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Expression and Activity of Citrus Phytoene Synthase and β-Carotene Hydroxylase in *Escherichia coli*

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Citrus phytoene synthase (CitPsy) and β-carotene hydroxylase (CitChx), which are involved in carotenoid biosynthesis, are distantly related to the corresponding bacterial enzymes from the point of view of amino acid sequence similarity. We investigated these enzyme activities using *Pantoea ananatis* carotenoid biosynthetic genes and *Escherichia coli* as a host cell. The genes were cloned into two vector systems controlled by the T7 promoter. SDS-polyacrylamide gel electrophoresis showed that CitPsy and CitChx proteins are normally expressed in *E. coli* in both soluble and insoluble forms. *In vivo* complementation using the *Pantoea ananatis* enzymes and HPLC analysis showed that β-carotene and zeaxanthin were produced in recombinant *E. coli*, which indicated that the citrus enzymes were functionally expressed in *E. coli* and assembled into a functional multi-enzyme complex with *Pantoea ananatis* enzymes. These observed activities well matched the results of other researchers on tomato phytoene synthase and Arabidopsis and pepper β-carotene hydroxylases. Thus, our results suggest that plant carotenoid biosynthetic enzymes can generally complement the bacterial enzymes and could be a means of carotenoid production by molecular breeding and fermentation in bacterial and plant systems.

Key words: carotenoid biosynthesis, phytoene synthase, β-carotene hydroxylase, β-carotene, zeaxanthin, *E. coli*

Carotenoids are one of the largest groups of natural pigments and are produced from all photosynthetic organisms including plants, cyanobacteria, and algae, as well as by numerous non-photosynthetic bacteria and fungi (Schmidt-Dannert, 2000). Carotenoids are important biological precursors of vitamin A and are generally believed to enhance human health. Several studies have reported an inverse relationship between carotenoid consumption and the incidence of some cancers (Bramley, 2000; Niles, 2000). Moreover, carotenoids have antioxidative activities and play important roles in reducing chronic diseases. Recently, it has been discovered that carotenoids play key roles in junctional communication and gene regulation attributed to tumor suppressing activity (Bertram, 1999). Carotenoids are currently used as food colorants, nutritional supplements, and for cosmetic and pharmaceutical purposes.

All carotenoids are derived from the isoprenoid or terpenoid pathways (Cunningham and Gantt, 1998). The condensation of three molecules of isopentenyl pyrophosphate and one molecule of dimethylallyl pyrophosphate produces the diterpene geranylgeranyl pyrophosphate (Fig. 1). The tail-to-tail condensation of two molecules of geranylgeranyl pyrophosphate results in the first carotenoid, phytoene, the production of which is catalyzed by phytoene synthase (Psy). This step is the first reaction in the pathway leading to carotenoid biosynthesis. Phytoene undergoes a series of four desaturations that result in the formation of lycopene. These desaturation reactions lengthen the conjugated double bond that constitutes the chromophore in carotenoid pigments. In higher plants, these steps are catalyzed by two enzymes, phytoene desaturase and ξ-carotene desaturase. Lycopene is converted into β-carotene or α-carotene by cyclization. Subsequent hydroxylation reactions produce zeaxanthin and lutein, respectively; zeaxanthin is produced from β-carotene via β-cryptoxanthin by β-carotene hydroxylase (Chx). Moreover, it has been reported that the carotenoid biosynthetic enzymes functionally form multi-enzyme complexes (Cunningham and Gantt, 1998).

A number of carotenoids have been isolated from natural sources and produced by microbial fermentation (Schmidt-Dannert, 2000). Recently, metabolic engineering has offered possible means of improving the level of carotenoid production (Wang *et al.*, 2000). In addition, the isolation of a number of carotenoid biosynthetic genes could contribute to the engineering of microorganisms

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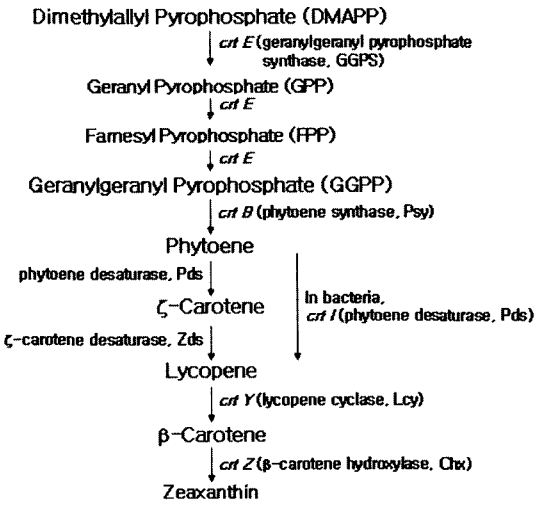

Fig. 1. Scheme of the carotenoid biosynthetic pathway in higher plants.

and crop plants (Römer *et al.*, 2000; Rosati *et al.*, 2000; Ye *et al.*, 2000).

