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## Article

# Hot Spots of Phytoene Desaturase from *Rhodobacter sphaeroides* Influencing the Desaturation of Phytoene

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**Abstract:** Phytoene desaturase (CrtI, E.C. 1.3.99.31) shows variable desaturation activity, thereby introducing different numbers of conjugated double bonds (CDB) into the substrate phytoene. In particular, *Rhodobacter sphaeroides* CrtI is known to introduce additional 6 CDBs into the phytoene with 3 CDBs, generating neurosporene with 9 CDBs. Although in-depth studies have been conducted on the function and phylogenetic evolution of CrtI, little information exists on its range of CDB-introducing capabilities. We investigated the relationship between the structure and CDB-introducing capability of CrtI. CrtI of *R. sphaeroides* KCTC 12085 was randomly mutagenized to produce carotenoids of different CDBs (neurosporene for 9 CDBs, lycopene for 11 CDBs, and 3,4-didehydrolycopene for 13 CDBs). From six CrtI mutants producing different ratios of neurosporene/lycopene/3,4-didehydrolycopene, three amino acids (Leu163, Ala171, and Ile454) were identified that significantly determined carotenoid profiles. While the L163P mutation was responsible for producing neurosporene as a major carotenoid, A171P and I454T produced lycopene as the major product. Finally, according to the in silico model, the mutated amino acids are gathered in the membrane-binding domain of CrtI, which could distantly influence the FAD binding region and consequently the degree of desaturation in phytoene.

**Keywords:** phytoene desaturase; CrtI; *Rhodobacter sphaeroides*; random mutagenesis



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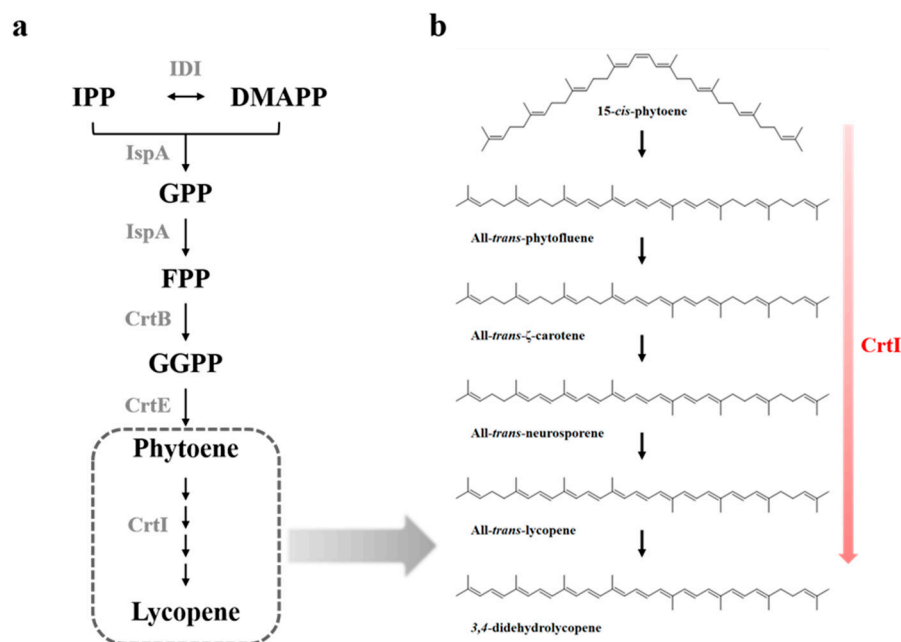
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## 1. Introduction

Carotenoids are a diverse group of colored isoprenoid derivatives that play distinct roles in nature [1]. More than 800 different carotenoids are synthesized in photosynthetic microorganisms, plants, and animals [2]. Naturally occurring carotenoids and their biosynthetic pathways are classified as C30, C40, and C50 based on the carbon numbers of their backbone structures [3]. Carotenoids serve several biological functions, including in coloration, photoprotective activities, and light harvesting, and are also the precursors for several plant hormones [4–6]. Carotenoids are widely used in the food, medical, pharmaceutical, and cosmetic industries as colorants and functional ingredients [7,8]. Despite the structural diversity and commercial importance of carotenoids, only a few simple-structured carotenoids, such as  $\beta$ -carotene and lycopene, are produced commercially by chemical synthesis or isolation. Their increasing industrial importance has led to renewed efforts to develop bioprocesses for the production of diverse carotenoids [8–10].

*Rhodobacter sphaeroides* is a Gram-negative purple bacterium that produces cellular energy through photosynthesis and synthesizes a C40 carotenoid spheroidene [11] through sequential reactions with seven pathway enzymes: geranylgeranyl diphosphate (GGPP) synthase (CrtE), phytoene synthase (CrtB), phytoene desaturase (CrtI), spheroidene monooxygenase (CrtA), methoxy-neurosporene desaturase (CrtD), hydroxy-neurosporene synthase (CrtC), and hydroxyneurosporene-O-methyltransferase (CrtF). *R. sphaeroides* is a well-known carotenogenic bacterium similar to *Pantoea agglomerans*, which was previously classified as *Erwinia herbicola* [12], and its carotenogenic pathway has been widely used as a model system [13].

