger than the relative protein levels reported previously in similar constructs with the reporter gene systems (Smolke *et al.*, 2000). One reason for this difference might be the application of this technology to an actual metabolic pathway, where the products of the enzymes in the pathway are actually substrates for other enzymes in the pathway. This may lead to amplification of relative trends in mRNA stability and protein production from those seen in reporter gene systems. This work demonstrates the application of directed mRNA processing and stability technologies for metabolic pathway design and optimization.

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