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**FAD- and NADPH-dependent carotenoid cyclases**

**Corresponding author:**
Florence Bouvier,
Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique and Université de Strasbourg, 67084 Strasbourg Cedex, France
Phone: 3 33 8841 7231, Fax: 3 33 88 61 44 42
E-mail: Florence.Bouvier@ibmp-cnrs.unistra.fr

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**Biochemical processes and macromolecular structures**
Characterization of plant carotenoid cyclases as members of the flavoprotein family functioning with no net redox change

Alexis Samba Mialoundama¹, Dimitri Heintz¹, Nurul Jadid¹,², Paul Nkeng³, Alain Rahier¹, Jozsef Deli⁴, Bilal Camara¹ and Florence Bouvier¹

¹Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique and Université de Strasbourg, 67084 Strasbourg Cedex, France.
²Department of Biology, Botanical and Plant Tissue Culture Laboratory Sepuluh Nopember Institut of Technology (ITS) Gedung H Kampus ITS Sukolilo Surabaya, 60111 East-Java, Indonesia
³Institut de Chimie, Université de Strasbourg, 67000 Strasbourg, France
⁴Department of Biochemistry and Medical Chemistry, University of Pécs, Medical School, Szigeti út 12, Box 99, H-7624 Pécs, Hungary
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Corresponding author with E-mail address:

Florence Bouvier
Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique and Université de Strasbourg, 67084 Strasbourg Cedex, France.
Phone: 3 33 8841 7231, Fax: 3 33 88 61 44 42
E-mail: Florence.Bouvier@ibmp-cnrs.unistra.fr

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Abstract

The later steps of carotenoid biosynthesis involve the formation of cyclic carotenoids. The reaction is catalyzed by lycopene beta-cyclase (LCY-B), which converts lycopene into β-carotene, and by capsanthin-capsorubin synthase (CCS), which is mainly dedicated to the synthesis of κ-cyclic carotenoids (capsanthin and capsorubin), but has also LCY-B activity. Although the peptide sequences of plant LCY-Bs and CCS contain a putative dinucleotide-binding motif, it is believed that these two carotenoid cyclases proceed via protic activation and stabilization of resulting carbocation intermediates. Using pepper CCS as a prototypic carotenoid cyclase, we show that the monomeric protein contains one noncovalently-bound FAD which is essential for enzyme activity only in the presence of NADPH, which functions as the FAD reductant. The reaction proceeds without transfer of hydrogen from the dinucleotide cofactors to β-carotene or capsanthin. Using site-directed mutagenesis, amino acids potentially involved in the protic activation were identified. Substitutions of Ala, Lys, and Arg, for Glu295 in the conserved 293FLEET297 motif of pepper CCS or LCY-B abolish the formation of β-carotene and κ-cyclic carotenoids. We also found that mutations of the equivalent Glu196 located in the 194LIEDT198 domain of structurally-divergent bacterial LCY-B, abolish the formation of β-carotene. The data herein reveal plant carotenoid cyclases to be novel enzymes that combine characteristics of non-metal-assisted terpene cyclases with those attributes typically found in flavoenzymes that catalyze reactions, with no net redox, such as type 2 isopentenyl diphosphate isomerase. Thus, FAD in its reduced form could be implicated in the stabilization of the carbocation intermediate.
INTRODUCTION

Later steps of carotenoid biosynthesis involve the formation of diverse cyclic carotenoids. For example, \( \beta \)-carotene, the vitamin A precursor, is synthesized de novo by photosynthetic organisms, limited non-phototrophic bacteria and fungi, and also by aphids (Moran and Jarvik, 2010) according to a multi-step pathway that ends with the cyclization of lycopene by lycopene beta-cyclase (LCY-B). Similarly, in pepper chromoplasts, antheraxanthin and violaxanthin are respectively converted into the \( \kappa \)-cyclic carotenoids capsanthin and capsorubin by capsanthin-capsorubin synthase (CCS). In both cases, the proposed mechanism involves a concerted protic attack and stabilization of a transient carbocation without any net redox change (Camara, 1980; Bouvier et al., 1994; Britton, 1998). Several cDNAs for LCY-B have been cloned from bacteria (Misawa et al., 1990; Cunningham et al., 1994; Armstrong, 1997; Cunningham and Gantt, 2001), fungi (Verdoes et al., 1999; Velayos et al., 2000; Arrach et al., 2001) and plants (Hugueney et al., 1995; Ronen et al., 2000) using functional complementation. Information available from primary structures suggest that the cyclization of lycopene is catalyzed by holomeric proteins in photosynthetic organisms (Cunningham et al., 1994; Maresca et al., 2007), by holomeric (Misawa et al., 1990) or heteromeric (Krubasik and Sandmann, 2000; Viveiros et al., 2000) proteins in non-photosynthetic bacteria; and, by holomeric, bifunctional proteins in fungi that combine the activities of phytoene synthase and lycopene cyclase (Verdoes et al., 1999; Velayos et al., 2000; Arrach et al., 2001). This structural diversity of LCY-Bs coupled to lack of significant amino acid sequence identity between the lycopene cyclases from bacteria, fungi and plants hinders our understanding of the catalytic mechanism of LCY-Bs and CCS. In addition, the amino terminus of plant LCY-B and CCS contains an amino sequence motif characteristic of a polypeptide predicted to adopt a Rossmann fold (Rossmann et al., 1974) and suggests the binding of an as yet unknown dinucleotide prosthetic ligand. It has been shown by using recombinant bacterial enzyme, that the cyclization of lycopene into \( \beta \)-carotene strictly requires NADPH, but proceeds without any net redox change (Schnurr et al., 1996; Hornero-Mendez and Britton, 2002). Under the same conditions, FAD alone could not sustain bacterial LCY-B activity (Schnurr et al., 1996). Much less is known about the
dinucleotide requirements of plant carotenoid cyclases, which are highly conserved within plants, but are extremely divergent in non-plant organisms. Previously, a crucial acidic domain for lycopene cyclase activity was identified using an affinity-labeling strategy, followed by site-directed mutagenesis (Bouvier et al., 1997), in the absence of any crystal structures. This so-called 293FLEET297 motif of LCY-B and CCS contained two tandem Glu295Glu296 residues that were essential for LCY-B- and \( \beta \)-cyclase activities (Bouvier et al., 1997). However, it still remains unclear how the protic mechanism is compatible with the requirement of dinucleotide cofactors.