In cyanobacteria and plants, a complex and multi-component pathway is employed by phytoene desaturase (PDS),  $\zeta$ -carotene desaturase (ZDS), carotene *cis-trans* isomerase (CRTISO), and  $\zeta$ -carotene *cis-trans* isomerase (Z-ISO) for desaturation of 15-*cis*-phytoene to all-*trans*-lycopene [14–16]. In contrast, bacteria and fungi employ a sole CrtI to generate all-*trans*-lycopene from 15-*cis*-phytoene [17]. During the catalytic desaturation reaction, CrtI uses flavin adenine dinucleotide (FAD) as an electron shuttle and oxygen as the acceptor [18]. In nature, C40 carotenoid structures have been diversified by CrtI, with different desaturation activities for 15-*cis*-phytoene. Depending on the carotenogenic microorganisms, CrtI can catalyze either a 3-step, 4-step, or 5-step desaturation reaction of 15-*cis*-phytoene, which is synthesized by CrtB from two moles of GGPP (Figure 1a). *R. sphaeroides* CrtI catalyzes 3-step desaturation, *P. agglomerans* CrtI catalyzes 4-step desaturation [19], and *Neurospora crassa* CrtI catalyzes 5-step desaturation [20]. The neurosporene with 9 conjugated double bonds (CDBs), lycopene with 11 CDBs, and 3,4-didehydrolycopene with 13 CDBs (or tetrahydrolycopene with 15 CDBs) are end-products in desaturation reactions, whereby 15-*cis*-phytoene is sequentially desaturated by different catalytic activities of CrtIs (Figure 1b). Most carotenogenic enzymes, including CrtI, can be functionally expressed in *Escherichia coli*; therefore, *E. coli* is a convenient heterologous host for the production of diverse carotenoids [21–25]. To date, several studies have considered the catalytic mechanism of CrtI (or PDS) in *E. coli* and native host strains. Our understanding of substrate binding sites, potential catalytic residues, and recognition regions of the hydrocarbon substrate is based largely on the crystal structure of the plant-type phytoene desaturase PDS via in silico docking experiments [26]; however, the exact mechanism for the desaturation process is still unclear.



**Figure 1.** The lycopene biosynthesis pathway and phytoene desaturation catalyzed by phytoene desaturase (CrtI): (a) lycopene biosynthesis pathway in recombinant *Escherichia coli*; (b) phytoene desaturation catalyzed by CrtI from *Rhodobacter sphaeroides*. Metabolite abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate. Pathway enzymes: IDI, isopentenyl diphosphate isomerase; IspA, farnesyl diphosphate synthase; CrtB, geranylgeranyl diphosphate synthase; CrtE, phytoene synthase; CrtI, phytoene desaturase. The dotted line indicates the multi-step desaturation process of phytoene to lycopene.

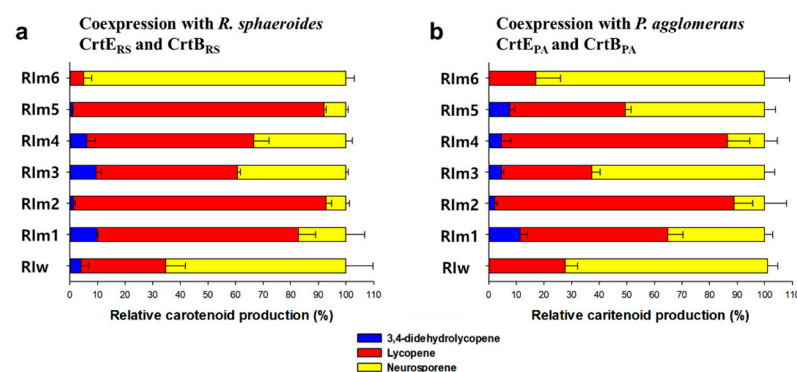
In the present study, *R. sphaeroides* CrtI was mutated using random mutagenesis to introduce different degrees of desaturation in phytoene in an *E. coli* strain expressing

*R. sphaeroides* CrtE and CrtB. Next, altered amino acids in the evolved CrtI mutants were analyzed in silico to investigate the relationship between the structural changes caused by mutated amino acids and phytoene desaturation.

## 2. Results

### 2.1. Reconstruction of Lycopene Biosynthesis Pathways and Directed Evolution of the *R. sphaeroides* Phytoene Desaturase (CrtI)

Three lycopene synthetic genes encoding CrtE<sub>RS/PA</sub>, CrtB<sub>RS/PA</sub>, and CrtI<sub>RS/PA</sub> from *R. sphaeroides* and *P. agglomerans* were modified to be modularly expressed and then coexpressed on plasmids in *E. coli* [26,27]. *R. sphaeroides* produces neurosporene from phytoene through a 3-step desaturation reaction catalyzed by CrtI<sub>RS</sub>; however, a recombinant *E. coli* coexpressing CrtE<sub>RS</sub>, CrtB<sub>RS</sub>, and CrtI<sub>RS</sub> as an individual expression module (pUCM\_I<sub>RS</sub> + pACM\_E<sub>RS</sub>\_B<sub>RS</sub>) produced further desaturated lycopene (11 CDBs, 30.7 ± 7.1%), 3,4-didehydrolycopene (13 CDBs, 4 ± 2.7%), as well as neurosporene (9 CDBs, 66 ± 10.1%) (Figure 2a). On the other hand, coexpression of CrtI<sub>RS</sub> with heterologous CrtE<sub>PA</sub> and CrtB<sub>PA</sub> (pUCM\_I<sub>RS</sub> + pACM\_E<sub>PA</sub>\_B<sub>PA</sub>) produced neurosporene (73.4 ± 3.7%) and lycopene (27.6 ± 4.7%), without formation of 3,4-didehydrolycopene (Figure 2b). This suggests that the heterologous carotenogenic enzyme complex can influence the desaturation of phytoene.