Previously we isolated cDNAs encoding Psy and Chx from citrus (Kim *et al.*, 2001a; Kim *et al.*, 2001b). In the carotenoid biosynthetic pathway, these two enzymes are important and/or regulatory enzymes in the production of carotenoids such as lycopene, β-carotene, zeaxanthin, and β-cryptoxanthin. It is known that almost all cloned carotenoid biosynthetic enzymes, with a few exceptions, are functionally expressed in *E. coli*, thus demonstrating that enzymes from phylogenetically distant species can be associated into a functional multi-enzyme complex. Although many genes have been isolated from various species of plants, only the enzyme activities of tomato Psy (Misawa *et al.*, 1994), and Arabidopsis and pepper Chx (Sun *et al.*, 1996; Bouvier *et al.*, 1998; Tian and DellaPenna, 2001) have been characterized in bacterial systems. We believed that citrus Psy (CitPsy) and Chx (CitChx) could also be functionally expressed in a bacterial system. To confirm the formation of the functional complexes of plant enzymes with bacterial enzymes, we believed that a study of citrus Psy and Chx would be useful.

This paper describes the individual expressions of the two enzymes, CitPsy and CitChx, in *E. coli* and demonstrates their ability to assemble a functional enzyme complex with *Pantoea ananatis* carotenoid biosynthetic enzymes. Our results suggest that citrus enzymes can be used as a means of engineering bacteria and plants for the production of valuable carotenoids.

Materials and Methods

Plasmid construction

To amplify *CitPsy* (Kim *et al.*, 2001b) and *CitChx* cDNAs (Kim *et al.*, 2001a), oligomeric primers were synthesized as follows: for *CitPsy* cDNA, CitPsy5, 5'-CCTAGCTAGCGCTAGCACTGCTGGA-3' and CitPsy3, 5'-CGGGATCCGTTTCAACTATATATGCTT-3'; for *CitChx* cDNA (*CHX1* and *CHX2*), CitChx5, 5'-CCTAGCTAGCTCGGGTACCAGATC-3' and CitChx3, 5'-CGGGATCCATTAAATTGATTATTTTGG-3'. CitPsy5 and CitChx5 were used as forward PCR primers. Their 5'-ends begins next to the site of the 89 and 81 amino acids from amino terminals of the *CitPsy* and *CitChx* structural genes, respectively, and which contains the mismatched bases of the *Nhe*I restriction site (underlined letter). CitPsy3 and CitChx3 were used as reverse PCR primers corresponding to 3'-UTR of the *CitPsy* and *CitChx* genes, respectively. They contain the mismatched bases of *Bam*HI restriction site (underlined letter). The introduced restriction sites allowed the PCR product to insert into the *Nhe*I and *Bam*HI sites of pRSET-A (Invitrogen, USA).

PCR amplification was performed using a DNA thermal cycler (Perkin-Elmer Cetus) in a 50 μl volume using *pfu* DNA polymerase (Promega, USA), 0.1 mM dNTP, and 20 pmole of each primer. Initial denaturation was carried out at 94°C for 5 min, and amplification was performed over 20 cycles of; 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. PCR products were visualized on 1.2% agarose gels and stained with ethidium bromide. The products were purified using a GenCleanII kit (BIO 101, Inc, USA), and then were digested with *Nhe*I and *Bam*HI. Products obtained by treating with *Nhe*I and *Bam*HI were cloned, in frame, in the *Nhe*I and *Bam*HI restriction sites, downstream of the pRSET-A T7 promoter. pRPSY vector carrying *CitPsy* and pRCHX carrying *CitChx*, both plasmids contained the carbenicillin-resistant gene.

Isolation of carotenoid biosynthetic genes from Pantoea ananatis (former name: Erwinia uredovora)

To assay the enzyme activity of phytoene synthase, we cloned the *Pantoea ananatis* genes *crtE*, *crtX*, *crtY*, and *crtI* (Misawa *et al.*, 1990) into the *Nhe*I and *Xho*I sites of pET24b (Novagen, USA), called pET-BZ, and then transformed pET-BZ into *E. coli* BL21 (DE3). To check the enzyme activity of β-carotene hydroxylase, we constructed a vector, pET-Z, containing *Pantoea ananatis* *crtE*, *crtX*, *crtY*, *crtI*, and *crtB* genes in *Nhe*I and *Xho*I sites of pET24b, which contained the kanamycin-resistant gene, and then transformed pET-Z into *E. coli* BL21(DE3).