To further explore the mechanism of plant carotenoid cyclases, we first choose pepper capsanthin-capsorubin synthase (CCS) as a prototypic enzyme because it displays a strong identity (52%) to pepper LCY-B, and we have shown previously that CCS could also catalyze the cyclization of lycopene into \( \beta \)-carotene (up to 25% of activity compared to LCY-B) (Hugueney et al., 1995). Herein, we have shown that monomeric CCS purified to homogeneity from plant chromoplasts or recombinant CCS purified from \textit{E. coli}-transformed cells, are typical flavoproteins containing one noncovalently-bound FAD. We also observed that CCS-bound FAD is required for enzyme activity in the presence of NADPH, which functions as a reductant of FAD. During this process, no hydrogen is transferred to \( \beta \)-carotene or \( \kappa \)-cyclic carotenoids. In addition to this cofactor requirement, we also show from extensive site-directed mutagenesis using pepper CCS and LCY-B and \textit{Erwinia herbicola} LCY-B (Mialoundama, 2009), that Glu295 of pepper CCS and LCY-B plays a key role in the formation of \( \beta \)-carotene and \( \kappa \)-cyclic carotenoids, and we demonstrate that a similar role is played in structurally-divergent bacterial LCY-Bs by Glu196. These characteristics suggest that plant CCS and LCY-Bs are mechanistically similar to non-metal-assisted terpene cyclases, such as squalene:hopene cyclase, and oxidosqualene cyclase, and additionally represent a new subfamily of flavoproteins like isopentenyl diphosphate isomerase type II, which catalyze carotenoid cyclization without any net redox modification of the substrate.

RESULTS

Identification of CCS as a typical flavoprotein
Amino acid sequence alignment indicates that plant type LCY-Bs and CCS contain dinucleotide-binding motifs characteristic of an N-terminal Rossmann fold of FAD-dependent oxidoreductases (Rossmann et al., 1974; Cunningham et al., 1994; Hugueney et al., 1995). To analyze the nature of the potential dinucleotide prosthetic group, we noted during the initial purification steps that it was more tightly bound to CCS prepared from pepper chromoplasts, than that of recombinant CCS produced in \textit{E. coli}. Based on this evidence, we first purified CCS from pepper chromoplasts using a previously described procedure (Bouvier et al., 1994). When homogenous fractions (Fig. 1A) were subjected to mild electrophoretic conditions, the band corresponding to homogenous CCS protein could be observed without further treatment due to its yellow color, as observed previously (Bouvier et al., 1994) (Fig. 1B). Further analysis of chromoplast-derived CCS showed UV-visible absorption (458 nm) and fluorescence (520 nm) spectra characteristic of flavoproteins (Fig. 1C).

To further characterize the binding and the structure of the flavin prosthetic group, the yellow gel bands (Fig. 1B) were subjected to in-gel digestion using trypsin. The resulting peptide mixture was then extracted and analyzed by LC-MS/MS. We could unambiguously identify CCS peptides, but no peptide fragments carrying covalently-linked FAD or FMN were detected (data not shown). Subsequently, we extracted the yellow gel band using acidic acetonitrile before chromatographic analysis, using FAD and FMN standards. Both cofactors provide the best signal in positive electrospray mode, and the best MS signal was obtained with 25V (data not shown). The best specific Multiple Reaction Monitoring (MRM) transitions were m/z (786 -> 348) for FAD, and (457 -> 439) for FMN (Fig. 2). MRM analysis revealed the presence of FAD in the yellow gel extract (Fig. 2), whereas no FMN was detected (Fig. 2). The broadening of FAD and FMN peaks is probably due to the phosphate group, which is known to be poorly ionized under electrospray conditions. Collectively, these data demonstrate that FAD is noncovalently bound to chromoplast CCS (ChrCCS). The stoichiometry of 0.95 FAD per monomeric CCS could be determined at 458 nm, assuming that free and bound FAD have the same millimolar absorbance coefficient (11.3 mM\(^{-1}\)cm\(^{-1}\)) (Whitby, 1953). Thus, one could postulate that each native CCS monomer (52 kD) contains one
noncovalently-bound FAD. Very similar data were obtained using purified recombinant pepper CCS (reCCS) (Supplemental Fig. S1) and recombinant LCY-B (reLCY-B) (data not shown). Binding affinities for FAD were determined for ChrCCs, reCCS and reLCY-B. The dissociation constant ($K_d$) values of 0.4 ± 0.02 μM, 0.6 ± 0.03 μM and 0.9 ± 0.02 μM were obtained for ChrCCs, reCCS and reLCY-B. These data collectively demonstrate that CCS and plant LCY-B are FAD-binding flavoproteins.

**Implication of FAD and NADPH during CCS catalysis**

To determine if FAD is implicated in the cyclization of lycopene into β-carotene or the formation of κ-cyclic carotenoids, we removed noncovalently-bound FAD from chromoplast CCS (ChrCCS) and recombinant CCS (reCCS) using hydrophobic interaction chromatography, and determined the activity of the resulting apoenzymes under diverse reconstitution conditions. Apo-ChrCCS and Apo-reCCS revealed no enzymatic activity toward carotenoid cyclization in the absence of added cofactors (Fig. 3A). Next, we analyzed the role of FAD following the reconstitution of apo-ChrCCS and apo-reCCS with FAD (Fig. 3A). The resulting holoenzyme catalyzed the cyclization of lycopene into β-carotene and the synthesis of κ-cyclic carotenoids only in the presence of FAD and NADPH (Fig. 3A). The NADPH requirement was also observed for purified ChrCCS and reCCS that contained noncovalently-bound FAD in their native form. Similar data were obtained for recombinant pepper reLCY-B (Fig. 3B). The $K_m$ values for NADPH were 0.15 ± 0.07 mM, 0.25 ± 0.03 mM and 0.22 ± 0.02 mM for ChrCCS, reCCS and reLCY-B respectively. Subsequently, we tested whether the requirement of FAD and NADPH is coupled to hydrogen transfer to the cyclic carotenoids. To this end, we determined the CCS activity in the presence of FAD and (4R)-[4-3H]NADPH prepared as described previously (McCracken et al., 2004). Under these conditions, we did not observe tritium incorporation in the purified β-carotene, or in capsanthin (data not shown). Thus no hydrogen from NADPH is transferred to β-carotene and capsanthin, in agreement with previous data (Hornero-Mendez and Britton, 2002). Finally, we noted that upon incubation of the holoenzyme with NADPH, a strong modification of the absorption spectrum of holo-CCS was observed under anaerobic condition (Fig. 3C).
This change is reminiscent of the direct reduction of CCS-bound FAD, as noted for isopentenyl diphosphate isomerase type II, where noncovalently-bound FMN could be reduced directly by NADH or NADPH (Hemmi et al., 2004; Rothman et al., 2007).