**Figure 2.** Relative carotenoid profiles in *E. coli* strains coexpressing six CrtI<sub>RS</sub> mutants with (a) *R. sphaeroides* CrtE<sub>RS</sub> and CrtB<sub>RS</sub> and (b) *P. agglomerans* CrtE<sub>PA</sub> and CrtB<sub>PA</sub>. Blue, red, and yellow bars represent 3,4-didehydrolycopene, lycopene, and neurosporene, respectively. RIw refers to a wild-type CrtI<sub>RS</sub>. Data are expressed as the mean ± SD.

Random mutation of the *crtI*<sub>RS</sub> gene was performed using PCR-based mutagenesis [28], and the resulting mutant *crtI*<sub>RS</sub> genes were transformed and coexpressed in a recombinant *E. coli* expressing the *R. sphaeroides* CrtE<sub>RS</sub> and CrtB<sub>RS</sub> as pACM\_E<sub>RS</sub>\_B<sub>RS</sub> (Table 1). From a colony library of approximately 10,000 colonies, colonies of dissimilar colors were visually screened, isolated, and analyzed. Among yellow (background carotenogenic clones) and white colonies (non-carotenogenic clones, due to inactivated CrtI<sub>RS</sub>), two red colonies, named RIm1 and RIm2, were selected, and carotenoid profiles of the clones were investigated by high-performance liquid chromatography (HPLC). RIm1 and RIm2 mutant clones produced significantly different carotenoid profiles: neurosporene (17.2 ± 6.7%), a major lycopene (72.9 ± 6.3%), and 3,4-didehydrolycopene (9.9 ± 0.4%) in RIm1 vs. neurosporene (7.2 ± 1.3%), a major lycopene (91.5 ± 2.0%), and 3,4-didehydrolycopene (1.3 ± 0.7%) in RIm2 mutant (Figure 2a and Table 2). Interestingly, one weak yellow colony (RIm6) produced a major neurosporene (95 ± 3.1%) and a small amount of lycopene (5.3 ± 2.4%) (Figure 2a and Table 2). Next, a 2nd round of random mutagenesis of RIm1 and RIm2 was carried out to generate the 2nd colony library of ca. 10,000 colonies. Among the 2nd library, two deep pink colonies (RIm3 and RIm4) from the parental RIm1 and one weak pink colony (RIm5) from the parental RIm2 were selected. HPLC analysis revealed that the mutants RIm3, RIm4, and RIm5 also showed unique carotenoid profile variations with a major lycopene (51.2 ± 1.2% in RIm3, 60.5 ± 5.5%

in RIm4, and  $90.8 \pm 0.9\%$  in RIm5), followed by neurosporene ( $39.4 \pm 0.9\%$  in RIm3,  $33.4 \pm 2.4\%$  in RIm4, and  $8.1 \pm 0.9\%$  in RIm5) and 3,4-didehydrolycopene ( $9.4 \pm 2.1\%$  in RIm3,  $6.2 \pm 3.2\%$  in RIm4, and  $1.1 \pm 0.1\%$  in RIm5) (Figure 2a and Table 2).