Carotenoid biosynthetic genes, *crtE*, *crtX*, *crtY*, *crtI* and *crtB* (GenBank accession number D90087) were isolated from *Pantoea ananatis* genomic DNA by PCR using LA *Taq* polymerase (Takara, Japan). Genomic DNA was extracted from *Pantoea ananatis* as described by Murray and Thompson (1980). PCR primers were synthesized as follows: to isolate *crtE*, *crtX*, *crtY*, and *crtI*, crt5(NheI), 5'-CCTAGCTAGCATGACGGTCTGCGCAAAA-3' and crt 3(XhoI)-Z, 5'-GCCGCTCGAGGGCGCTAGAGCGGGC GCT-3'; for isolation of *crtE*, *crtX*, *crtY*, *crtI*, and *crtB*, crt5(NheI) and crt3(XhoI)-BZ, 5'-GCCG- CTCGAGGATTATTCAAATCAGATC-3'.

Table 1. Plasmids used for the production of β-carotene and zeaxanthin in *E. coli* BL21(DE3)

Plasmid	Expressed enzyme	Carotenoids	Source (GenBank Accession No.)
pRPSY	CitPsy	None	
pRCHX	CitChx (CHX1, CHX2)	None	AF220218
pET-BZ	*Pantoea* CrtE, CrtX, CrtY, CrtI	None	AF296158, AF315289
pET-Z	*Pantoea* CrtE, CrtX, CrtY, CrtI, CrtB	β-Carotene	D90087
pRPSY+pET-BZ	CrtE, CrtX, CrtY, CrtI, CitPsy	β-Carotene	D90087
pRCHX+pET-Z	CrtE, CrtX, CrtY, CrtI, CrtB, CitChx	Zeaxanthin	

Cell growth

To analyze the expressions of CitPsy and CitChx protein, and carotenoid production, cultures of *E. coli* BL21(DE3) containing various plasmids (Table 1) were grown in 5 mL of LB medium containing carbenicillin and/or kanamycin. The cells were incubated at 37°C with gentle shaking until the absorbency of the culture at 600 nm reached about 0.5. The culture was then induced by adding 1 mM (final concentration) isopropyl β-D-thiogalactopyranoside (IPTG) and then incubated for 8 h. Cells were harvested by centrifugation at 6000 rpm for 15 min.

SDS-polyacrylamide gel electrophoresis

E. coli cells resuspended in 20 mM Tris-HCl buffer, pH 8.0, were completely disrupted by sonication on ice. The insoluble fraction was pelleted by centrifugation; the supernatant is referred to as the soluble fraction. Protein samples were analyzed using an SDS-containing discontinuous polyacrylamide gel (15%) electrophoresis system, as described by Laemmli (1970). Samples of the insoluble fraction were mixed with 1x sample buffer and boiled for 15 min. Electrophoresis was carried out using an SE250 Mighty Shell II Vertical Slab Gel Unit (Hoeffer Scientific Instruments, USA). After running at a constant current of 20 mA, the gels were stained by submerging them for 1 h into 0.05% Coomassie Brilliant Blue solution (dissolved in 50% methanol, 10% acetic acid, and 40% DDW).

Carotenoid pigment analysis

Cell harvest, pigment extraction, and HPLC analysis of the carotenoid pigments were similar to those previously described (Cunningham *et al.*, 1994) with a few modifications. In brief, after the cell growth phase, the cells were harvested, resuspended in 50 μl of water, and then 100 ml of 6% KOH in methanol was added. The insoluble components were pelleted by centrifugation, and the pellets were extracted with methanol, then with methanol/diethyl ether (1/1, v/v), and finally with diethyl ether until they became colorless. The extracts were combined, and the carotenoid pigments were transferred to diethyl ether and washed free of alkali. The ether layer was reduced to dryness on a rotary evaporator at 30°C. The saponified samples were then dissolved in an appropriate volume of methyl tert-butyl ether (MTBE)/methanol (1/1, v/v) for HPLC analysis and filtered through a 0.45 μm polytetrafluoroethylene syringe filter (Micro Filtration Systems, USA).

The separation was carried out using a μBondapak™ C18 reverse phase column (3.9×30 mm, particle size 10 μm) (Waters Chromatography, USA) and a guard column containing C18. The major components in the solvent extracts obtained from *E. coli* were identified by comparing their HPLC retention times and UV-visible absorption spectra with those of known reference compounds. Carotenoids were separated at room temperature on reverse phase column using an MTBE/methanol/water gradient from (95:1:4) to (25:71:4) over 13 min. Peak responses were measured at 445 nm.