**Implication of the acidic FLEET motif in CCS and LCY-B activity**

Based on the indirect implication of FAD and NADPH in the cyclization of carotenoid, we decided to explore further the role of amino acids potentially involved in the protic activation during carotenoid cyclization. A homology-based, structural alignment reveals that lycopene cyclases identified to date cannot be placed into a single homogenous group. The 293FLEET297 motif in LCY-Bs and in CCS (Supplemental Fig. S2) was previously shown to be crucial for cyclization of lycopene to β-carotene (Bouvier et al., 1997). Because neither crystal structure nor homology modeling is yet possible for LCY-B and CCS, we further analyzed the contribution of this motif in recombinant pepper CCS (reCCS) and LCY-B (reLCY-B) (Mialoundama, 2009), by substituting Ala for targeted residues, and by substituting Lys and Arg for selected acidic amino acids to convert them to positive charges. We also substituted alanine for conserved Asp127, Asp259, Glu128, Glu332 and His360 residues, which are conserved between highly-divergent plant and bacterial carotenoid cyclases (Supplemental Fig. S2 ). This strategy was used because Ala substitutions generate low distortions in protein structure (Fersht, 1987), while Lys and Arg substitutions eliminate the protic activation potential of acidic amino acid residues. Before mutant proteins were expressed in *E. coli*, these substitutions were verified by DNA sequencing. Recombinant LCY-B and CCS were then purified by His-Tag-affinity chromatography as described in the experimental section. They migrated as a 66 kD band and were immunoreactive to antibodies directed against LCY-B or CCS (Fig. 4). The intensity of the protein signals as shown revealed that mutations of LCY-B and CCS have no effect on expression or stability of proteins. Through the use of an FAD-Agarose affinity column, we observed that the mutant and the wild-type reCCS and reLCY-B proteins had practically the same affinity for FAD (data not shown). The effects of different mutations on cyclization of lycopene into β-carotene were compared to wild-type enzymes, and the kinetic properties were determined from Lineweaver-Burk plots.
Substituting Ala, Lys or Arg for Glu295 completely abolishes reLCY-B (Table I) and reCCS (Table II) activity. Substituting Ala for E296 reduces the lycopene cyclase activities of reLCY-B (Table I) and reCCS (Table II) to residual levels. Similarly, substituting Ala for D127, E128, D259, and E332 drastically reduced the activity up to 90%. In the case of reLCY-B H360A (Table I) or reCCSH360A (Table II), the activity was abolished but was partially recovered in reLCY-B H360K/R and reCCSH360K/R. Compared to the wild-type cyclases, the apparent $V_{\text{max}}$ values of the different mutant cyclases were reduced while the $K_m$ values were not altered (Table I and II). Thus the reduction of the catalytic activity was probably not due to a modified binding of lycopene. This indicates that the D127, E128, D259, and H360 residues of reLCY-B and reCCS have an important catalytic role, but are not likely to directly initiate cyclization.

Examination of protein sequence data revealed that plant LCY-B or CCS Glu295 is structurally equivalent to bacterial Glu196 of *Erwinia* LCY-B (Fig. 5). This led us to envisage that this invariant residue may have an identical function. This idea was explored by site-directed mutations substituting Glu196 of *Erwinia* LCY-B to Ala, Lys and Arg. To test these mutant strains an *in vitro* complementation procedure was employed using *E. coli* that synthesized lycopene. This offered an indirect and rapid mean of screening for lycopene cyclase activity by measuring $\beta$-carotene accumulation following transformation of *E. coli* with plasmids that contained wild-type or mutated lycopene cyclase. Under these conditions, plasmids containing lycopene cyclase allowed up to 80% conversion of endogenous lycopene into $\beta$-carotene. However, none of the plasmids containing the *Erwinia* Glu196Ala, Glu196Lys or Glu196Arg mutants of lycopene cyclases were able to direct $\beta$-carotene synthesis (Fig. 5). Thus, an active glutamate residue was needed to initiate lycopene cyclization by pepper CCS, plant type LCY-Bs, and bacterial LCY-B.

To further analyze the catalytic mechanism of plant carotenoid cyclase, we incubated wild-type and mutant reCCS with different 5,6-epoxy-xanthophylls and further analyzed the reaction products using a sensitive liquid chromatography-tandem mass spectrometry (UPLC-APCI+ MS-MS). When wild-type reCCS was incubated with
antheraxanthin, we noted that in addition to capsanthin, the normal product, a new xanthophyll (peak 1) was formed (Fig. 6A). The polarity, absorption maxima in the eluting solvent, and the total ion chromatogram (TIC) of the new xanthophylls (Supplemental Fig. S3), showed it to be 5,6-diepikarpoxanthin (peak 1) (Deli et al., 1998). In a similar vein, we incubated wild-type reCCS with violaxanthin. HPLC and MS analysis of reaction products showed that when violaxanthin was used as a substrate, capsanthin-5,6-epoxide (peak 6) and capsorubin (peak 5) were formed as expected, and a new xanthophyll (peak 4) appeared (Fig. 6B). The relative elution of peak 4 on HPLC, and its spectral characteristics in the eluting solvent is consistent with peak 4 being 5,6-diepilatoxanthin (Deli et al., 1998), but we could not further analyze its structure. The formation of these new xanthophylls were abolished when reCCSAla295, reCCSLys295 and reCCSArg295 mutants were used for the enzymatic tests (data not shown). As these new xanthophylls might represent trapped intermediates during the formation of κ-cyclic carotenoids, they were also tested as possible substrates of reCCS. The results revealed that they were not converted into κ-cyclic xanthophylls (data not shown). Thus these new xanthophylls are not transient intermediates en route to κ-cyclic carotenoid synthesis. They most probably represent dead-end products of the trapped carbocation produced during the formation of capsanthin and capsorubin.