**Table 1.** Strains and plasmids used in this study.

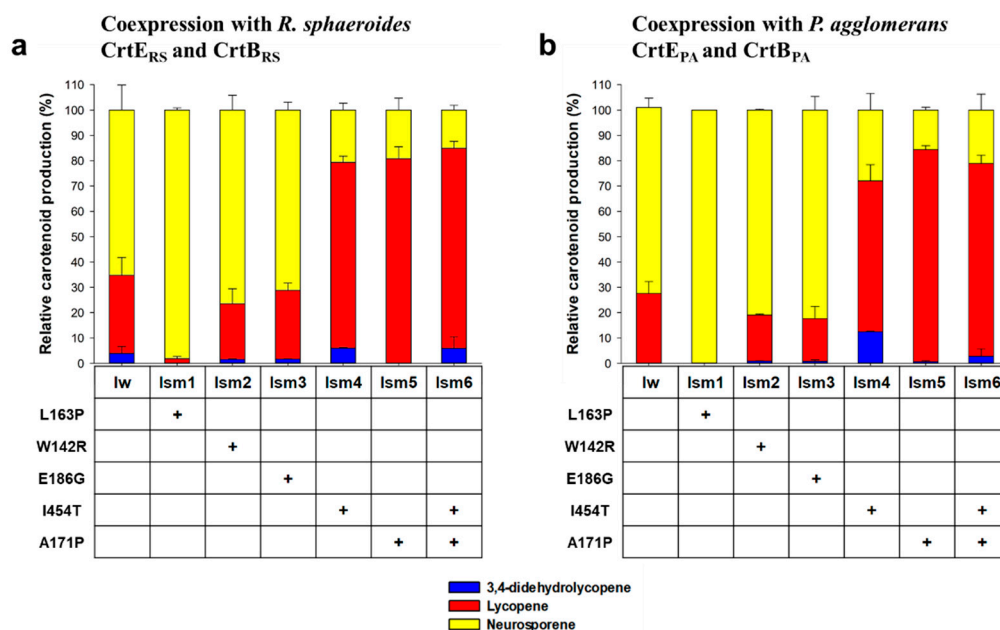
Plasmid	Description	Source or Reference
Strains		
<i>Rhodobacter sphaeroides</i>	Microbial source for C <sub>40</sub> carotenoid pathway genes	KCTC 12085
<i>Pantoea agglomerans</i>		KCTC 2479
Plasmids		
pGEM <sup>®</sup> T-easy vector	Cloning vector	Promega
pT_crtE <sub>RS</sub>	Vector containing <i>crtE</i> gene from <i>R. sphaeroides</i>	This study
pT_crtB <sub>RS</sub>	Vector containing <i>crtB</i> gene from <i>R. sphaeroides</i>	This study
pT_crtI <sub>RS</sub>	Vector containing <i>crtI</i> gene from <i>R. sphaeroides</i>	This study
pUCM	Cloning vector modified from pUC19. constitutive <i>lac</i> promoter, Amp	[8]
pUCM_E <sub>RS</sub>	Constitutively expressed <i>crtE</i> gene from <i>R. sphaeroides</i>	This study
pUCM_B <sub>RS</sub>	Constitutively expressed <i>crtB</i> gene from <i>R. sphaeroides</i>	This study
pUCM_I <sub>RS</sub>	Constitutively expressed <i>crtI</i> gene from <i>R. sphaeroides</i>	This study
pACM_E <sub>RS</sub>	Constitutively expressed <i>crtE</i> gene from <i>R. sphaeroides</i>	This study
pACM_E <sub>RS</sub> _B <sub>RS</sub>	Constitutively expressed <i>crtE</i> and <i>crtB</i> genes from <i>R. sphaeroides</i>	This study
pACM_E <sub>PA</sub> _B <sub>PA</sub>	Constitutively expressed <i>crtE</i> and <i>crtB</i> genes from <i>P. agglomerans</i>	[22]
pUCM_I <sub>RS</sub> _Im1	Constitutively expressed mutant <i>crtI</i> gene (I454T)	This study
pUCM_I <sub>RS</sub> _Im2	Constitutively expressed mutant <i>crtI</i> gene (A171P)	This study
pUCM_I <sub>RS</sub> _Im3	Constitutively expressed mutant <i>crtI</i> gene (I454T and E186G)	This study
pUCM_I <sub>RS</sub> _Im4	Constitutively expressed mutant <i>crtI</i> gene (I454T and L429L)	This study
pUCM_I <sub>RS</sub> _Im5	Constitutively expressed mutant <i>crtI</i> gene (A171P and W142R)	This study
pUCM_I <sub>RS</sub> _Im6	Constitutively expressed mutant <i>crtI</i> gene (L163P)	This study
pUCM_I <sub>RS</sub> _ImA171P	Constitutively expressed mutant <i>crtI</i> (Site-directed mutation)	This study
pUCM_I <sub>RS</sub> _ImL163P	Constitutively expressed mutant <i>crtI</i> (Site-directed mutation)	This study
pUCM-I <sub>RS</sub> _ImW142R	Constitutively expressed mutant <i>crtI</i> (Site-directed mutation)	This study
pUCM-I <sub>RS</sub> _ImE186G	Constitutively expressed mutant <i>crtI</i> (Site-directed mutation)	This study
pUCM-I <sub>RS</sub> _ImI454T	Constitutively expressed mutant <i>crtI</i> (Site-directed mutation)	This study
pUCM-I <sub>RS</sub> _ImA171P&I454T	Constitutively expressed mutant <i>crtI</i> (Site-directed mutation)	This study

**Table 2.** CrtI<sub>RS</sub> mutants generated by random mutagenesis and altered carotenoid profiles when complemented with the *R. sphaeroides* CrtB<sub>RS</sub> and CrtE<sub>RS</sub> and the *P. agglomerans* CrtB<sub>PA</sub> and CrtE<sub>PA</sub>. \* Neu, neurosporene; Lyc, lycopene; Ddl, didehydrolycopene. Proportions reported in parentheses: relative ratio of carotenoid profiles.

Mutants	Parental Gene	Nucleotide Changes	Amino Acid Changes	Carotenoid Profiles with CrtE <sub>RS</sub> and CrtB <sub>RS</sub>	Carotenoid Profiles with CrtE <sub>PA</sub> and CrtB <sub>PA</sub>
RIm1	WT	T1361C (ATC→ACC)	I454T	* Ddl (9.9%) Lyc (72.9%) Neu (17.2%)	Ddl (11.3%) Lyc (53.5%) Neu (35.1%)
RIm2	WT	G511C (GCC→CCC)	A171P	Ddl (1.3%) Lyc (91.5%) Neu (7.2%)	Ddl (2.1%) Lyc (86.7%) Neu (11.2%)
RIm3	RIm1	T1361C (ATC→ACC) A557G (GAG→GGG)	I454T  E186G	Ddl (9.4%) Lyc (51.2%) Neu (39.4%)	Ddl (4.7%) Lyc (32.6%) Neu (62.7%)
RIm4	RIm1	T1361C (ATC→ACC) C1287T (CTC→CTT)	I454T  Silent mutation	Ddl (6.2%) Lyc (60.5%) Neu (33.4%)	Ddl (4.6%) Lyc (82.0%) Neu (13.4%)
RIm5	RIm2	G511C (GCC→CCC) T424A (TGG→AGG)	A171P  W142R	Ddl (1.1%) Lyc (90.8%) Neu (8.1%)	Ddl (7.6%) Lyc (41.9%) Neu (50.5%)
RIm6	WT	T488C (CTG→CCG)	L163P	Ddl (0%) Lyc (5.3%) Neu (94.7%)	Ddl (0%) Lyc (0%) Neu (100%)