Results and Discussion

Sequence comparisons between CitPsy and CitChx and those of other organisms

The deduced amino acid sequences of *CitPsy* and *CitChx* (CHX1) were compared to those of other organisms (Kim *et al.*, 2001a; Kim *et al.*, 2001b) (Fig. 2 and Fig. 3). The CitPsy sequence showed a high degree of similarity with the Psy sequences of higher plants including tomato (84% and 75%), pepper (75%), muskmelon (73%), Arabidopsis (70%), daffodil (68%), carrot (65%), and maize (64%). However, the sequence did not show significant homology with bacterial Psy (4-31%) (Fig. 2). CitChx showed similar homologous patterns with CitPsy in higher plants (57-68%) and bacteria (6-32%) (Fig. 3). Nine histidine residues essential for enzymatic function, were found to be conserved in citrus and bacterial enzymes (Fig. 3, boxed), the residues of which are putative iron ligands of carotenoid hydroxylases, or may participate in the electron transfer process (Bouvier *et al.*, 1998). Plants enzymes have longer N-terminal regions than bacterial enzymes (Fig. 2 and Fig. 3). This region is important for protein targeting and/or dimerization of the enzyme (Sun *et al.*, 1996; Cunningham and Gantt, 1998). Although no significant homology was found between plants and bacterial enzymes, it has been reported that the enzymes of Arabidopsis, pepper, and tomato can complement the bacterial carotenoid biosynthetic enzymes in *E. coli* (Misawa *et al.*, 1994; Sun *et al.*, 1996; Bouvier *et al.*, 1998). Moreover, as the citrus enzymes have a high degree of similarity with those of Arabidopsis, tomato, and pepper, it was believed that citrus enzymes could be functionally expressed in *E. coli*.