DISCUSSION

FAD and NADPH dependence of plant carotenoid cyclases

Amino acid sequence alignment of plant type LCY-Bs suggests that the N-terminal region could adopt a Rossmann-fold characteristic of flavoproteins (Dym and Eisenberg, 2001). The nature of the putative prosthetic flavin group had remained elusive. Previously, a strict requirement of exogenous NADPH, and non-essentially, of exogenous FAD, have been reported for the enzymatic cyclization of lycopene into β-carotene using E. uredovora LCY-B (Schnurr et al., 1996). However, although E. uredovora LCY-B functions in the presence of NADPH, no hydrogen is transferred from NADPH to β-
carotene (Hornero-Mendez and Britton, 2002). This reinforced the accepted protic cyclization mechanism of carotenoids triggered by unknown amino acid residues, but leaves unexplained the indirect role played by NADPH. Using CCS as a carotenoid cyclase prototype has allowed us to identify non-bound FAD as the prosthetic group implicated in the synthesis of β-carotene and κ-cyclic carotenoids. Our results also reveal that the cyclization reaction takes place only in the presence of FAD and NADPH (Fig. 3AB). Data further revealed that when NADPH is incubated with CCS holoenzyme, its absorption spectrum is drastically modified (Fig 3C). The characteristic absorption at ca 450 nm was lost (Fig. 3C). A similar change was observed when dithionite was used instead of NADPH (data not shown). The spectral change suggests a direct reduction of FAD by NADPH (Fig. 3C). In Erwinia pantoea, FAD reduced by Ti(III)-citrate in the absence of NADPH could sustain the conversion of lycopene into β-carotene (P. Beyer, Freiburg, personal communication) (Yu et al., 2010). Very similar observations have been described for isopentenyl diphosphate isomerase (IPP)-type II (Hemmi et al., 2004; Rothman et al., 2007), for which the reaction requires the reduction of FMN-bound enzyme by NADPH. In this context it is interesting to note that the characteristics of CCS are reminiscent of those described for isopentenyl diphosphate isomerase type II, which is a flavoenzyme, and requires FMN and NADPH for activity, but functions without net redox modifications of the substrate. Because flavoproteins usually catalyze redox reactions, the question arises how do CCS-bound FAD function? With respect to data obtained from IPP-type II which has been crystallized, two likely explanations could be offered (Rothman et al., 2008; Unno et al., 2009). In one case, CCS-bound FAD when reduced by NADPH, could act as an acid-base catalyst (Unno et al., 2009). Alternatively, reduced CCS-bound FAD could be involved in the stabilization of the transient carbocation formed during the reaction (Rothman et al., 2008). The latter conclusion is supported by the recent finding, showing that E. pantoea LCY-B could function with reduced 1- and 5-deazaflavin analogs that have probably limited potential to trigger the protic activation of lycopene (Yu et al., 2010).

**Importance of invariant acidic residues in plant carotenoid cyclases**

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LCY-Bs cloned from bacteria, plants and fungi do not contain consensus motifs. We have previously used a site-directed mutant approach to identify several amino acid residues critical for differential inhibition of plant LCY-B and CCS (Bouvier et al., 1997). In the present study, we made use of alanine scanning mutagenesis strategy combined to the introduction of positive or negative residues to replace several conserved residues between plant cyclases LCY-B, CCS and bacterial LCY-B. Stably-expressed recombinant proteins were purified and used for enzyme assays. The mutant proteins allowed us to define Glu295 as a critical residue for the cyclization of lycopene into β-carotene, and also for the formation of κ-cyclic carotenoids in plants. Glu295A/K/R failed to synthesize β-carotene or κ-cyclic carotenoids. In addition, the mutation of Glu196 of *E. herbicola* (Fig. 5) or *E. pantoea* (P. Beyer, Freiburg, personal communication) (Yu et al., 2010), equivalent to Glu295 of plant type LCY-Bs, abolished the cyclization of lycopene into β-carotene. Therefore, the presence of a Glu residue at position 295 in plant enzymes or 196 in bacteria is essential for the catalytic activity of carotenoid cyclases. Cloning of fungal LCY-B from wild-type and mutant strains of *Phycomyces blakesleeanus* (Arrach et al., 2001) supports our conclusion. Fungal LCY-Bs have no homology with plant cyclases, however, massive amounts of lycopene accumulated in the *Phycomyces* Car23 mutant that has a Glu77-to-Lys77 substitution. Glu77 represents the acidic residue in the PxE(E/D) motif found in lycopene β-cyclases of different organisms including, *Mycobacterium aurum*, *Sulfolobus solfataricus* and fungi. Thus, although bacterial, fungal and plant type LCY-Bs are highly divergent, one could suggest that they have evolved to convert lycopene into β-carotene via an acid-catalyzed cyclization mechanism. Indeed, the formation of 5,6-diepikarpoxanthin, and of putative 5,6-diepilatoxanthin catalyzed by CCS (Fig. 6) could be rationalized by considering that following the protic attack, the incipient carotenoid carbocation generated is flexibly stabilized to yield different structures (Fig. 7). This has been hypothesized previously, because these new xanthophylls are present in trace amounts in red pepper fruits and absent in yellow varieties, which are known to be devoid of CCS (Bouvier et al., 1994).

Prootic activations of carbon-carbon double bonds are uncommon in nature. Typical examples are Δ8-Δ7 cholesterol isomerase (Wilton et al., 1969), isopentenyl...
diphosphate (IPP) isomerase (Street et al., 1990), squalene cyclase (Wendt et al., 2000). While Cys67 has been identified as the protic activator in IPP isomerase (Street et al., 1990), aspartic acid residues are the proton initiators in squalene cyclase. For squalene cyclase, protonation of the terminal double bond of squalene is triggered by Asp376 of the D376DTAVV motif, and assisted by His451 (Wendt et al., 2000). It has been suggested that the tandem aspartic residues in the DDTAVV motif of squalene cyclase offer higher acidity which is required for protonating the terminal double bond of squalene compared to the terminal oxirane of oxidosqualene, the phytosterol precursor, which is more easily protonated (Wendt et al., 2000). It is possible that the tandem glutamate residues in the FLEET motif of LCY-Bs and CCS could play the same role. Interestingly, the similarity between the catalytic mechanism of squalene cyclase, and LCY-B or CCS is supported by the fact that aza-squalene, a potent transient-state inhibitor of squalene cyclase (Reinert et al., 2004), is also a strong inhibitor of pepper CCS and LCY-B (Camara et al., 1985; Bouvier et al., 1997). Due to these characteristics, and because flavin prosthetic groups are not involved in squalene cyclase catalysis, one may suggest that CCS- or plant LCY-B -bound FAD, in its reduced form, is probably involved in the stabilization of the incipient carotenoid carbocation (Yu et al., 2010) and could function as previously envisioned “negative point charges” (Bouvier et al., 1997). Given previous biochemical data (Schnurr et al., 1996; Hornero-Mendez and Britton, 2002) indicating the requirement of NADPH for LCY-B activity, we show that FAD is likely reduced by NADPH. Further studies are required to fully evaluate this hypothesis.

