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Escherichia coli as a platform for functional expression of plant P450 carotene hydroxylases

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Abstract

Carotenoids and their derivatives are essential for growth, development, and signaling in plants and have an added benefit as nutraceuticals in food crops. Despite the importance of the biosynthetic pathway, there remain open questions regarding some of the later enzymes in the pathway. The CYP97 family of P450 enzymes was predicted to function in carotene ring hydroxylation, to convert provitamin A carotenes to non-provitamin A xanthophylls. However, substrate specificity was difficult to investigate directly in plants, which mask enzyme activities by a complex and dynamic metabolic network. To characterize the enzymes more directly, we amplified cDNAs from a model crop, *Oryza sativa*, and used functional complementation in *Escherichia coli* to test activity and specificity of members of Clans A and C. This heterologous system will be valuable for further study of enzyme interactions and substrate utilization needed to understand better the role of CYP97 hydroxylases in plant carotenoid biosynthesis.

Keywords: Carotene; P450; CYP97; Hydroxylases; Vitamin A; *Oryza sativa*; Metabolic engineering; Xanthophylls; Plants; *Escherichia coli* functional complementation

Carotenoids are an abundant class of isoprenoid compounds found in all plants where they are synthesized in plastids by nuclear-encoded enzymes. Their numerous roles in plant growth and development include functions as accessory pigments in photosynthesis and as photoprotectors [1–3]. Carotenoids such as β-carotene and zeaxanthin, among others, serve as precursors to cleavage products including the hormone abscisic acid (ABA) [4] and to other apocarotenoids, some of which are gaining attention for their roles in internal and external signaling [5–11]. The importance of certain carotenoids in human health has led to efforts to breed or metabolically engineer carotenoid content and composition [12–15].

Plants are an important dietary source of provitamin A carotenoids; predictable efforts to use breeding of vitamin-rich crops to address human vitamin A deficiency will require elucidation of those mechanisms controlling conversion of provitamin A carotenes to non-provitamin A xanthophylls. For example, in maize endosperm, there is wide variation in carotenoid content and composition [16,17] making this tissue a target for improvement of carotenoid content and provitamin A levels [1,18,19]. Like maize, wheat is another of many crops in the Poaceae that exhibit diversity in yellow seed color and are attractive breeding targets for manipulating endosperm carotenoids [20,21]. Endosperm carotenoids are synthesized and accumulate on amyloplast envelope membranes whereas in chloroplasts, carotenoids are found on envelope and thylakoid membranes. Targeting of carotenoid enzymes to specific plastid membranes and metabolon biogenesis/maintenance are poorly understood phenomena, especially given that many carotenoid enzymes are encoded by single copy genes and are destined for multiple suborganellar locations. Moreover, there is limited understanding of

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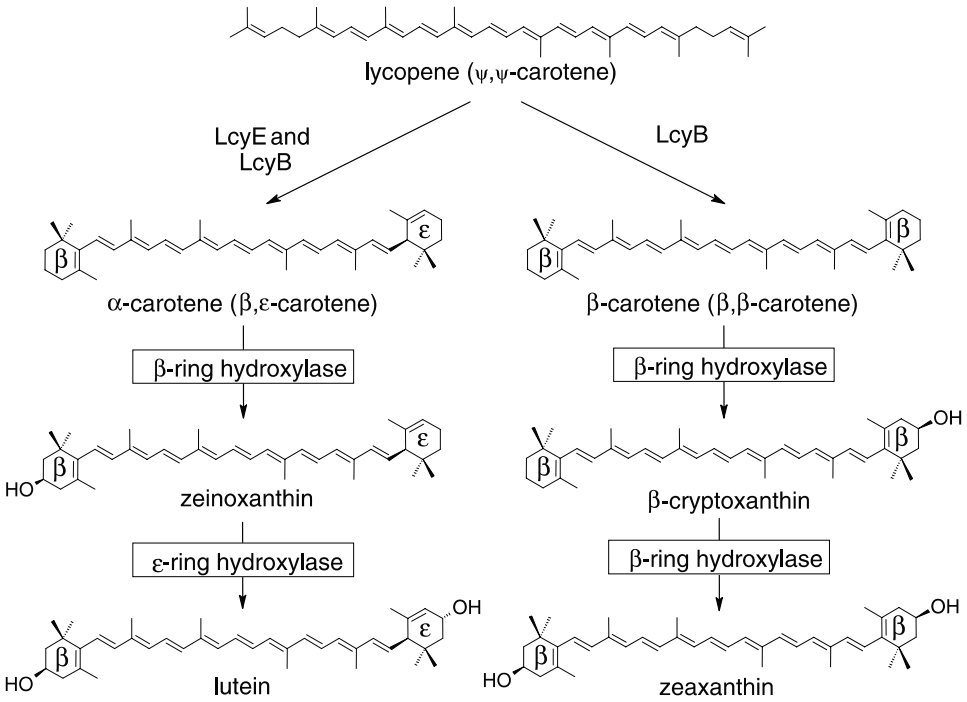


Fig. 1. Schematic illustration of the cyclohexene ring hydroxylation in β- and α-carotene. Conversion of lycopene to β-carotene requires lycopene β-cyclase (LcyB) alone, while conversion of lycopene to α-carotene requires lycopene β- and ε-cyclases (LcyE). Formation of the hydroxylated xanthophylls zeaxanthin and lutein is mediated by two separate stereospecific β- and ε-ring hydroxylases. In hydroxylation of α-carotene, the two hydroxylases may act in the reverse order shown, to yield the mono-hydroxy intermediate α-cryptoxanthin, in which the ε-ring is hydroxylated.

how pathway intermediates are channeled to downstream products including photosynthetic pigments or signaling molecule apocarotenoids.

In all plants, the biosynthesis of carotenoids begins with the formation of the 40-carbon phytoene, followed by desaturation steps leading to synthesis of lycopene, after which point the pathway diverges to form either β-carotene (having two β rings) or α-carotene (having one β-ring and one ε-ring) (Fig. 1). Further hydroxylation of the carotenes leads to biosynthesis of the xanthophylls. For example, hydroxylation of the C3 and/or C3′ on one or both β-rings of β-carotene leads to β-cryptoxanthin (β,β-carotene-3-ol) and zeaxanthin (β,β-carotene-3,3′-diol). In contrast, hydroxylation of the ε-ring, β-ring, or both rings of α-carotene generates α-cryptoxanthin (β,ε-carotene-3′-ol), zeinoxanthin (β,ε-carotene-3-ol), and lutein (β,ε-carotene-3,3′-diol), respectively [22]. The carotenes with β-rings have provitamin A activity, whereas their hydroxylated β-ring products do not [23].