## 2.2. Identification of Mutation in Mutant CrtI<sub>RS</sub> by Sequencing Analysis

To reveal the genetic alterations of the six mutant CrtI<sub>RS</sub>, genes encoding RIm1, RIm2, RIm3, RIm4, RIm5, and RIm6 were sequenced using the Sanger method (Table 2). A single-nucleotide point mutation was found in RIm1 and RIm2, causing an I454T amino acid change and an A171P change, respectively. To confirm the effect of the single I454T and A171P mutations on the degree of desaturation on phytoene, site-directed mutagenesis (SDM) of wild-type CrtI<sub>RS</sub> was applied to generate Ism4 (SDM\_I454T) and Ism5 (SDM\_A171P). HPLC analysis revealed that the two SDM mutants, Ism4 and Ism5, produced very similar carotenoid profiles to those of RIm1 and RIm2:  $14.6 \pm 2.7\%$  of neurosporene,  $79.4 \pm 2.5\%$  of lycopene,  $6 \pm 0.2\%$  of 3,4-didehydrolycopene in the SDM mutant Ism4, and  $19.2 \pm 4.6\%$  of neurosporene and  $80.8 \pm 4.7\%$  of lycopene without 3,4-didehydrolycopene in the SDM mutant Ism5 (Figure 3a). The very similar carotenoid profiles observed between random mutants and SDM mutants strongly indicate that the single amino acid mutations (I454T and A171P) intrinsically altered the catalytic activity of CrtI<sub>RS</sub> toward phytoene. Similarly, an SDM mutant (Ism1, SDM\_L163P) of the major neurosporene-producing RIm6 (Figure 3a) showed a carotenoid profile very similar to that of RIm6, demonstrating the intrinsically altered catalytic activity of CrtI<sub>RS</sub>.



**Figure 3.** Relative carotenoid profiles in *E. coli* strains coexpressing site-directed mutant CrtI<sub>RS</sub> with (a) the *R. sphaeroides* CrtE<sub>RS</sub> and CrtB<sub>RS</sub> and (b) the *P. agglomerans* CrtE<sub>PA</sub> and CrtB<sub>PA</sub>. Blue, red, and yellow bars represent 3,4-didehydrolycopene, lycopene, and neurosporene, respectively. Iw refers to a wild-type CrtI<sub>RS</sub>. + symbol indicates the amino acid change in the mutant CrtI. Data are expressed as the mean  $\pm$  SD.

One additional nucleotide point mutation was observed in the 2nd round mutants RIm3, RIm4, and RIm5. The 2nd single amino acid change of E186G in RIm3 led to double amino acid mutations (I454T and E186G). Similarly, the 2nd single W142R change in RIm5 generated a double amino acid mutation (A171P and W142R). Compared to RIm3 and RIm5, the single nucleotide point mutation in RIm4 caused a silent mutation. Notably, although RIm4 shared a single amino acid A171P mutation with RIm2, except for an additional single silent mutation in RIm4, the two mutants produced different carotenoid profiles (Table 2 and Figure 2a); however, the difference in carotenoid profiles between the two mutants was negligible.

### 2.3. Verification of the Effect of E186g and W142r Mutations on Activity of Phytoene Desaturase by Site-Directed Mutagenesis

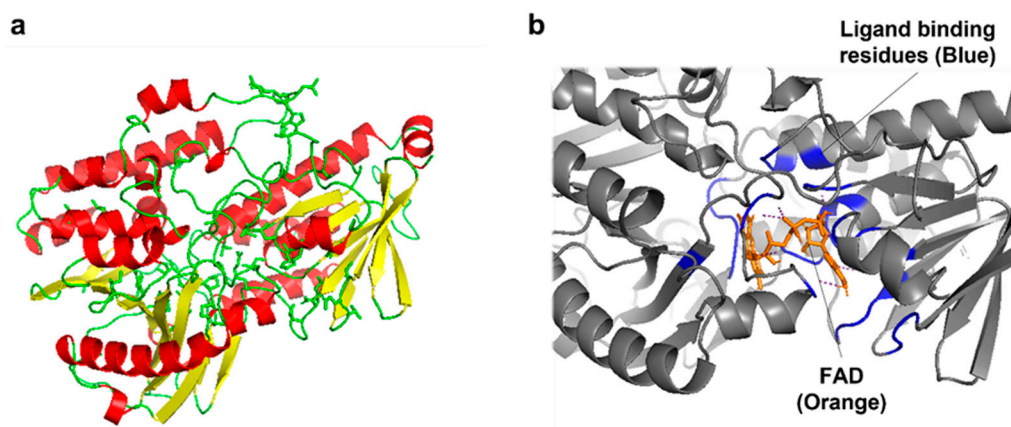
As RIm3 and RIm5 mutants had additional amino acid mutations (E186G in RIm3 and W142R in RIm5) in comparison with RIm1 (I454T) and RIm2 (A171P) (Table 2). The individual effects of the W142R and E186G mutations were investigated by generating two SDM mutants, Ism2 (SDM\_W142R) and Ism3 (SDM\_E186G). HPLC analysis revealed that both Ism2 and Ism3 produced slightly less lycopene in comparison with that of the wild-type CrtI<sub>RS</sub> (Figure 3a), suggesting a marginal effect of the W142R and E186G mutations on the degree of phytoene desaturation by RIm3 (E186G and I454T) and RIm5 (W142R and A171P). Since I454T and A171P mutations significantly influenced the degree of desaturation of phytoene, an SDM mutant Ism6 (SDM\_I454T\_A171P) was generated and its carotenoid profile was investigated. As expected, dramatical alteration of the carotenoid profile ( $10.1 \pm 1.9\%$  of neurosporene,  $84.5 \pm 2.7\%$  of lycopene, and  $5.4 \pm 4.6\%$  of 3,4-didehydrolycopene) was observed in comparison with that of the wild-type CrtI<sub>RS</sub> (Figure 3a).