In conclusion, the data presented herein reveal that plant type carotenoid cyclases have evolved to integrate two characteristics. First, they belong to FAD flavoproteins that catalyze reactions without net redox changes (Bornemann, 2002). In the terpene series, this feature is shared with isopentenyl diphosphate isomerase II. Second, they belong to the class of non-metal assisted cyclases such as squalene and oxidosqualene cyclases that operate via a protic attack. With respect to the latter mechanism, we show that Glu295 of plant LCY-B and CCS or the equivalent Glu196 from bacterial LCY-B play a key role. Further work based on the CCS crystal is required to delineate the catalytic mechanism.

MATERIAL AND METHODS
Materials

All *trans* lycopene extracted from red tomato fruits and all *trans* xanthophylls extracted from red pepper fruits and *Viola tricolor* flowers (Deli et al., 1998) were purified (Davies 1976) and used as substrates. *Erwinia herbicola* (renamed *Pantoea agglomerans*) DNA was used as the source for the amplification of bacterial LCY-B by polymerase chain reaction. CCS and LCY-B antibodies were prepared as described previously (Bouvier et al., 1994). The FAD-Agarose affinity column and all chemicals were obtained from Sigma-Aldrich and GE Healthcare unless otherwise stated.

Site-directed Mutagenesis

The different point mutations within the peptide sequence of pepper cyclase, pepper capsanthin capsorubin synthase and *E. herbicola* lycopene cyclase were available from previous work (Mialoundama, 2009) using the QuickChange site-directed mutagenesis (Stratagene) as previously described (Bouvier et al., 1997). The introduced mutations were verified by DNA sequencing using an automatic DNA sequencer. For *Capsicum* LCY-B (Accession number CAA60119) the following mutagenic oligonucleotides were used: D127A, GTTGGGTGGATGCGATTTGAGGCT; E128A, ATGGTTTTATGCGATTTGAGGCT; D259A, ATGGTTTTATGCGATTTGAGGCT; E295A, AGGATATTTCTTGAAGAACCTCACC; E295K, AGGATATTTCTTGAAGAACCTCACC; E296A, ATATTTCTTGAAGAACCTCACC; E296K, ATATTTCTTGAAGAACCTCACC; E296R, ATATTTCTTGAAGAACCTCACC; E332A, ATATTTCTTGAAGAACCTCACC; H360A, GCCGGTATGCTCCATCCACCGGT; H360K, GCCGGTATGCTCCATCCACCGGT; H360R, GCCGGTATGCTCCATCCACCGGT. For *Capsicum* CCS (Accession number CAA53759) the following mutagenic oligonucleotides were used: D127A, GTTGGGTGGATGCGATTTGAGGCT; E128A, ATGGTTTTATGCGATTTGAGGCT; D259A, ATGGTTTTATGCGATTTGAGGCT; E295A, TTGGTATTCTTGGAGAGACTTCTT; E295K, TTGGTATTCTTGGAGAGACTTCTT; E295R, TTGGTATTCTTGGAGAGACTTCTT; E296A, TTGGTATTCTTGGAGAGACTTCTT; H360A, GCCGGTATGCTCCATCCACCGGT; H360K, GCCGGTATGCTCCATCCACCGGT; H360R, GCCGGTATGCTCCATCCACCGGT. For *Capsicum* CCS (Accession number CAA53759) the following mutagenic oligonucleotides were used: D127A, GTTGGGTGGATGCGATTTGAGGCT; E128A, ATGGTTTTATGCGATTTGAGGCT; D259A, ATGGTTTTATGCGATTTGAGGCT; E295A, TTGGTATTCTTGGAGAGACTTCTT; E295K, TTGGTATTCTTGGAGAGACTTCTT; E295R, TTGGTATTCTTGGAGAGACTTCTT; E296A, TTGGTATTCTTGGAGAGACTTCTT.
GTATTCTTGGAAGCGACTCTCTTAGT; E296K, GTATTCTTGGAAGACCTTTTATTAGT; E296R, GTATTCTTGGAACGGGACCTCTTTTATTAGT; E332A, GTCTTTAGGAAGCGAAGTGTTGATC; H360A, TCAGGGATAGTTGCTCCATCGTCTGGG; H360K, TCAGGGATAGTTAAACCATCGTCTGGG; H360R, TCAGGGATAGTTGCTCCATCGTCTGGG. For Erwinia LCY-B (Accession number Q01331) the following mutagenic oligonucleotides were used: E196A, ACGCTGCTGATCGC; E196K, ACGCTGCTGATCAAGGATACG; E196R, ACGCTGCTGATCGGATACGCT; E196R, ACGCTGCTGATCAGGATACGCTAC. Mutated positions are underlined.

**Expression and Characterization of Recombinant Enzymes**

Recombinant LCY-B and CCS (reLCY-B and reCCS) devoid of transit peptide sequences were expressed in *E. coli* (TOPO10) using pBAD/TOPOThiofusion vector (Invitrogen) before purification by metal affinity chromatography on TALON™ resin (Clontech, Inc.) (Bouvier et al., 1997). The mature part of pepper LCY-B (amino acid residues 54 to 498 of the precursor protein) and the mature part of CCS (amino acid residues 41 to 498 of the precursor protein) were amplified by PCR using the forward primer GCTCTTTTGGAGCTTGTACCTGAGAC and the reverse primer TTCTTTATCCTGTAACAAATTGTTGAT for LCY-B. For CCS the forward primer TTCCATTATAGAAACAAAAGCAGTAC and the reverse primer AAGGCTCTCATTGCTATTAGATTGCCCAG were used. The protein composition of each fraction was analyzed by SDS-PAGE and Western blotting using the ECL Western blotting detection kit (GE Healthcare) and anti-LCY-B or anti-CCS antibodies (Bouvier et al., 1994; Bouvier et al., 1997) followed by densitometry scanning using UN-SCAN-IT gel™ software (Silk Scientific). Protein concentrations were determined according to Smith et al. (Smith et al., 1985).