In plants, the hydroxylation of provitamin A carotenes to form non-provitamin A xanthophylls is thought to be mediated by two structurally distinct classes of mixed function oxygenases. The first class of hydroxylases is comprised of the β-ring non-heme diiron monooxygenases. Members of this class were identified from a wide range of bacterial, algal, and plant species [24–26]. Plant diiron β-ring hydroxylases contain four transmembrane helices and four conserved iron-binding histidine clusters. Histidine residues in these clusters coordinate the orientation of two iron atoms to form oxo-bridged diferric centers [27], which are involved in the formation of a substrate based alkyl-radical and the subsequent hydroxylation of the substrate via an oxygen-rebound mechanism [28]. Enzyme activity of the diiron β-ring hydroxylases has been demonstrated in an *Escherichia coli*[1] functional complementation system [24]. The second class consists of P450 enzymes from the CYP97 clan, which on the basis of genetic evidence, are hypothesized to hydroxylate carotene ε- and β-rings, respectively [29,30]. These predicted P450 heme-thiolate hydroxylases contain a single transmembrane anchoring sequence, typical of other eukaryotic P450 enzymes [31,32]. In addition, each enzyme has a P450 domain, a conserved oxygen binding signature, and a conserved heme-thiolate-binding signature that binds a single heme group with one iron atom in the center [25,31,32]. Similar to diiron hydroxylation reactions, P450 hydroxylations are also redox-sensitive and involve the formation of a substrate based alkyl-radical that is immediately trapped by the $HO^{\cdot}$ from the iron atom [33]. A major limitation for the genetic evidence used to support substrate specificity of the CYP97 carotene hydroxylases is that biochemical profiling of mutants is complicated by poorly understood compensatory mechanisms, including changes in transcriptional activity of genes encoding other carotenoid biosynthetic enzymes such as the non-heme

[1] *Abbreviations used: E. coli*, *Escherichia coli*; CD, conserved domain; ORF, open reading frame; NUE, near-upstream element; FUE, far upstream element; CDD, conserved domain database; UTR, untranslated region.

diiron carotene hydroxylases, which may ultimately impact pathway flux [29,30].

Progress in characterizing the full complement of enzymes and identifying the corresponding genes has been significantly advanced with use of *E. coli* to functionally test activity of the membrane bound carotenoid pathway enzymes, an approach adopted by the carotenoid community [e.g. [24,34–38]]. The bacterial system could potentially provide a simple platform to manipulate and study the CYP97 carotene hydroxylases. Therefore, we isolated cDNAs from *Oryza sativa*, a representative member of the Poaceae, and used *E. coli* complementation to demonstrate activity and investigate carotene ring specificity of the putative P450 β- and ε- ring hydroxylases. Since only a subset of P450 enzymes function in *E. coli*, the widely used heterologous bacterial system was not necessarily a feasible approach. [39–41]. Our demonstration that these members of the CYP97 clan function in *E. coli* indicates that this system will be valuable in further dissecting the structural basis for ring specificity and other molecular applications. Given that β-ring hydroxylation is alternatively possible with either CYP97 or diiron enzymes raises the question of which are the appropriate genes/enzymes to choose as breeding targets or in metabolic engineering of this pathway; whether it is to achieve enhanced levels of endosperm provitamin A carotenoids in food crops, or increased abiotic and/or biotic resistance in plants in general.

Materials and methods

Phylogenetic and sequence analyses

Nucleotide and corresponding protein sequences, highly similar to the putative CYP97A4 coding mRNA from *O. sativa* L. (AK068163) [42], were obtained using BLAST analyses from all available public databases in NCBI GenBank and Institute of Genomic Research (TIGR) gene indices: CYP97A3 (#AAL25587), CYP97B3 (#AAL32753), CYP97C1 (#AAR83120) [29], and CYP86A1 (#NM_116260) from *Arabidopsis thaliana* (L.) Heynh.; CYP97B4 (#XP_464306) and CYP97C2 (#AK065689) from *Oryza sativa* L.; #BJ234910 and #CA501638 from *Triticum aestivum*; # TC69886, #TC76166 and #BM816653 from *Hordeum vulgare* L.; CYP97B1; synonym CYP97A2 (#Z49263) from *Pisum sativum* L. [43]; CYP97B2 (#AAB94586) and #TC228439 from *Glycine max* (L.) Merr. [44]; #BQ971938 from *Helianthus annuus* L.; #BE552887 from *Zea mays* L.; #TC101515 and #TC109838 from *Medicago truncatula* Gaertn; and #BT012891 from *Lycopersicon esculentum* Mill.

Protein sequences were screened for chloroplast targeting signal peptides using two prediction algorithms: GENOPLANTE™ PREDOTAR (Predotar for Prediction of organelle targeting sequences) at http://genoplante-info.infobiogen.fr/predotar/ and ChloroP 1.1 Server at http://www.cbs.dtu.dk/services/ChloroP/ [45]. Rice protein sequences were compared against the P450 database at http://132.192.64.52/blast/P450.html using the P450 blast server. Prior to alignment, protein sequences were truncated to include the P450 conserved domain (CD) and exclude the chloroplast targeting sequence. Boundaries of conserved cytochrome P450 domains were identified using NCBI Conserved Domain Search tool (RPS-BLAST). Amino acid sequences were aligned by ClustalW, and a neighbor-joining tree was constructed with a 500 bootstrap replication support using MEGA3 software [46].

Total RNA extraction and isolation of cDNAs

Total RNA was extracted from *Oryza sativa* L. cv Nipponbare (*japonica*) leaves (approx. 6 weeks old) using the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Approximately 2 μg of total RNA, in a 20 μl reaction, was used for cDNA synthesis using the SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen Corporation, Carlsbad, CA). GenBank #AK068163 has a 1929 bp ORF between nucleotide number 54 and 1982. GenBank #AK065689 has a 1683 bp ORF between nucleotide number 105 and 1787. Gene specific primers, with terminal *Eco*RI and *Xho*I restriction sites, (forward, 5′-CCG GAA TTC A_{54}TG AGC TCA GCG ACG TCA GTG AGT_{77}-3′ and reverse, 5′-ACC GCT CGA GT_{1985}C AGA TTC GAG TTG CTG AGA CTT G_{1962}-3′, MWG-Biotech Inc., Oaks Parkway, NC) were used to amplify the full-length coding sequence of CYP97A4 (#AK068163) [42]. The coding sequence (excluding the first 21 bp) of CYP97C2 (#AK065689) was amplified using gene specific primers with terminal *Eco*RI and *Xho*I restriction sites, (forward, 5′-CCG GAA TTC C_{105}CG TCC CGT GCG TAC CAT TC_{124} -3′ and reverse, 5′-ACC GCT CGA G_{1793}TC ATC TGG ACC CAC TGA GTG CA_{1774}-3′, MWG-Biotech Inc., Oaks Parkway, NC) (50). Fifty microlitre reactions each contained 4 μl cDNA, 1 μl of each primer (20 μM), 19 μl H_2O, and 25 μl of a master mix (50 mM $MgCl_2$, 10 mM dNTP mix, 50 U/ml *Taq* polymerase, and 2× PCR buffer) (Promega Corp., WI). Amplification conditions were: 1 cycle, 95 °C, 3 min; 40 cycles, 95 °C, 30 s, 58.1 °C, 40 s, 72 °C 2 min; 1 cycle, 72 °C, 8 min.

Construction of expression vectors and functional analysis

The CYP97A4 (#AK068163) and CYP97C2 (#AK065689) sequences amplified as above were subcloned in-frame into *Eco*RI and *Xho*I sites of the pCOLADuet™-1 vector (Novagen, WI), renamed pRT-A4 and pTR-C2, respectively. For testing of β-ring substrate specificity, constructs were transformed into *E. coli* BL21 (DE3) cells (Novagen, WI) harboring pAC BETA-O4 which confers β-carotene accumulation [24]. pRT-A4 and pTR-C2 ORFs were also introduced into *E. coli* BL21 (DE3) cells carrying the pACCRT-EIB [47] and plasmid y2 [48] which carries the *Arabidopsis* lycopene ε cyclase, which together confer accumulation of lycopene and δ-carotene (ε,ψ-carotene), along with some ε-carotene (ε,ε-carotene) (minor product) [48]. For carotenoid analyses, saturated cultures in LB medium were diluted 100-fold into 25 ml fresh medium in 250 ml flasks, then grown in the dark at 300 rpm at 21 °C, 28 °C, or 37 °C (cultures containing pTR-C2 were grown at 37 °C) until OD 0.6 at which point they were induced with 10 mM IPTG (unless otherwise noted) and further cultured for a total of three days.