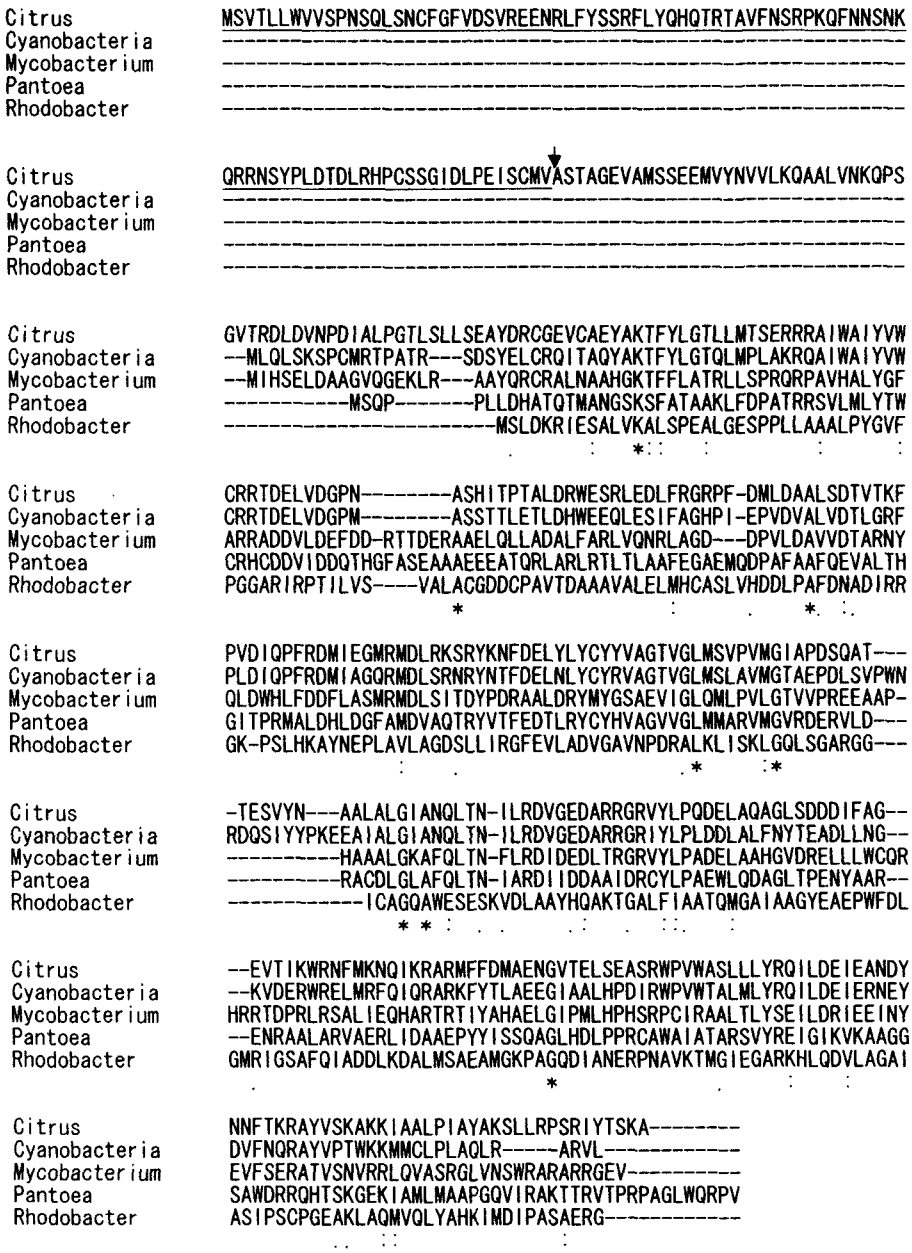

Fig. 2. Alignment of the deduced amino acid sequences of citrus phytoene synthase (*CitPsy*) with those of bacterial enzymes. Alignment was performed using the deduced amino acid sequences of each gene and was generated by Clustal X (version 1.81). The underlined sequence corresponds to the putative transit peptide region predicted by PCGENE software (IntelliGenetics Inc., Release 6.60). The arrow indicates the starting point of the *CitPsy* cDNA PCR product. The gene sequences used for amino acid translation and the GenBank accession numbers are: *Mycobacterium* (AJ133724), *Rhodobacter* (X52291), *Pantoea* (M38423), and *Cyanobacteria* (AB001284). An asterisk indicates identical amino acids.

Protein expression of CitPsy and CitChx in E. coli

In order to determine the normal expressions of *CitPsy* and *CitChx* cDNAs, constructs were prepared that lacked a putative chloroplast transit peptide in the N-terminal region (Sun *et al.*, 1996) (Fig. 2 and Fig. 3). This construct encodes the truncated *CitPsy* and *CitChx* gene products fused to several amino acids derived from vector sequence that contained the polyhistidine tag at the N-terminal. By IPTG induction, each protein was detected by SDS-PAGE as a single band of the expected size (Fig. 4). Protein bands of the same size were also detected in other vector systems including pET11C and pET21a (data not shown). In particular, most of the CitPsy protein was expressed in an insoluble form, i.e., as inclusion bodies, and a small amount of this protein was detected in the soluble fraction (Fig. 4). CitPsy protein was more abundantly expressed than CitChx protein (Fig. 4A), which was similar pattern to that found using vectors (data not shown). This phenomenon may have, at least partly, resulted from different codon usage in bacteria and citrus. The codon