### 2.4. Complementation of Mutant Crtirs with the *P. agglomerans* CrtEpa and CrtBpa

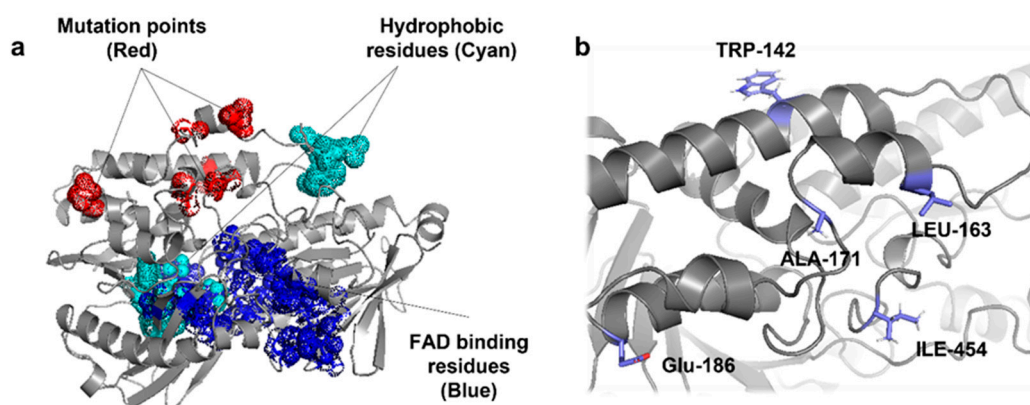
Even though carotenogenic enzymes have the same catalytic function, carotenoid profiles tend to vary depending on the source of the enzymes when expressed in a heterologous host [22]. To better understand the function of the mutant CrtI<sub>RS</sub>, random and SDM mutants were coexpressed in *E. coli* with the heterologous *P. agglomerans* CrtE<sub>PA</sub> and CrtB<sub>PA</sub>. HPLC analysis revealed that the three single amino acid mutants (RIm1, RIm2, and RIm6) when coexpressed with the heterologous CrtE<sub>PA</sub> and CrtB<sub>PA</sub> produced carotenoid profiles similar to those of RIm1, RIm2, and RIm6 when coexpressed with the native CrtB<sub>RS</sub> and CrtE<sub>RS</sub> (Table 2 and Figure 2b); however, RIm3, RIm5 (two double amino acid mutants) and RIm6 (a single amino acid mutant) when coexpressed with the heterologous CrtE<sub>PA</sub> and CrtB<sub>PA</sub> produced different carotenoid profiles in comparison with those coexpressed with native CrtB<sub>RS</sub> and CrtE<sub>RS</sub> (Table 2 and Figure 2b). This suggests that the 2nd amino acid alteration of E186G in RIm3 and W142R in RIm5 might influence the conformation of the enzyme complex structure, which consequently alters the catalytic activity of mutant CrtI<sub>RS</sub> toward phytoene. Notably, unlike the single amino acid mutants (RIm1, RIm2, and RIm6), RIm4 produced different carotenoid profiles when coexpressed with heterologous and native CrtE and CrtB. Three SDM mutants Ism2 (SDM\_W142R), Ism3 (SDM\_E186G), and Ism6 (SDM\_I454T\_A171P) produced similar carotenoid profiles when coexpressed with heterologous and native CrtE and CrtB (Figure 3b).

### 2.5. Structural Evaluation of Mutant Crtirs Using Computational Model Analysis

To understand the correlation between the structural changes and the observed activity of mutant CrtI<sub>RS</sub>, an in silico model of CrtI<sub>RS</sub> was created using the I-TASSER program [29] with the Protein Data Bank (PDB) templates of *Nonlabens dokdonensis* DSW-6  $\gamma$ -carotenoid desaturase (4REP, [30]) and *Pantoea ananatis* phytoene desaturase (4DGK, [18]) (Figure 4a). As FAD, a redox-active cofactor, is present in the active site region of CrtI<sub>RS</sub>, in silico ligand docking was simulated using COACH-D [31] with the PDB of FAD binding residues of *Pseudomonas savastanoi* pv. *phaseolicola* oxidoreductase. Predicted FAD binding sites of CrtI<sub>RS</sub> were the residues 16–17, 19–21, 40–42, 47–49, 61–65, 251–523, 282–284, 287, 315, 385, 433, 476–477, 483–485, and 488 (blue in Figures 4b and 5a). Interestingly, the five mutated amino acids of CrtI<sub>RS</sub> (Trp142, Leu163, Ala171, Glu186, and Ile454) were present in the putative membrane-binding domain (red in Figure 5a and blue in Figure 5b). The membrane-binding domain was previously predicted to influence the hydrophobic residues (cyan in Figure 5a), which are involved in the FAD-associated tunnel of CrtI from *P. ananatis* [18]; therefore, different degrees of phytoene desaturation (the observed differences in carotenoid profiles) in mutant CrtI<sub>RS</sub> could be attributed to the alteration of the FAD binding environment in the active site region of CrtI<sub>RS</sub>.