Extraction of carotenoids from E. coli cells, HPLC separation and identification

Carotenoids were extracted in ethanol:ether (1:2 v/v), essentially, as described [49]. Bacterial cell pellets were extracted in 10 volumes of the ethanol:ether mix using a Dounce homogenizer, incubated at room temperature for 5–10 min, and centrifuged at 10,000*g*. Supernatants were frozen at −80 °C for 30 min, centrifuged at 10,000*g* at 4 °C, supernatants dried under nitrogen gas, dissolved in HPLC mobile phase, and stored at −20 °C.

Separation was carried out using a Waters HPLC system equipped with a 2695 Alliance separation module, a 996 photodiode array detector, a column heater, a fraction collector II, Empower software (Millipore, Franklin, MA), and a Nucleosil 5 C_{18} (5 μm, 250 × 4.6 mm) column with a Nucleosil C_{18} (5 μm, 4 × 3.0 mm) guard column (Phenomenex, Torrance, CA), mobile phase of 100 parts acetone: 4 parts water, column flow rate of 0.6 ml/min, and sample and column temperature of 30 ± 5 °C. Peaks were identified on the basis of retention times/spectra matching those of authentic standards (INDOFINE Chemical Company Inc., Sommerville, NJ), and standards purified from bacteria expressing genes encoding carotenoid biosynthetic enzymes [48]. Authentic lactucaxanthin, purified

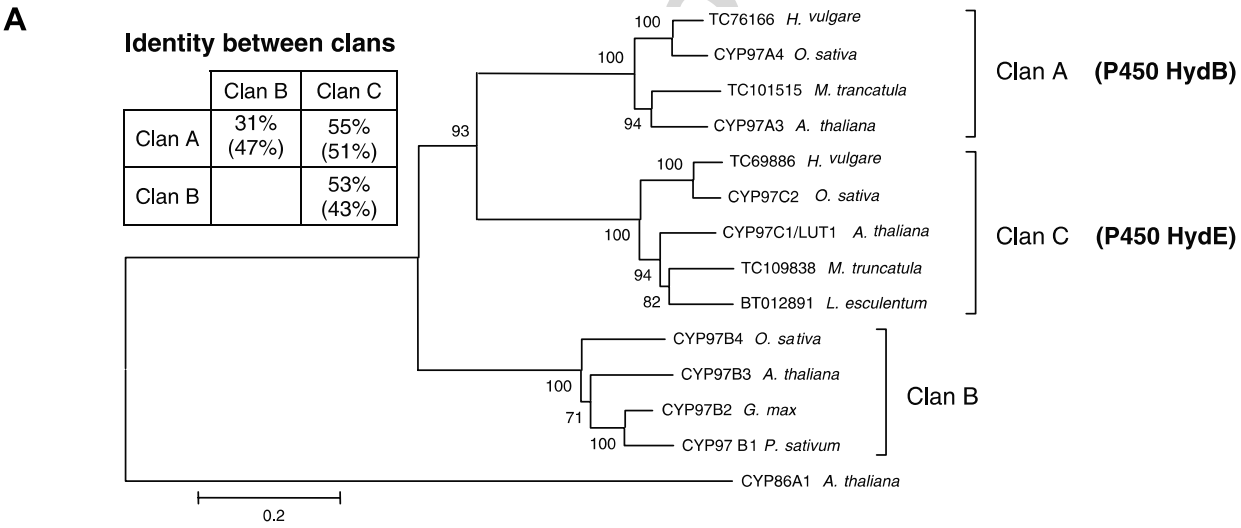
from *Lactuca sativa* leaves [50] and distinguished as a novel HPLC peak in comparison to a maize leaf profile, exhibited a spectrum matching reported values [51]. Identification of ε,ε-carotene and lactucaxanthin were confirmed by comparison with reported λ_{max} (wavelength of maximum absorption) spectral values [51] and comparison to standards chromatographed in the identical system. For quantification of extracted metabolites, data were collected at λ_{max} for individual metabolites and integrated peak areas calculated.

Results

Phylogenetic analysis

Among all known CYP97 P450 enzyme family members, three distinctive, yet closely related, clans (i.e., A, B, and C) can be discerned [52]. The amino acid alignment and phylogenetic tree construction for a selection of thirteen protein sequences from seven different plant species (monocots and dicots) is in agreement with the three clans designation (Fig. 2A). Clan A and C enzyme sequences are more closely related with three blocks of amino acid sequence insertions that differentiate members of Clan B from those in Clans A and C (Fig. 2B). Block 1 is about 100 residues downstream of the P450 domain; blocks 2 and 3 are 37 aa apart and located between the oxygen- and heme thiolate-binding signatures. Members of the CYP97 clan, Clan B, do not have any functionally demonstrated representatives and as shown in the sequence comparisons, are more distant from Clans A and C. Clan B sequences do possess a potential chloroplast targeting sequence [45] and transcripts are present in shoots as reflected by ESTs, suggesting localization in chloroplasts as predicted for Clan A and C enzymes. Clan B homologs are found in angiosperms (Fig. 2), in *Ginkgo biloba* (AAT28222) and the diatom, *Skeletonema costatum* (AAL73435) [53]. This taxonomically broad distribution suggests that Clan B enzymes may function in all plants rather than catalyze synthesis of taxon-specific metabolites.

To test carotene hydroxylase activity of Clan A and C enzymes, we identified sequences that were most closely related to *Arabidopsis* LUT1 (CYP97C1) using the genome of *Oryza sativa*, a representative of the Poaceae, and amplified cDNAs from *O. sativa* L. cv Nipponbare leaves. The



B

	Block 1	Block 2	Block 3	
CYP97B1 *P. sativum*	LLEG-EGRDGQKSVE	DVLPGGHKGDKDGYTI	LVQNNNEEVEGWAGFDPSRSPGALY	Clan B
CYP97B2 *G. max*	LLEG-EGYDGPDSIE	DVLPGGHKGEKDGYTI	LVQNKNEEIEGWAGLDPSRSPGALY	
CYP97B3 *A. thaliana*	LIREKETSSGEDTIE	ETLPGGHKGEKEGHKV	LRTKESNGIEGWAGFDPSRSPGALY	
CYP97B4 *O. sativa*	LIER--GEHGEKYTI	DKLPGGHNGAKEGYEI	SVPKKDESIEGWAGFDPDRSPGAMY	
BT012891 *L. esculentum*	PDAI-------SGSA	DVLPG-------NYKV	DLE----------GPV	Clan C
TC109838 *M. truncatula*	ADAV-------NGTA	DELPG-------DYKI	DLD----------GPV	
CYP97C1 *A. thaliana*	PYAE-------DGSA	DILPG-------NYKV	DID----------GAI	
CYP97C2 *O. sativa*	TSAL-------SGKP	DVLPG-------NYKI	DLE----------GPV	
TC69886 *H. vulgare*	TYAL-------SGEP	DVLPG-------NYKV	DLE----------GPI	
CYP97A3 *A. thaliana*	AAAL-------KGEE	DILG--------EYPI	PLD----------GPN	Clan A
TC101515 *M. trancatula*	TAAS-------DGED	DVLG--------EYPI	PLD----------GPN	
CYP97A4 O. sativa	KAAT-------DGED	DMLG--------GYPI	PLD----------GPN	
TC76166 *H. vulgare*	KAAS-------DGED	DMLG--------EYPI	PLD----------GPN	

Fig. 2. Comparison between the conserved P450 domain of the three clans in the CYP97 family. (A) A rooted neighbor-joining tree was constructed using the fatty acid ω-hydroxylase (CYP86A1) from *A. thaliana* as an outgroup. Numbers adjacent to branches are bootstrap values supporting the presented final tree. Average identities among clans are shown in the table in comparison to pair wise comparisons between *A. thaliana* and rice sequences, which are enclosed in parentheses. (B) Three sequence blocks distinguish members of Clan B from those in Clans A and C and are shown in excerpts from the alignment of the conserved P450 domains.