**Purification of Chromoplast CCS (ChrCCS) and Analysis of Flavin Prosthetic Groups**

ChrCCS was purified essentially as described previously from pepper chromoplasts (Bouvier et al., 1994) using sequentially Q-Sepharose, Affigel 501, Mono-P and
Sephacryl S-200 HR column chromatography and a last step including size-exclusion chromatography on a Superose-6 column using a Waters 650 FPLC system (Bouvier et al., 1994). Because the homogenous protein preparation displayed a yellow color that was preserved under mild SDS-PAGE (lowering by half the SDS concentration in the solubilization buffer and separation at 6°C), we first adopted an in-gel digestion procedure followed by mass spectrometry analysis using an automated protein digestion system (MassPREP Station, Waters) as described previously (Heintz et al., 2009) to determine whether the prosthetic group was covalently bound. The gel was subsequently extracted using acetonitrile acidified with 1% formic acid. The resulting yellow supernatant was clarified by centrifugation and dried under argon and solubilized in 200 µl of water/acetonitrile (90/10, v/v) before Ultra performance liquid chromatography (UPLC) analysis on an Acquity UPLC system (Waters), using authentific FAD and FMN standards. The column was eluted with water containing 0.1 % of formic acid (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B). The flow rate was 0.40 ml min⁻¹. Chromatographic conditions were: linear gradient 95% A to 5% B (2 min); 95% A to 100% B (6 min); 100 %B (2 min); 100% B to 95% A (1 min); 95%A to 100% A (2 min). UV-visible spectra were recorded from 200 nm to 500 nm. The ESI source was used in positive and negative mode with capillary voltage 3.4 kV; RF lens at 0V, resolution (LM1, HM1, LM2, HM2) 15, ion energy 1 and 2; 0.5. Source and desolvatation temperature were 135 and 400°C. Multiple reaction monitoring (MRM) with positive electrospray ionization (ESI+) was used for qualitative analysis. Cone tensions and collision energy were optimized at 25V and 20V for each compound. For FAD the MRM transition was \( m/z \) 785.8 -> 348 and for FMN the MRM transition was \( m/z \) 457 -> 439. The prosthetic group of recombinant pepper CCS (reCCS) or recombinant pepper LCY-B (reLCY-B) was extracted and analyzed using the same procedure. Purified CCS in phosphate buffer (pH 7) containing 10% glycerol and 0.5% Tween 80, was used for UV-visible absorption spectrophotometry using a NanoDrop 2000 (Thermo Scientific) or a Shimadzu UV2401 spectrophotometer and fluorescence emission spectra were recorded with a Shimadzu RF5302 fluorescence spectrophotometer at room temperature following excitation at 458 nm. The flavin content was determined as described previously (Whitby, 1953).
Deflavinylation and Reconstitution of Carotenoid Cyclases

To prepare the apoenzyme of carotenoid cyclases, we modified a previously described procedure (Van Berkel et al., 1988). Purified carotenoid cyclases were adsorbed onto a PhenylSepharose column (1x5 cm) equilibrated with 50 mM K₂HPO₄ buffer (pH7) containing 1 mM EDTA and 2M NaCl and 1M KBr. FAD was released using the same buffer adjusted to pH 5. The apoproteins were subsequently eluted with 50 mM Tris-maleate buffer (pH 6.8) containing 15% glycerol and 40% DMSO. DMSO was eliminated by dialysis against the same buffer devoid of DMSO and the fluorescence of the apoenzymes was recorded at 520 nm following excitation at 458 nm to assess the elimination of free FAD. For the reconstitution and the determination of the binding affinity of FAD, carotenoid cyclase apoenzymes in 0.5 ml of Tris-maleate pH 6.8 containing 15% glycerol were incubated with 5% molar excess of FAD for 12h at 4°C. Excess FAD was removed using Sephadex G-25 column (PD-10) (GE Healthcare). The binding affinities were determined using GraphPad Prism 4.0 program.

Carotenoid Cyclase Assay

Unless otherwise stated, assays were performed in a reaction mixture (final volume of 250 μl) containing 50 mM Tris-maleate buffer pH 6.8, 1 mM DTT, 2 mM NADPH, 10 μM FAD, 1 mg of Tween 80, 50 nmol of carotenoid substrates and a definite amount of enzyme. The reaction mixture was flushed with argon and incubated at 25°C for 30 min to 1 h. The reaction products were isolated following the addition of 500 μl of chloroform/methanol (2:1, v/v) before HPLC analysis of the lipid phase (Camara, 1985; Bouvier et al., 2000). Alternatively, a plasmid based complementation procedure was used to determine the conversion of lycopene into β-carotene using *E. coli* which produces lycopene (Bouvier et al., 1997). After transformation with plasmids encoding wild type and mutant LCY-Bs and CCSs and induction with 0.5 mM isopropyl-β-
thiogalactopyranoside, total carotenoids were extracted from the bacterial pellets and subjected to HPLC analysis (Camara, 1985). General methods, including carotenoid analysis and identification were as described previously (Davies, 1976). The HPLC system consisted of SpectraSYSTEM (ThermoFinnigan, France) comprising a SCM1000 solvent degasser, a P1-1000XR gradient pump, an AS3000 autosampler and a UV6000LP diode array detector. Data acquisition and processing were done using ChromQuest Version 3 software (ThermoFinnigan, France). The reaction products were collected, evaporated to dryness and further characterized by UPLC coupled to tandem mass spectrometry (UPLC-MS/MS) using the MS mode. The analysis was performed on a Waters Quattro Premier XE (Waters, Mildorf, MA USA) equipped with an Atmospheric Pressure Chemical Ionisation (APCI) source and coupled to an Acquity UPLC system (Waters) with diode array detector (DAD). UV spectra were recorded from 200 to 500 nm. Chromatographic separation was achieved using an Acquity UPLC BEH C18 column (100 x 2.1 mm, 1.7µm; Waters), coupled to a an Acquity UPLC BEH C18 pre-column (2.1 x 5 mm, 1.7µm; Waters). The mobile phases were 75% methanol in water (solvent A) and 100% isopropanol (solvent B).The following separation sequence was adopted: 100% A (5min); 100% A to 50% B (3min); 50% A + 50% B (4 min); 50% A to 100% B (5min); 100% B (10 min); 100% B to 50%A (2min); 50% B + 50% A (5min); 50% B to 100% A (6 min). The parameters for MS detection and APCI ionization were as follow: nebulizer gas flow was set to approximately 50 l h\(^{-1}\), and the desolvatation gas flow at 150 l h\(^{-1}\). The APCI probe temperature was set to 500\(^\circ\)C, and the source temperature to 120\(^\circ\)C. The capillary voltage was set at 1.5 kV and cone voltage after optimisation for each carotenoid was set to 25V. The ionization mode was positive. Low mass and high mass resolution were 15 for both mass analyzers, ion energies 1 and 2 were 1V, entrance and exit potential were 50V and detector (multiplier) gain was 650V. Mass spectra of carotenoids were acquired with a scan range of \(m/z\) range of 350-750 amu. The combination of the chromatography retention time (\(t_R\)) and the parent mass was used to selectively monitor the different carotenoids as follow: Antheraxanthin ([M+H\(^+\)] = 586, \(t_R\) = 8.47), violaxanthin ([M+H\(^+\)] = 602, \(t_R\) = 8.13), capsanthin ([M+H\(^+\)] = 586, \(t_R\) = 8.32), capsanthin-5,6 epoxide ([M+H\(^+\)] = 602, \(t_R\) = 7.96), capsorubin ([M+H\(^+\)] = 584, \(t_R\) = 7.81), and 5,6-diepikarpoxanthin([M+H\(^+\)] = 602, \(t_R\) = 7.59). Data acquisition and analysis were
performed with the MassLynx software (ver.4.1) running under Windows XP professional on a Pentium PC. The kinetic parameters were obtained by non-linear regression analysis using the GraphPad Prism 4.0 program (GraphPad Software, La Jolla CA).