**Figure 4.** The in-silico-predicted structure of CrtI<sub>RS</sub> (a) and partial view of molecular docking with FAD (b). The FAD ligand binding residues are shown in blue and FAD is shown in orange.



**Figure 5.** The in-silico-predicted structure of FAD binding residues and hydrophobic residues in CrtI<sub>RS</sub> (a) and five amino acid mutation sites of CrtI<sub>RS</sub> (b).

### 3. Materials and Methods

#### 3.1. Strains, Plasmids, and Culture Conditions

All strains and plasmids used in this study are listed in Table 1. *E. coli* XL-Blue was grown in Luria–Bertani (LB, 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) or M9 minimal medium (2 g/L glucose, 3 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/L NaCl, 6 g/L Na<sub>2</sub>HPO<sub>4</sub>, 1 g/L NH<sub>4</sub>Cl, 0.5 g/L CaCl<sub>2</sub>, 0.5 g/L MgSO<sub>4</sub>). Genomic DNA of *R. sphaeroides* KCTC 12085 grown in LB medium was isolated using the genomic DNA extraction kit GeneAll® Exgene™ cell SV mini (GeneAll, Seoul, South Korea). The *crtE* gene (encoding GGPP synthase), *crtB* (phytoene synthase), and *crtI* (phytoene desaturase) were amplified from genomic DNA by PCR using gene-specific primers (Table 3) and cloned into the pGEM® T-easy vector (Promega Corporation, Madison, WI, USA), resulting in pT\_crtE<sub>RS</sub>, pT\_crtB<sub>RS</sub>, and pT\_crtI<sub>RS</sub>. The cloned gene was amplified from pT\_crtE<sub>RS</sub>, pT\_crtB<sub>RS</sub>, and pT\_crtI<sub>RS</sub> by PCR, then subcloned into the constitutive expression vector pUCM [22], generating pUCM\_E<sub>RS</sub>, pUCM\_B<sub>RS</sub>, and pUCM\_I<sub>RS</sub>. To assemble the individual expression modules of the *crtE*, *crtB*, and *crtI* genes on pUCM, each gene module was amplified by PCR and subsequently cloned into pACM plasmid, generating pACM-E<sub>RS</sub>, pACM-E<sub>RS</sub>-B<sub>RS</sub>, and pACM-E<sub>RS</sub>-B<sub>RS</sub>-I<sub>RS</sub>.

**Table 3.** Primers used in this study. The underlined sections indicate restriction enzyme sites.

Gene	Primer Sequence	Enzyme Site
<i>crtE</i>	F: 5' <u>GCTCTAGA</u> AAGGAGGATTACAAAATGGCGTTTGAACAGCGGATTG 3' R: 5' GGAATTCTCAGACGCGGGCCGCGACCT 3'	<i>XbaI</i> <i>EcoRI</i>
<i>crtB</i>	F: 5' <u>GGAATTC</u> AGGAGGATTACAAAATGATTGCCTCTGCCGATCT3' R: 5' CATGCCATGGCTAGATCGGGTTGGCCCG3'	<i>EcoRI</i> <i>NcoI</i>
<i>crtI</i>	F: 5' <u>GCTCTAGA</u> AAGGAGGATTACAAAATGCCCTCGATCTCGCCC 3' R: 5' <u>CGGAATTCT</u> CATTCCGCGGCAAGCCT 3'	<i>XbaI</i> <i>EcoRI</i>

### 3.2. Error-Prone PCR Mutagenesis

Random mutagenesis of the *R. sphaeroides crtI* gene on the plasmid pUCM was performed using a previously reported error-prone PCR method [28]. Briefly, the mutagenic PCR condition of 1.5 mM MgCl<sub>2</sub>, unbalanced dNTP ratio (ATP:TTP:CTP:GTP, 1:1:1:4), and *Taq* polymerase were utilized to incorporate mismatched bases into the *crtI* gene with primers (5'-GCTCTAGAAGGATTACAAAATGCCCTCGATCTCGCCC-3' and 5'-CGGAATTCTCATTCCGCGGCAAGCCT-3') flanking the *crtI* gene on the pUCM vector. The PCR products were purified using a gel DNA extraction kit (Macrogen, Inc., Seoul, Korea), followed by digestion with the restriction enzymes *EcoRI* and *XbaI*. The fragments were cloned into the corresponding site of vector pUCM and then transformed into *E. coli* harboring pACM-E<sub>RS</sub>-B<sub>RS</sub>. The cells grown on M9 agar plates were supplemented with ampicillin (100 µg/mL) and chloramphenicol (50 µg/mL) at 37 °C for 24 h and then incubated at 20 °C until colonies developed. Colonies on M9 agar plates were visually screened, and those with color changes were selected and restreaked on LB agar plates to isolate pure colonies. The isolated cells were cultured in LB medium supplemented with ampicillin (100 µg/mL) and chloramphenicol (50 µg/mL), and pUCM plasmids containing the mutagenic *crtI* gene were isolated from 1% (*v/v*) agarose gel with a gel DNA extraction kit (Macrogen). Mutagenic *crtI* gene sequences were verified by Sanger sequencing (Macrogen). Fresh transformed *E. coli* strains harboring pACM-E<sub>RS</sub>-B<sub>RS</sub> and pUCM\_mutant\_crtI were prepared and cultured in Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 0.17 M KH<sub>2</sub>PO<sub>4</sub>, 0.72 M K<sub>2</sub>HPO<sub>4</sub>, and 10 g/L glycerol) supplemented with ampicillin (100 µg/mL) and chloramphenicol (50 µg/mL) to investigate the carotenoid profile.