O. sativa AK065689 and AK068163 cDNAs, correspond to CYP97C2 (P450 HydE, putative ε-ring carotene hydroxylase) and CYP97A4 (P450 HydB, putative β-ring carotene hydroxylase). They encode conceptually translated proteins sharing 84% and 76% similarity (excluding the non-conserved transit peptide sequences) with the *A. thaliana* CYP97C1 (LUT1) and CYP97A3 (LUT5) [30] candidates for ε- and β-ring hydroxylases. Rice CYP97A4 and CYP97C2 map to chromosomes 2 and 10, respectively. CYP assignment of rice clones is based on BLAST searches using the Rice P450 BLAST Server, which implements the standardized system of cytochrome P450 nomenclature [52].

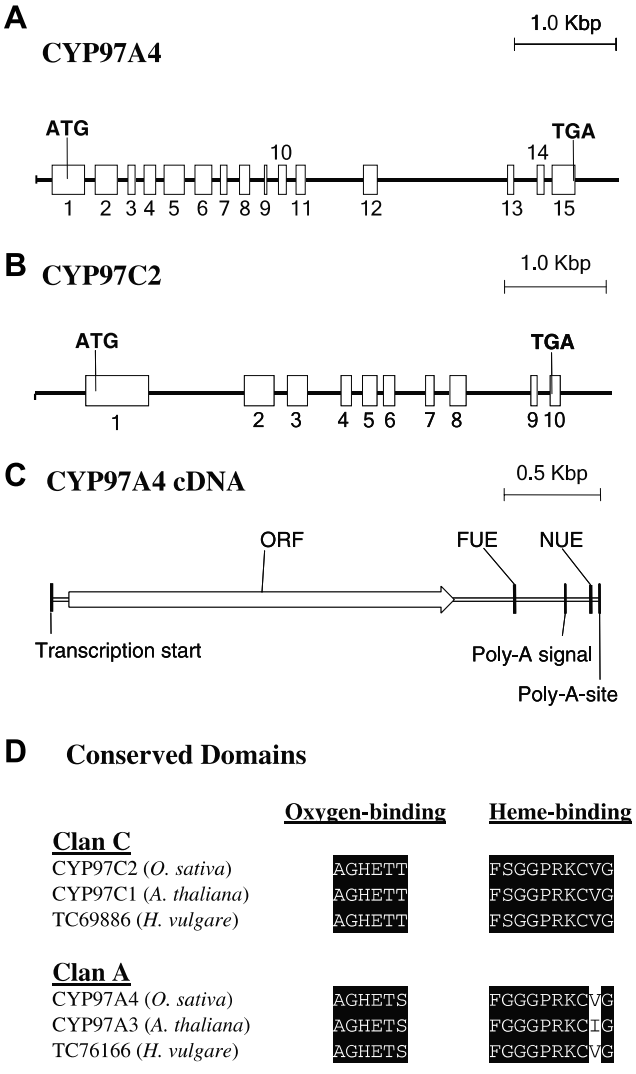


Fig. 3. Gene structure and P450 domains of CYP97 Clan A and C enzymes. A and B, gene structures for rice CYP97A4 and CYP97C2, respectively. Open boxes indicate exons; C, revised cDNA structure of CYP97A4 cDNA. The transcribed cDNA is a predicted 2414 bp fragment with an 88 bp 5′-UTR, a 1929 bp ORF, and a 397 bp 3′-UTR with conserved NUE, FUE, and poly-A site; D, comparison of conserved oxygen and heme-binding domains for CYP97 Clan A and C enzymes of rice compared with those in other plant species.

P450 DNA sequence analysis

The *O. sativa* CYP97A4 (P450 *HydB*) is comprised of 15 exons and 14 introns, while the CYP97C2 (P450 *HydE*) gene consists of 10 exons and 9 introns (Figs. 3A and B). All intron boundaries are consistent with the conserved 5′-AG and 3′-GT flanking element rule [54]. The CYP97C2 cDNA is described in the NCBI GenBank # AK065689 as a 1876 bp long cDNA with a 1683 bp open reading frame (ORF), a 104 bp 5′-untranslated region (5′-UTR), and an 89 bp 3′-UTR [42]. The CYP97A4 cDNA (GenBank #AK068163) is indicated as a 4217 bp long cDNA with a 1929 bp ORF, a 53 bp 5′-UTR, and a 2235 bp 3′-UTR [42]. However, analysis of AK068163 cDNA and corresponding genomic sequence (GenBank # NT_107182) support a 2414 bp full-length cDNA with a 1929 bp ORF, an 88 bp 5′-UTR, a 397 bp 3′-UTR with conserved near-upstream element (NUE; poly-A addition signal), far upstream element (FUE), and terminal "Pyr(T)A" poly-A site (Fig. 3C) [55–57]. The modified full-length cDNA structure (Fig. 3C) is based on the finding that several rice 3′-end ESTs map around the proposed poly-A addition site, whereas there are no ESTs that map to the 3′-end of the model presented by Kikuchi and coworkers [42].

Conserved domain characteristics of the CYP97C2 and CYP97A4 amino acid sequences

Significant *E* values from PSI- and PHI-BLAST searches against the conserved domain database (CDD) at NCBI confirm that CYP97C2 and CYP97A4 are P450 proteins [58]. The conserved heme-thiolate-binding signature (FXXGXXXCXG) in plants corresponds to Phe_{488}-Ser-Gly-Gly-Pro-Arg-Lys-Cys-Val-Gly_{497} in CYP97C2 and to Phe_{534}-Gly-Gly-Gly-Pro-Arg-Lys-Cys-Val-Gly_{543} in CYP97A4 and as compared with those of other related plant enzymes (Fig. 3D). The underlined conserved cysteine in each of these motifs is an essential residue since it contributes the thiol group, which is the fifth ligand that binds the iron atom in these hemoproteins. Similarly, near the middle of the P450 conserved domain, a conserved oxygen binding signature (A/G)GX(D/E)T(T/S) is detected with the corresponding amino acid sequence of Ala_{361}-Gly-His-Glu-Thr-Thr_{366} for the CYP97C2 and Ala_{402}-Gly-His-Glu-Thr-Ser_{407} for the CYP97A4 (Fig. 3D) and as compared with those of other related plant enzymes. The underlined threonine in these respective sequences is involved in the binding of an oxygen molecule, which is essential for catalysis.