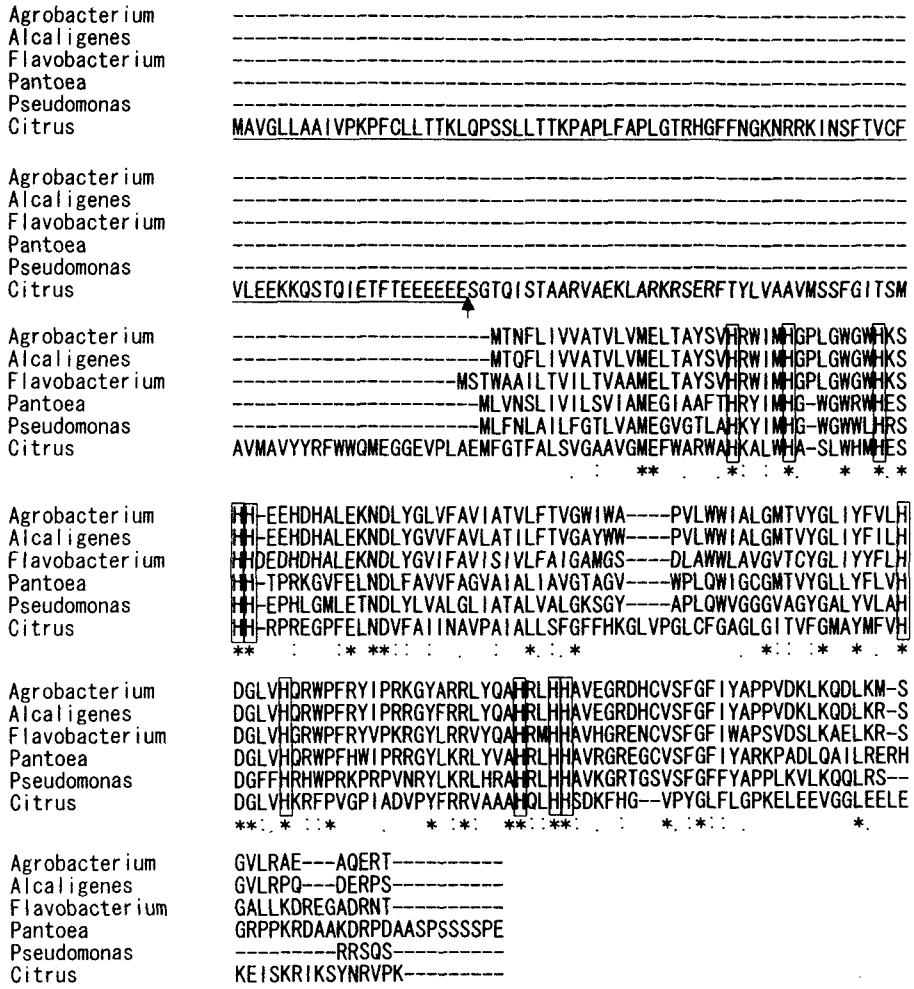

Fig. 3. Alignment of the deduced amino acid sequences of the citrus β-carotene hydroxylase (*CitChx*) with those of bacterial enzymes. Alignment was performed using the deduced amino acid sequences of each gene, and was generated using Clustal X (version 1.81). The underli ned sequence corresponds to the putative transit peptide region predicted by PCGENE software (IntelliGenetics Inc., Release 6.60). The arrow indicates the starting point of the *CitPsy* cDNA PCR product. The gene sequences used for amino acid translation and their GenBank accession numbers are: *Flavobacterium* (U62808), *Agrobacterium* (D58420), *Alcaligenes* (D58422), *Pantoea* (M87280), and *Pseudomonas* (NC_002947). An asterisk indicates identical amino acids and the boxed letters are histidine (H) residues, which are important for iron coordination.

usage of the *CitPsy* gene better matched that of bacteria than that of the *CitChx* gene, as determined by analysis at 'www.kazusa.or.jp/codon'.

Enzymatic activities of CitPsy and CitChx in E. coli

To determine whether CitPsy and CitChx were catalytically active, *E. coli* carrying the *Pantoea ananatis* gene clusters devoid of *crtB* and *crtZ* or *crtZ* genes was complemented with *CitPsy* (pRPSY) or *CitChx* (pRCHX) constructs, respectively (Table 1). Specifically, using carotenoid biosynthetic genes from *Pantoea ananatis*, we constructed the plasmids pET-BZ and pET-Z not to produce carotenoid and to produce β-carotene, respectively. When the *CitPsy* gene (pRPSY) was transformed into bacteria harboring pET-BZ, it was expected that β-carotene would accumulate in the cell. Zeaxanthin or cryptoxanthin would be produced only in transformed bacteria

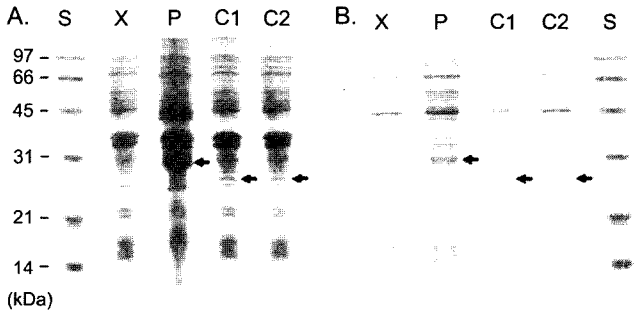

Fig. 4. Expression of CitPsy and CitChx in *E. coli* carrying pRPSY (P) or pRCHX (C1 and C2), respectively. Control (X) is the pattern of the expressed proteins of *E. coli* carrying pRSET-A, as a negative control. SDS-PAGE gels showed the band patterns of the soluble (A) and insoluble (B) fractions of the expressed proteins. *E. coli* BL21(DE3) was used as a host cell. Arrows indicate bands corresponding to CitPsy and CitChx proteins. S represents the low molecular standard (Bio-Rad, USA). Each lane contained about 20 μg of protein.