### 3.3. Site-Directed Mutagenesis

Phusion High-Fidelity DNA Polymerase (New England BioLabs, Inc., Ipswich, MA, USA) was used to perform SDM of the *crtI* gene. Mutagenesis primers were designed according to the desired *crtI* gene mutations. After PCR amplification, the PCR product was digested with *DpnI* for 5 h and transformed into *E. coli*. The sequence changes in SDM were verified by Sanger sequencing (Macrogen).

### 3.4. Analysis of Carotenoid Production

Carotenoid extraction was performed using a previously described extraction method [22]. Briefly, 50 mL of culture was harvested and separated into a cell pellet and culture medium. Carotenoids were repeatedly extracted with a total of 20 mL of acetone until all visible color disappeared from the cell pellet. Equal volumes of water and hexane were added to the acetone extract and vortex-mixed. The upper carotenoid-containing solvent layer was carefully collected and dehydrated with 0.1 g anhydrous sodium sulfate (Sigma-Aldrich, St-Louis, MO, USA) for 20 min. After centrifugation (4 °C and 13,000 rpm), the supernatant was collected and completely dried using a Genevac EZ2 centrifugal evaporator (Genevac, Inc., Vally Center, NY, USA). The dried residue was resuspended in 500 µL acetone and 20 µL of aliquot was subjected to an Agilent 1260 series HPLC (Agilent technologies, Palo Alto, CA, USA) system equipped with an Agilent photodiode array detector and Zorbax eclipse XDB-C18 column (4.6 × 150 mm, silica particle, 80 Å, 5 µm; Agilent Technologies). The column temperature was maintained at 35 °C and the flow rate was 1 mL/min.

Acetonitrile, methanol, and isopropanol (80:15:5, *v/v/v*) were used for isocratic elution. UV/Vis analysis of neurosporene, lycopene, and 3,4-didehydrolycopene was carried out at wavelengths of 440 nm, 470 nm, and 490 nm, respectively. The relative ratio of each carotenoid profile was calculated by comparing the peak area of each carotenoid in the LC chromatogram generated by OpenLab ChemStation<sup>®</sup> software (Agilent Technologies). The results are expressed as means  $\pm$  standard deviations of three replicates.

### 3.5. Computational Modeling of Phytoene Desaturase

To compare the functional differences of mutant CrtI enzymes, protein structures of CrtI mutants were computationally predicted using I-TASSER [29]. Two protein templates were used to construct CrtI protein models:  $\gamma$ -carotenoid desaturase (PDB ID: 4repA) from *N. dokdonensis* DSW-6 [30] and phytoene desaturase (PDB ID: 4dgkA) from *P. ananatis* [18]. Starting with the protein structures, FAD was docked into the crystal structures using COACH-D [31]. The structure of the FAD ligand was prepared using ChemsSketch [32] prior to performing docking simulations. The model structures were visualized using the PyMol Molecular Graphics System (ver 2.0.4, Schrödinger, LLC, New York, NY, USA).

## 4. Conclusions

In this study, phytoene desaturase of *R. sphaeroides* (CrtI<sub>RS</sub>), a catalyst in the 3-step desaturation of phytoene, was randomly mutated to alter its catalytic activity towards phytoene. CrtI<sub>RS</sub> mutants produced different ratios of neurosporene (9 CDBs)/lycopene (11 CDBs)/3,4-didehydrolycopene (13 CDBs). Leu163, Ala171, and Ile454 were particularly important residues in determining product alteration between neurosporene, lycopene, and 3,4-didehydrolycopene (Table 2). The evaluation of an *in silico* model of CrtI<sub>RS</sub> concluded that the mutated amino acids were gathered in the membrane-binding domain, which could distantly influence the FAD binding region [18]. As CrtI<sub>RS</sub> is a bacterial carotene desaturase, the microenvironment of the cofactor FAD-binding region is important for the desaturation of phytoene catalyzed by a sole CrtI [33]. Although the distant influence of mutated residues on the FAD binding region of CrtI<sub>RS</sub> was demonstrated through the altered activity of mutant CrtI<sub>RS</sub>, the mechanism of FAD reoxidation in successive phytoene desaturation requires further investigation. Notably, phytoene desaturation and FAD reoxidation by quinones are separate events in plant-type phytoene desaturase [34]. This can provide insights into how bacterial CrtI simultaneously modulate phytoene desaturation and FAD reoxidation.

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