Supporting Data

Supplemental Figure S1. Spectral analysis of purified recombinant CCS (reCCS). Absorption (1) and fluorescence (2) of reCCS (2 μM) in 10 mM phosphate buffer (pH 7) containing 15% glycerol and 0.5% Tween 80. Excitation was set at 458 nm.

Supplemental Figure S2. Multiple alignment of deduced amino acid sequence of selected lycopene beta-cyclases (LCYs) and capsanthin-capsorubin synthase (CCS) Capsicum annuum (Ca). Ca LCY (Accession CAA60119), Ca CCS (Accession CAA53759), Synechocystis Sy LCY (Accession CAA52677) and Erwinia herbicola Eh LCY (Accession QO1331) were used. The positions of the first and last amino acids in each sequence are shown. Identical residues are shown on a black background and residues selected for point mutations are indicated by an asterisk.

Supplemental Figure S3. Total ion chromatogram analysis of carotenoid products isolated from the incubation of reCCS with xanthophyll epoxides. Peaks refer to antheraxanthin (substrate) or violaxanthin (substrate) and identified products: capsanthin, 5,6-diepiparoxanthin, capsanthin 5,6 epoxide and capsorubin.

ACKNOWLEDGEMENTS

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LITERATURE CITED


Camara B, Dogbo O, d'Harlingue A, Bardat F (1985) Inhibition of lycopene cyclization by Capsicum chromoplast membranes by 2-aza-2,3-dihydroviolalen. Phytochemistry 24: 2751-2752


FIGURE LEGENDS

Figure 1. SDS-PAGE analysis and spectral analysis of CCS purified from chromoplasts (ChrCCS). A, Chromoplast membranes proteins (MB) and purified ChrCCS fractions (lanes 1-8) eluted after the Superose 6 column were separated by SDS-PAGE and stained with Coomassie blue. Molecular weight markers (PageRuler™ Prestained Protein Ladder, Fermentas) are shown on the left. B, Unstained gel showing the yellow ChrCCS band corresponding to fractions (5-7) from the Superose 6 column shown in A. The SDS concentration in the solubilization buffer was reduced by half and the separation was carried out at 6°C. Molecular weight markers are shown on the left. C, Visible absorption (1) of purified ChrCCS (5.5 μM) in 10 mM phosphate buffer (pH 7)
containing 15% glycerol and 0.5% Tween 80. Fluorescence spectrum (2) of purified ChrCCS (2 μM) in the same buffer. Excitation was set at 458 nm.

**Figure 2.** Analysis of prosthetic flavin bound to purified ChrCCS by UPLC-MS/MS. A From top to bottom, UPLC-MS/MS chromatograms showing the identification of FAD and FMN using multiple reaction monitoring (MRM) with positive electrospray ionization (ESI+). Flavin extract obtained from homogenous ChrCCS separated from SDS-PAGE as described in Figure 1B was used for the identification of FAD (1) using the transition of m/z (785.8 -> 348), t<sub>R</sub>=3.47 and t<sub>R</sub>= 3.63. The absence of FMN (2) in the gel extract was based on the FMN transition (m/z 457- > 439) obtained for FMN standard (3) with the transition of m/z (457- > 439), t<sub>R</sub>=3.66 and t<sub>R</sub>= 3.93, compared to FAD standard with the transition of m/z (785.8- > 348), t<sub>R</sub>=3.47 and t<sub>R</sub>= 3.63. B, UPLC-MS/MS spectra representing the fragmentation pattern determined for FAD and FMN standards using daughter scan to validate the above transitions FAD: transition of m/z (785.8 -> 348), FMN: transition of m/z (457 - 439).

**Figure 3.** Effect of FAD and NADPH on the activity of ChrCCS, reCCS and reLCY-B using different reconstitution conditions. The different holoenzymes were deflavinylated as described under “Materials and Methods” before incubation in a reaction mixture containing 50 μg of protein and appropriate cofactors as indicated. A, For CCS the lycopene cyclase (conversion of lycopene to β-carotene) and the κ-cyclase (conversion of antheraxanthin into capsanthin) activities were determined. Values were normalized with respect to the holo-enzymes or apo-enzymes incubated with the substrate in the presence of FAD and NADPH. The 100% values refer to: ChrCCS (β-carotene: 81 ± 7.2 nmol mg<sup>-1</sup>h<sup>-1</sup>; capsanthin: 232 ± 15.6 nmol mg<sup>-1</sup>h<sup>-1</sup>); Apo-ChrCCS (β-carotene: 67 ± 5.2 nmol mg<sup>-1</sup>h<sup>-1</sup>; capsanthin: 178 ± 11.6 nmol mg<sup>-1</sup>h<sup>-1</sup>); reCCS (β-carotene: 72 ± 6.3 nmol mg<sup>-1</sup>h<sup>-1</sup>; capsanthin: 156 ± 10.4 nmol mg<sup>-1</sup>h<sup>-1</sup>); Apo-reCCS (β-carotene: 40 ± 5.7 nmol mg<sup>-1</sup>h<sup>-1</sup>; capsanthin: 125 ± 15.7 nmol mg<sup>-1</sup>h<sup>-1</sup>); reLCY-B (β-carotene: 115 ± 12.3 nmol mg<sup>-1</sup>h<sup>-1</sup>) and apo-reLCY-B (β-carotene: 77 ± 6.7 nmol mg<sup>-1</sup>h<sup>-1</sup>). Abbreviations refer to purified Chromplast CCS (ChrCCS) and the corresponding apoprotein devoid of FAD (Apo-ChrCCS), recombinant CCS (reCCS) and its apoprotein (Apo-reCCS). B,
Recombinant pepper LCY-B (reLCY-B) and its apoprotein (Apo-reLCY-B) were used to determine the conversion of lycopene into β-carotene. C, UV-visible absorption of purified ChrCCS incubated with 5 mM NADPH under argon atmosphere. Absorption of oxidized (1) ChrCCS (5.5 μM) in 10 mM phosphate buffer (pH 7) containing 15% glycerol, 0.5% Tween 80 and 5 mM NADPH (2).