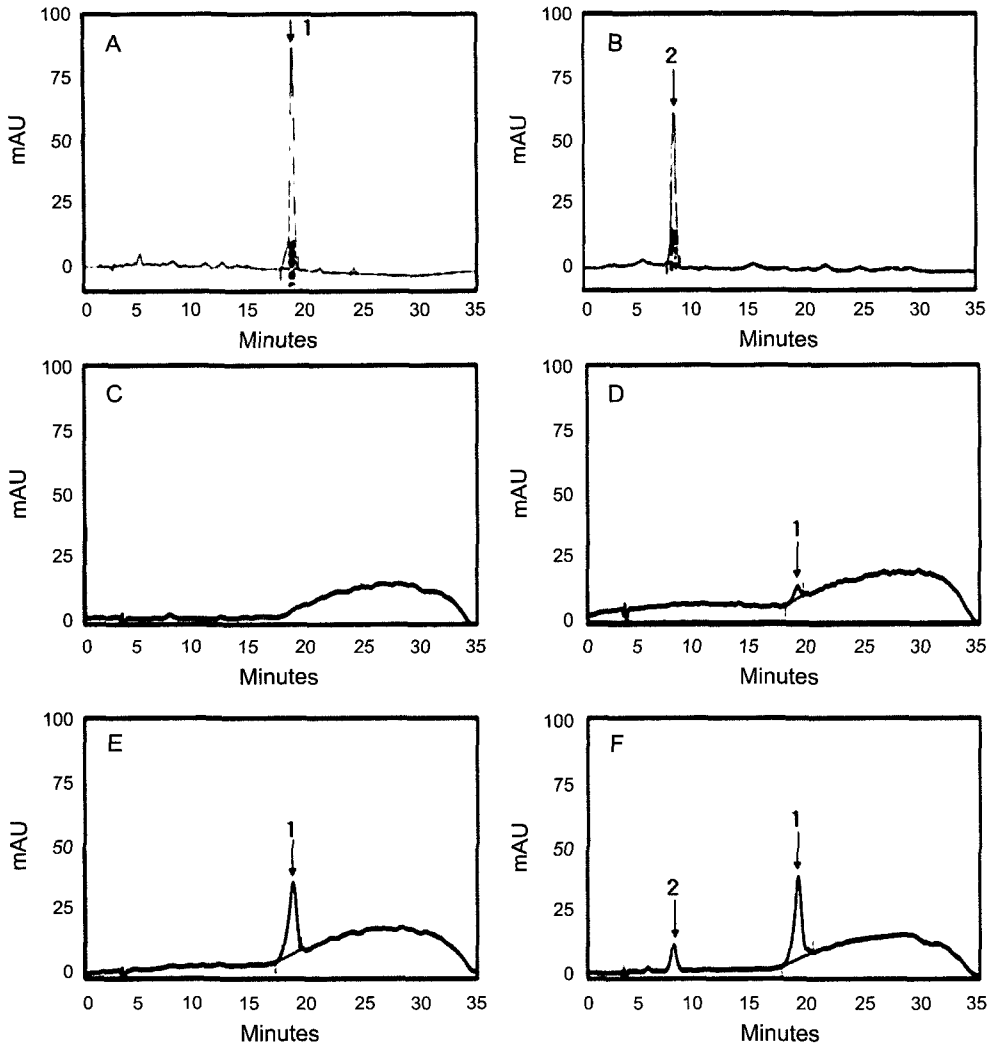

Fig. 5. HPLC analysis of carotenoid pigments produced during *in vivo* complementation in *E. coli*. Following IPTG induction and 8 h culture, the pigment extract of *E. coli* (C) transformed with pET-BZ and pRSET-A, (D) transformed with pET-BZ and pRPSY, (E) transformed with pET-Z and pRSET-A, and (F) transformed with pET-Z and pRCHX. The different peaks correspond to β-carotene (1) and zeaxanthin (2), as deduced from chromatograms of standard compounds (A and B).

with pET-Z and *CitChx* (pRCHX). *E. coli* colonies containing both pET-BZ and pRPSY were distinguishable from those containing both pET-Z and pRCHX, using the method of Sun *et al.* (1996), i.e., *E. coli* colonies containing β-carotene were orange/yellow in color and the colonies accumulating zeaxanthin were bright yellow (data not shown).

HPLC analysis of the carotenoid pigments in *E. coli* cultures inoculated with transformed cells containing pET-BZ and pRPSY showed a peak corresponding to β-carotene (Fig. 5D). The amount of β-carotene produced by CitPsy in *E. coli* was very much lower (9-10%) than that produced by *Pantoea ananatis crtB* (Fig. 5E). This might have been caused by the different solubilities between CitPsy and CrtB proteins (Fig. 4A) or enzymatic activities. A control culture, containing pET-BZ and pRSET-A vectors, did not produce any carotenoid peak (Fig. 5C). HPLC analysis of cell cultures harboring pET-Z and pRCHX showed that β-carotene is partly converted into zeaxanthin by CitChx, though a major portion (75-80%) was left (Fig. 5E and 5F). Thus, not all of the β-carotene was found to be transformed into zeaxanthin, which differs from results obtained using Arabidopsis β-carotene hydroxylase (Sun *et al.*, 1996). This difference could be caused by double transformation using two vector systems, which may induce a lower β-carotene hydroxylase expression level (Fig. 4) than the single vector system (Ruther *et al.*, 1997). Though only a portion (20-25%) of the β-carotene was converted to zeaxanthin, no peak corresponding to β-cryptoxanthin was detected in the HPLC elution profile. This may have been due to the presence of an undetectably small amount of β-cryptoxanthin. Previous studies have shown that the production ratio of zeaxanthin to β-cryptoxanthin can vary according to the

enzyme source and activity (Sun *et al.*, 1996; Bouvier *et al.*, 1998). In Arabidopsis, a small amount (1-5% of the total carotenoid) of β-cryptoxanthin was produced (Sun *et al.*, 1996). Thus, if we should perform carotenoid production in large scale culture using *E. coli* carrying pET-Z and pRCHX, a peak corresponding to β-cryptoxanthin could be detected.