The overwhelming majority of eukaryotic P450 heme-thiolate enzymes are membrane bound through an anchoring transmembrane helix. Conversely, all P450s in prokaryotes are cytosolic. CYP97C2 is predicted to be a 62.1 kDa (561 residue) preprotein with a 40 residue N-terminal transit peptide that is cleaved upon import to yield a chloroplast-localized 57.8 kDa, 521 residue, mature protein [45]. CYP97A4 is predicted to be a

69.9 kDa (643 residue) preprotein containing a 41 residue N-terminal transit sequence that is removed upon chloroplast import to yield a chloroplast-localized 65.9 kDa, 602 residue, mature protein. In addition, both enzymes harbor a transmembrane helix sequence at the N-terminus of the P450 domain, as predicted by the HMMTOP server [59] to membrane anchor the mature P450 protein.

Heterologous expression of CYP97 genes in E. coli and substrate specificity

CYP97A4. For functional testing of β-ring carotene hydroxylase activity, we engineered *E. coli* cells as described in the methods to produce the β-carotene (β,β-carotene) substrate, which contains two β-rings for potential hydroxylation. For testing of a Clan A enzyme (a putative β-ring carotene hydroxylase), we amplified and cloned the full-length rice CYP97A4 cDNA (including its putative transit sequence) into pCOLADuet™-1, and introduced the resulting construct into the β-carotene accumulating cells. Accumulated pigments from transformed cells with or without the cDNA (Figs. 4A(e) and (d)) along with standards for β-carotene (Fig. 4A(c), peak 3) and hydroxylated products, β-cryptoxanthin (one hydroxylated ring, Fig. 4A(b), peak 2) and zeaxanthin (two hydroxylated rings, Fig. 4A(a), peak 1) were analyzed by HPLC. Extracted pigments were identified based on HPLC retention times (Fig. 4A) and spectra (Fig. 4B) matching those of authentic standards. As seen in Fig. 4A(e), only the doubly transformed cells accumulated β-cryptoxanthin (peak 2) and zeaxanthin (peak 1), in addition to the substrate β-carotene (peak 3). Cells that lacked the CYP97A4 cDNA did not have any hydroxylated β-carotene derivatives (Fig. 4A(d)).

We next tested the effect of culturing temperature and IPTG concentration on CYP97A4 hydroxylation activity in *E. coli*. At 21 °C and 10 mM IPTG, ~62% hydroxylated product accumulated, of which 52% was β-cryptoxanthin (mono-hydroxy intermediate) and 10% was zeaxanthin (di-hydroxy product) (Fig. 5A), a combined equivalent of 36% total hydroxylated β-rings (Fig. 5B). Increasing the culturing temperature from 21 °C to 37 °C increased production of β-carotene substrate but reduced the amount of hydroxylated products by 20% at 5 mM IPTG (data not shown) and by 40% at 10 mM IPTG (Fig. 5). Our initial attempts using overnight cultures grown at 37 °C and lower concentration of IPTG (i.e., 0.4 and 1 mM) did not result in accumulation of detectable xanthophylls. Interestingly, the ratio of β-cryptoxanthin (one hydroxylated ring) to zeaxanthin (two hydroxylated rings) decreased from 5:1 for growth at 21 °C to almost equimolar amounts for growth at 37 °C. In summary, CYP97A4 is more effective at lower temperature (21 °C) when expressed in *E. coli*, an organism having optimal growth at 37 °C.

CYP97C2. We next tested whether a Clan C enzyme, a putative ε-ring carotene hydroxylase, could utilize the β-ring substrate. We similarly amplified and cloned the rice

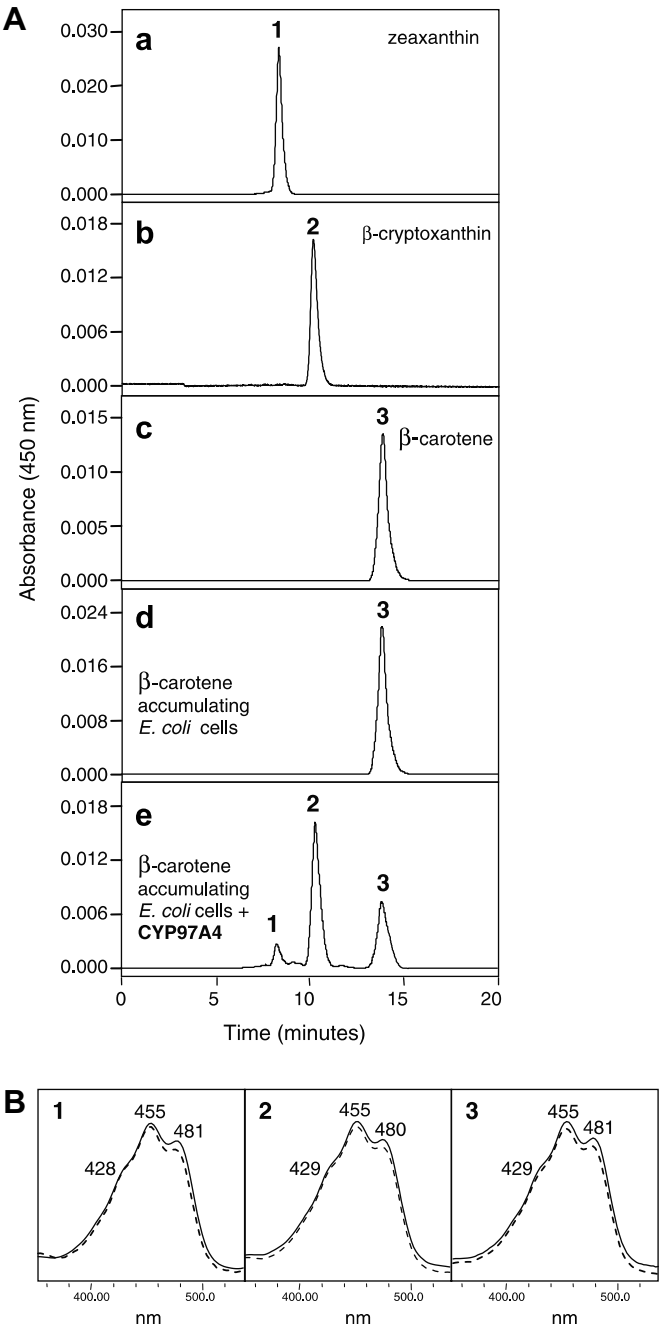


Fig. 4. Functional complementation of putative β-ring hydroxylase CYP97A4 cDNA in cells accumulating β-carotene. Bacterial cells were grown at 21 °C and extracted pigments were separated by reversed phase HPLC. (A) Chromatograms showing elution profiles: (a–c) authentic standards: 1, zeaxanthin; 2, β-cryptoxanthin; 3, β,β-carotene; (d) extracts from *E. coli* BL21 (DE3) cells accumulating β,β-carotene; (e) extracts from cells in (d) that were additionally transformed with the CYP97A4 cDNA. Peak numbers and identities in (d) and (e) correspond to those in (a–c). (B) Spectral profiles of standards (solid line) shown in (a–c, peaks 1–3) are superimposed on corresponding spectra (dashed line) obtained from peaks 1–3 shown in (e).

CYP97C2 cDNA, missing only 7 codons of the predicted 40 residue transit sequence, into pCOLADuet™-1 and introduced this construct into β-carotene accumulating cells; the pigment profile was compared with that obtained

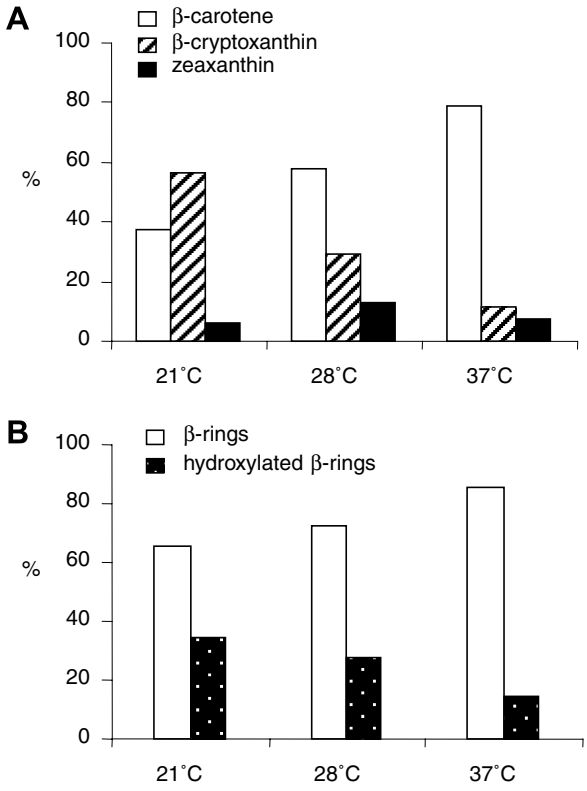


Fig. 5. Effect of temperature on product distribution and CYP97A4 enzyme activity *in vivo*. (A) Increasing the culturing temperature from 21 °C to 37 °C shifts the ratio of β-cryptoxanthin (one hydroxylated β-ring) to zeaxanthin (two hydroxylated β-rings) from 10:1 to ~1:1. (B) Overall % hydroxylation activity of CYP97A4 decreases from 35% at 21 °C to 15% at 37 °C.

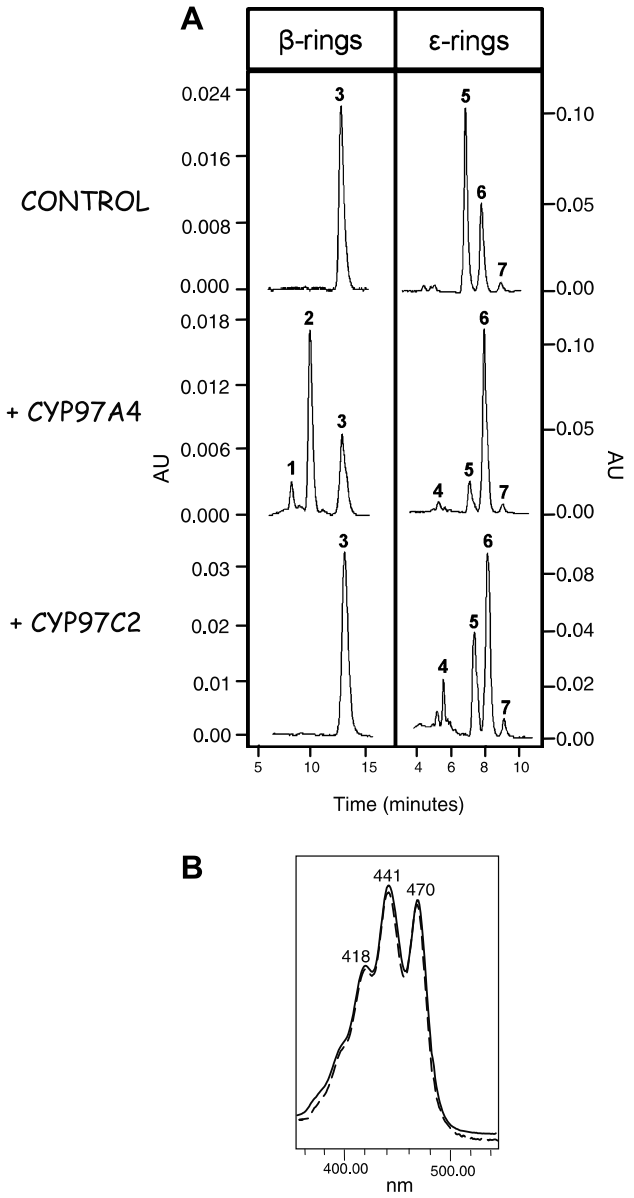


Fig. 6. Substrate specificity of P450 carotene hydroxylases. (A) *E. coli* cells engineered to accumulate carotenes with β rings (left panel) or ε-rings (right panel) were further transformed with test plasmids encoding CYP97A4 (putative β-ring hydroxylase) or CYP97C2 (putative ε-ring hydroxylase). HPLC chromatograms (left panel, 450 nm; right panel, 470 nm) show composition of accumulating carotenoid substrates and products. Left panel, *E. coli* cells transformed with pAC Beta-O4 (control) plus indicated test gene; Right panel, *E. coli* cells transformed with pACCRT-EIB+y2 (control) plus indicated test gene. Peaks: 1 = zeaxanthin, 2 = β-cryptoxanthin, 3 = β,β-carotene, 4 = lactucaxanthin, 5 = lycopene, 6 = δ-carotene, 7 = ε,ε-carotene. (B) Spectrum of peak 4 product of CYP97C2 (dashed line) overlayed with spectrum of authentic lactucaxanthin (solid line) chromatographed under identical conditions.

from CYP97A4 double transformed cells as above. The HPLC chromatogram (Fig. 6A, left panel, bottom) shows that when CYP97C2 was introduced into β-carotene accumulating cells, β-carotene substrate (peak 3) was the sole accumulating pigment as seen in the control (Fig. 6A, left panel, top, peak 3) and no hydroxylated products were detected. In comparison, introduction of CYP97A4 cDNA resulted in the mono- and di-hydroxylated products (left panel, middle, peaks 1 and 2).

In contrast, if the CYP97C2 cDNA was introduced into cells engineered with ε-ring substrates δ-carotene and ε,ε-carotene (Fig. 6A right panel, top, peaks 6 and 7), we found that CYP97C2 did confer hydroxylation of ε-rings. This was seen by accumulation of the more polar peaks including lactucaxanthin, a di-hydroxylated product (Fig. 6A, right panel, bottom, peak 4) with retention time and corresponding spectrum matching the authentic lactucaxanthin standard chromatographed in the same HPLC system (Fig. 6B). Hydroxylated products accumulated only when cells were grown at 37 °C and not at lower temperatures (data not shown). When the β-ring hydroxylase CYP97A4 was introduced into cells with the ε-ring substrates, there was a barely detectable amount of accumulated lactucaxanthin (Fig. 6 right panel, middle, peak 4). Spectra corresponding to the small bumps seen in the control (empty vector) do not match those for peak 4 nor for any carotenoid. Therefore, lactucaxanthin is only detected when the plant enzymes are present. These results demonstrate that the Clan A enzyme hydroxylates carotene β-rings and has weak activity towards carotene ε-rings while the Clan C enzyme only hydroxylates carotene ε-rings.