From these results, we conclude that CitPsy and CitChx are functionally expressed in *E. coli* and that they can assemble into functional multi-enzyme complex with *Pantoea ananatis* carotenoid biosynthetic enzyme regardless of there being no significant amino acid sequence similarity. Also, the results suggest that CitPsy and CitChx are functionally operational in citrus plants. By changing culture conditions, and plasmid reconstruction, in combination with modifications of vector systems, and proteins, valuable carotenoid compounds like, β-carotene, zeaxanthin, and β-cryptoxanthin could be more efficiently produced by microbial fermentation and in crop plants.

Acknowledgments

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References

Bertram, J.S. 1999. Carotenoids and gene regulation. *Nutr. Rev.* 57, 182-191.

Bouvier, F., Y. Keller, A. d'Harlingue, and B. Camara. 1998. Xanthophyll biosynthesis: molecular and functional characterization of carotenoid hydroxylases from pepper fruits (*Capsicum annuum* L.). *Biochim. Biophys. Acta* 1391, 320-328.

Bramley, P.M. 2000. Is lycopene beneficial to human health? *Phytochemistry* 54, 233-236.

Cunningham, F.X., Jr. and E. Gantt. 1998. Genes and enzymes of carotenoid biosynthesis in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49, 557-583.

Cunningham, F.X., Jr., S. Sun, D. Chamovitz, J. Hirschberg, and E. Gantt. 1994. Molecular structure and enzymatic function of lycopene cyclase from the *Cyanobacterium synechococcus* sp Strain PCC7942. *Plant Cell* 6, 1107-1121.

Kim, I.-J., K.-C. Ko, C.-S. Kim, and W.I. Chung. 2001a. Isolation and characterization of cDNAs encoding β-carotene hydroxylase in *Citrus*. *Plant Science* 161, 1005-1010.

Kim, I.-J., K.-C. Ko, C.-S. Kim, and W.I. Chung. 2001b. Isolation and expression patterns of a cDNA encoding phytoene synthase in *Citrus*. *J. Plant Physiol.* 158, 795-800.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Misawa, N., M. Nakagawa, K. Kovayashi, S. Yamano, Y. Izawa, K. Nakamura, and K. Harashima. 1990. Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. *J. Bacteriol.* 172, 6704-6712.

Misawa, N., M.R. Truesdale, G. Sandmann, P.D. Fraser, C. Bird, W. Schuch, and P.M. Bramley. 1994. Expression of a tomato cDNA coding for phytoene synthase in *Escherichia coli*, phytoene formation *in vivo* and *in vitro*, and functional analysis of the various truncated gene products. *J. Biochem.* 116, 980-985.

Murray, M.G. and W.F. Thompson. 1980. Rapid isolation of high-molecular-weight plant DNA. *Nucleic. Acids Res.* 8, 4321-4325.

Niles, R.M. 2000. Vitamin A and cancer. *Nutrition* 16, 573-576.

Römer, S., P.D. Fraser, J.W. Kiano, C.A. Shipton, N. Misawa, W. Schuch, and P.M. Bramley. 2000. Elevation of the provitamin A content of transgenic tomato plants. *Nature Biotechnol.* 18, 666-669.

Rosati, C., R. Aquilani, S. Dharmapuri, P. Pallara, C. Marusic, R. Tavazza, F. Bouvier, B. Camara, and G. Giuliano. 2000. Metabolic engineering of beta-carotene and lycopene content in tomato fruit. *Plant J.* 24, 413-419.

Ruther, A., N. Misawa, P. Böger, and G. Sandmann. 1997. Production of zeaxanthin in *Escherichia coli* transformed with different carotenogenic plasmid. *Appl. Microbiol. Biotechnol.* 48, 162-167.

Schmidt-Dannert, C. 2000. Engineering novel carotenoids in microorganisms. *Curr. Opin. Biotechnol.* 11, 255-261.

Sun, Z., E. Gantt, and F.X. Cunningham. 1996. Cloning and functional analysis of the β-carotene hydroxylase of *Arabidopsis thaliana*. *J. Biol. Chem.* 271, 24349-24352.

Tian, L. and D. DellaPenna. 2001. Characterization of a second carotenoid β-hydroxylase gene from Arabidopsis and its relationship to the *LUT1* locus. *Plant Mol. Biol.* 47, 379-388.

Wang, C., M.-K. Oh, and J.C. Liao. 2000. Directed evolution of metabolically engineered *Escherichia coli* for carotenoid production. *Biotechnol. Prog.* 16, 922-926.

Ye, X., S. Al-Babili, A. Klöti, J. Zhang, P. Lucca, P. Beyer, and I. Potrykus. 2000. Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287, 303-305.