Discussion

Phylogenetic analysis of CYP97 genes

Predictive metabolic engineering or marker-based breeding of enhanced provitamin A levels depends on controlling conversion of provitamin A carotenes to non-provitamin A xanthophylls in endosperm and other tissues. To assess this conversion requires that we have a means to investigate activities and specificities of the putative hydroxylase enzymes. We focused on the recently discovered CYP97 family encoding P450 enzymes for which enzyme activities have been implied but never demonstrated. We first constructed an enzyme tree of CYP97 P450 enzymes to identify candidate sequences for ε- and β-ring hydroxylases in rice, a member of the Poaceae and representative of many agronomically important crops. Enzymes clustered into three clans, A, B, and C, and appeared to be encoded by single copy genes based on the finding that in each case, only one orthologous sequence was found in each of four different plant species, two being among the most exhaustively sequenced genomes (i.e., *A. thaliana* and *O. sativa*).

Functional complementation results integrated with earlier genetic studies

We isolated cDNAs encoding both β- and ε-ring hydroxylases in the *Oryza sativa* CYP97 family and demonstrated that *E. coli* cells were suitable for expression of functional enzymes to allow testing of substrate specificity. We conducted a preliminary examination of substrate specificity with regard to β- and ε-ring-containing substrates. Using *E. coli* functional complementation, we demonstrated that a Clan A enzyme, CYP97A4, is a β-ring carotene hydroxylase with some minor activity towards ε-rings. This minor ε-ring activity of the Clan A enzyme likely contributes to the residual lutein that accumulated in *Arabidopsis* plants carrying a *lut1* null allele that blocks expression of a Clan C ε-ring hydroxylase [30]. The phenotype of the null *lut1* allele also indicates that the minor ε-ring activity observed in the *E. coli* system is consistent with minor activity in the plant; almost no lutein accumulates in the null mutant and therefore the minor activity of the Clan A enzyme is indeed minor and cannot compensate for the Clan C deficiency. Studies on the non-heme diiron β-ring hydroxylases also showed the enzymes to display some activity towards ε- rings [24]. Given this observation in two structurally distinct enzymes (the P450 and diiron β-ring hydroxylases) suggests that this plasticity may be a function of the relationship between the extracted hydrogen and the configuration of the double bond in the β-ring as compared to the ε-ring [29].

Previous knockout mutagenesis in *A. thaliana* of the two genes encoding non-heme diiron β-carotene hydroxylases and one encoding the ε-ring hydroxylase (*LUT1*, a Clan C enzyme) [60] led the authors to suggest that the LUT1 ε-ring hydroxylase may be active towards β-rings. Using *E. coli* we showed that a Clan C enzyme, CYP97C2, lacked detectable activity towards β-rings. Therefore, our results do not support the hypothesis that further reduction in β-ring hydroxylation caused by a lesion in *Lut1* triple mutants compared to defects in the diiron enzymes alone, is attributed to a defect in the Clan C enzyme (which did not exhibit such activity under the conditions tested). Therefore, the observation in the triple mutant must have some other explanation. One possibility is that the Clan C and A enzymes form a complex, and interference in the expression of one affects expression/stability of the other, and hence the additional impact on β-ring hydroxylation activity in the triple mutants.

When comparing the activity of the rice enzymes in *E. coli*, we noted that the carotene ε-ring hydroxylation mediated by the Clan C enzyme was weak. One possibility is that the ε-ring substrate (one or two ε-rings) is not the best substrate as suggested by biochemical profiles of plant CYP97 mutants. *Arabidopsis* mutants defective in a Clan C ε-ring hydroxylase (*lut1*) conditioned accumulation of zeinoxanthin (hydroxylated β-ring of α-carotene) while plants defective in a Clan A β-ring hydroxylase (*lut5*) accumulated α-carotene and not α-cryptoxanthin (hydroxylated ε-ring of α-carotene) which would be expected if the available Clan C ε-ring hydroxylase could accept α-carotene directly. These combined observations suggest that the preferred *in planta* substrate of the Clan C ε-ring hydroxylases is zeinoxanthin (the mono-hydroxylated β-ring) and that the enzymes function in an ordered manner with the Clan A enzyme hydroxylating the carotene β-ring to produce zeinoxanthin followed by the Clan C enzyme hydroxylating the ε-ring [30,61]. Therefore, Clan C enzyme activity in *E. coli* might be improved with another substrate, the mono-hydroxylated β-ring of α-carotene (zeinoxanthin) and/or association with the Clan A enzyme. Enhancement of Clan C activity, by co-expression of the Clan A enzyme needed to produce zeinoxanthin, is now testable given demonstrated utility of the *E. coli* platform to express each individual enzyme.

We also observed that expression of the Clan A enzyme in *E. coli* led to significant accumulation of β-cryptoxanthin, even when different conditions were tested; this compound is not usually found in *Arabidopsis*, although it is found in other tissues of other species such as maize [16]. While we demonstrated hydroxylation of β-rings in *E. coli* by Clan A enzymes using β-carotene (two β-rings), the Clan A enzyme might prefer α-carotene (mixed β-ring and ε-ring) as the substrate. Therefore, the *E. coli* platform will be valuable in testing different types of substrates and combinations of enzymes.

What other factors are required for successful function in E. coli?

P450 enzyme activity is generally predicated on availability of an electron donor partner [62]. Such partners include NADPH flavodoxin reductases of various forms that are found in plant plastids, among other locations, and which function as electron carries for photosynthetic and non-photosynthetic processes such as sterol and fatty acid biosynthesis [63,64]. In *E. coli*, a bacterium which lacks P450 enzymes, flavodoxin: NADPH flavodoxin reductase can serve as a replacement for the natural redox partner to facilitate function of some plant P450 enzymes but not others [63,65]. For example, sorghum CYP71E1, required for cyanogenic glycoside biosynthesis, could both function *in vivo* in *E. coli* using the *E. coli* flavodoxin: NADPH flavodoxin reductase or reconstituted *in vitro* with a plant NADPH cytochrome P450 reductase [40]. In comparison, CYP79B2, an enzyme required for indole glucosinolate biosynthesis in *Arabidopsis*, functioned in *E. coli* only with support of the plant reductase [66] as found in other cases [39]. The CYP97 Clan A and Clan C enzymes were functional in *E. coli* because they could accept electrons from the endogenous *E. coli* flavodoxin reductase [63,65].

Why is CYP97 carotene hydroxylase activity suboptimal in E. coli?

Hydroxylase activity of the CYP97 enzymes in *E. coli* could be improved over the modest conversion of monohydroxylated intermediate to di-hydroxylated product. In addition to substrate choice as mentioned above, another factor that might limit enzyme activity might be the basal level expression of the endogenous reductase. Bacterial flavodoxin reductase, encoded by *E. coli fpr*, is induced about 20-fold from basal levels as part of a global response to oxidative stress that is mediated by the *soxRS* regulon [67,68]. In the absence of such induction, reductase expression may limit activity of the plant CYP. Other possible enhancements might include optimization of codon usage of the plant cDNA and/or removal of upstream plastid targeting sequences. However, posttranslational modification (e.g., glycosylation) is an unlikely prerequisite since the CYP97 enzymes did function in *E. coli*.

What is the structural basis for enzyme specificity?

Understanding the structural basis for β- and ε-ring specificity as demonstrated here will require modeling in combination with directed enzyme evolution. Modeling alone is insufficient to predict structural determinants of activity as seen in the characterization of other P450 enzymes. For example, directed evolution of *Bacillus subtilis* CYP102A2 enhanced its activity towards fatty acids and other aromatic substrates; affected amino acids were remote from the active site and unpredicted from modeling on related crystal structures to affect substrate specificity [69]. The *E. coli* platform used here will be valuable in addressing the issue of substrate specificity as well as to produce the protein crystals needed to elucidate a three dimensional structure of these crucial enzymes.

There are some recently described bacterial and fungal genes that encode P450 carotene ring hydroxylases. CrtS, is a 62.63 kDa (5.75 p*I*) fungal P450 that hydroxylates β-carotene to form astaxanthin. However, it is unrelated to the plant enzyme [70]. A P450 β-ring hydroxylase gene was also found in the carotenoid-containing thermophilic bacterium, *Thermus thermophilus* and the crystal structure has been solved [71]. The location of this single P450 gene on the thermophile's megaplasmid suggests that the gene may have been acquired through horizontal transfer and perhaps conferred some advantage to heat stress. The bacterial gene encodes a 44 kDa (9.72 p*I*) enzyme in comparison to the predicted mature 66 kDa (5.44 p*I*) plant Clan A enzyme [72,73], though they share 21% similar residues and cluster in a neighbor-joining tree [74]. While the plant and bacterial enzymes are sufficiently different, and perhaps cases of convergent evolution, they both functioned in *E. coli*.

Metabolic engineering in plants-challenges

From the results shown here and from prior studies, it is evident that higher plants contain one P450 ε carotene hydroxylase that mediates carotene ε-ring hydroxylation and two structurally distinct (P450 and diiron) β-ring hydroxylases, both of which catalyze hydroxylation of carotene β-rings leading to xanthophyll formation. A fundamental issue to address is how do the P450 and non-P450 enzymes contribute to metabolon structure(s) whose biogenesis may be differentially controlled whether the pathway is located on the envelope or thylakoid membranes; each location leads to intermediates and products with multiple roles in plant development and physiology. In addition, how do these enzymes contribute to biogenesis and control of a pathway that also diverges to form alternate end-products, one via β-carotene (the carotenoid with highest provitamin A value) and the other via α-carotene? Indeed, to develop rational strategies for metabolic engineering of specific carotenoids such as the provitamin A β-carotene or any other of the numerous carotenoids found in nature, will require a deeper understanding of how these hydroxylases participate in metabolon biogenesis and substrate channeling.

In rice, as in *Arabidopsis*, in addition to CYP97C2, there appear to be at least two non-heme diiron β-carotene hydroxylases (GenBank #XM_473611, AK060559). Further investigation of their suborganellar membrane localization will help to elucidate the specific roles of these β-ring hydroxylase classes on envelope and thylakoid membrane carotenoid biosynthetic pathways. Structurally they are unique: the diiron β-ring hydroxylases contain four transmembrane helices causing the enzymes to embed in

the membrane while the P450 hydroxylases are peripheral membrane enzymes due to their single transmembrane anchoring sequence. Other structural distinctions seen in the enzymes are their isoelectric points (p*I*); the p*I* of the mature rice Clan A enzyme is 5.44, while the rice diiron enzymes are 6.98 and 9.04. Given the differences in enzyme structure and membrane association, the enzymes may respond differentially to pH gradients that form across the thylakoid lumen at high light irradiance (lumenal pH 5/stromal pH 8). If, as is the case of the xanthophyll cycle de-epoxidase enzyme, activities of the carotene hydroxylases are pH-dependent, localization/orientation on the thylakoid membranes may be critical. At high light irradiance, acidification of the thylakoid lumen activates membrane association of the violaxanthin de-epoxidase, an enzyme having a p*I* of 4.57 [75], to convert violaxanthin to zeaxanthin, a product which dissipates the high light energy; in the dark violaxanthin accumulates. Perhaps diiron carotene β-ring hydroxylases function in the dark or on different plastid membranes (including envelope) for apocarotenoid biosynthesis, while P450 β-ring hydroxylases are light activated or function on the thylakoid membranes in association with the photosynthetic apparatus.

If on the other hand, the two structurally distinct carotene hydroxylases exist on the same membrane system, it is compelling to consider how they contribute to metabolon biogenesis and substrate channeling. Early modeling of Cunningham [22] portrayed separate complexes for production of α-carotene and β-carotene, precursors of lutein and zeaxanthin, respectively. However, some observations agree and others conflict with this model and suggest that the enzymes might play some compensatory role. For example, in studies of potato tuber endosperm, overexpression of a bacterial phytoene synthase gene led to increases in lutein and β-carotene but not in zeaxanthin [76]. The result suggests that there were adequate levels of the P450 CYP97 ε-ring and β-ring activities to accommodate the increased pathway flux to lutein. However, the Clan A P450 β-ring hydroxylase was not accessible for hydroxylation of the β-carotene needed to produce zeaxanthin which suggests that cells were limited for specific β-carotene hydroxylase activity required for zeaxanthin biosynthesis, presumably that of the non-heme diiron enzyme. Therefore, despite the fact that we demonstrated that the P450 Clan A enzyme could utilize β-carotene as a substrate in *E. coli*, it may be that the enzyme is not in a biochemical context to utilize this substrate in potato tuber. Our interpretation of the potato experiments is that they support Cunningham's view of separate submetabolons for the α-carotene and β-carotene sides of the pathway. However, if the Clan A enzyme cannot compensate for the diiron enzyme, then one would predict that a Clan A knockout would affect lutein accumulation only and not that of the β-carotene derived xanthophylls. This was not the case for the *Arabidopsis lut5* (Clan A) mutation which caused both reduced lutein and β-carotene derived xanthophylls [30], thus suggesting that the Clan A enzyme is not limited to α-carotene derived hydroxylation activity but may also play a role in hydroxylation of β-carotene. In another example, a double T-DNA knockout of the two genes encoding *Arabidopsis* diiron hydroxylases conferred no increase in β-carotene, an increase in lutein, and reduced β-carotene derived xanthophylls [60]. Given that β-carotene was not increased and β-carotene derived xanthophylls were not completely eliminated, suggests that the P450 Clan A enzyme could partially compensate for the missing diiron enzymes to catalyze synthesis of zeaxanthin from β-carotene. Interestingly, the double knockout in the diiron enzymes did affect lutein levels in the seed, suggesting that differences in plastid membranes in the seed and in the leaf may be associated with different mechanisms with respect to compensation between the P450 CYP97 and diiron β-ring hydroxylases. In summary, the literature holds conflicting examples regarding interchangeability between the P450 Clan A and diiron carotene β-ring hydroxylases. Plant mutations may have a pleiotropic effect on expression of other genes in the pathway, thereby confusing the interpretation of the resulting biochemical profile. Moreover, the observations made in *Arabidopsis* leaves may not translate to similar effects in cereal endosperm where plastid structure and gene family structure are different [1,37]. However, despite individual limitations, with the combined use of the bacterial system reported here, together with the plant genetic studies, we can derive a deeper understanding of plant carotenogenesis.

Conclusion

In conclusion, we demonstrated feasibility of the *E. coli* system as a platform to assess substrate specificity for representative members of the CYP97 A and C clans. This heterologous system will be valuable for further study and manipulation of these enzymes. The roles and topologies of the carotenoid metabolons are still an open question and important topics of research in identifying components that may be key targets for metabolic engineering of either "arm" of the carotenoid biosynthetic pathway. For example, to confer accumulation of the pathway intermediate, β-carotene, would necessitate a block in β-carotene hydroxylase expression/activity. At this point, we cannot predict the appropriate class of enzyme target, the P450 or the diiron. Future elucidation of components of such complexes will be especially significant for metabolic engineering of enhanced levels of carotenes, given that β-carotene and α-carotene have provitamin A activity, whereas their hydroxylated xanthophyll products zeaxanthin and lutein do not.

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