**Figure 4.** Expression of plant type LCY-Bs. Soluble lysate from cells harboring the control vector and lines expressing wild or mutated plant recombinant LCY-B (reLCY-B) or CCS (reCCS) were used for affinity purification as described in the experimental section. Affinity purified wild and mutant reLCY-B or reCCS were probed with anti-LCY-B or anti-CCS. The arrowheads refer to the signal at approximately 66 kD.

**Figure 5.** Functional characterization of bacterial wild type and mutant LCY-Bs. Following the identification of Glu196 as an active site residue of Erwinia lycopene cyclase a plasmid-based assay was used to test wild-type and mutant Erwinia LCY-B. E. coli JM101 harboring the plasmid pACYC-EBI (chloramphenicol^R) which produces lycopene was used. After transformation of E. coli (JM101-pACYC-EBI (chloramphenicol^R)) with plasmids encoding wild-type or mutant Erwinia LCY-Bs and induction with 0.5 mM isopropyl-β-thiogalactopyranoside, total carotenoids were extracted from the bacterial pellets and analyzed by HPLC as described in the experimental section. Data shown represent the mean ± SD for 2 independent experiments.

**Figure 6.** HPLC analysis of κ-cyclic carotenoids synthesized by reCCS from xanthophylls epoxides. A, antheraxanthin conversion into κ-cyclic and 3,5,6-trihydroxy xanthophyll by reCCS. reCCS was incubated with antheraxanthin as described in the experimental section and products were analyzed by HPLC and on-line diode array detection. Peaks refer to 1, 5,6-diepipipoxanthin; 2, capsanthin; 3, antheraxanthin. B, violaxanthin conversion into κ-cyclic and 3,5,6-trihydroxy xanthophyll by reCCS. reCCS was incubated with violaxanthin and products were analyzed as described in A. Peaks
refer to 4, putative 5,6-diepilatoxanthin; 5, capsorubin; 6, capsanthin-5,6-epoxide; 7, violaxanthin.

**Figure 7.** Molecular diversity of carotenoids generated by CCS. A, Flexible stabilization of incipient carbocation generated by CCS. B, Possible pathways of carotenoids produced by CCS during the formation of κ-cyclic carotenoids.
Table I. Kinetic parameters of recombinant wild type and mutant lycopene cyclase (reLCY-B)

The values shown are mean ± SE for 3 determinations. ND, not determined.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$V_{\text{max}}$ (nmol mg$^{-1}$ h$^{-1}$)</th>
<th>$K_{\text{m}}$ (μM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
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<tr>
<td>Wild type</td>
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<tr>
<td>D259A</td>
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<td>4.2 ± 0.02</td>
<td>25.0 ± 2</td>
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<tr>
<td>E295A</td>
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<td>0</td>
</tr>
<tr>
<td>E295K</td>
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</tr>
<tr>
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Table II. *Kinetic parameters of recombinant wild type and mutant capsanthin-capsorubin synthase (reCCS)*

The values shown are mean ± SE for 3 determinations. ND, not determined.

<table>
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<th>Enzyme</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>Relative activity</th>
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</thead>
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<td></td>
<td>nmol mg (^{-1}) h (^{-1})</td>
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<td>%</td>
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<td>E296R</td>
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<tr>
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<td>H360R</td>
<td>22.0 ± 2</td>
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</table>
Figure 1. SDS-PAGE analysis and spectral analysis of CCS purified from chromoplasts (ChrCCS). A, Chromoplast membranes proteins (MB) and purified ChrCCS fraction (lanes 1-8) eluted after the Superose 6 column were separated by SDS-PAGE and stained with Coomassie blue. Molecular weight markers (PageRuler™ Prestained Protein Ladder, Fermentas) are shown on the left. B, Unstained gel showing the yellow ChrCCS band corresponding to fractions (5 -7) from the Superose 6 column shown in A. The SDS concentration in the solubilization buffer was reduced by half and the separation was carried out at 6°C. Molecular weight markers are shown on the left. C, Visible absorption (1) of purified ChrCCS (5.5 µM) in 10 mM phosphate buffer (pH7) containing 15% glycerol. Fluorescence spectrum (2) of purified ChrCCS (2 µM) in 10 mM phosphate buffer (pH7) containing 15% glycerol and 0.5% Tween 80. Fluorescence spectrum (2) of purified ChrCCS (2 µM) in the same buffer. Excitation was set at 458 nm.
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Figure 4. Expression of plant type LCY-Bs. Soluble lysate from cells harboring the control vector and lines expressing wild or mutated plant recombinant LCY-B (reLCY-B) or CCS (reCCS) were used for affinity purification as described in the experimental section. Affinity purified wild and mutant reLCY-B or reCCS were probed with anti-LCY-B or anti-CCS. The arrowheads refer to the signal at approximately 66 kD.
**Figure 5.** Functional characterization of bacterial wild type and mutant LCY-Bs. Following the identification of Glu_{196} as an active site residue of *Erwinia* lycopene cyclase a plasmid-based assay was used to test wild-type and mutant *Erwinia* LCY-B. *E. coli* JM101 harboring the plasmid pACYC-EBI (chloramphenicol\(^R\)) which produces lycopene was used. After transformation of *E. coli* (JM101- pACYC-EBI (chloramphenicol\(^R\))) with plasmids encoding wild-type or mutant *Erwinia* LCY-Bs and induction with 0.5 mM isopropyl-\(\beta\)-thiogalactopyranoside, total carotenoids were extracted from the bacterial pellets and analyzed by HPLC as described in the experimental section. Data shown represent the mean ± SD for 2 independent experiments.
Figure 6. HPLC analysis of κ-cyclic carotenoids synthesized by reCCS from xanthophylls epoxides. A, Antheraxanthin conversion into κ-cyclic and 3,5,6-trihydroxy xanthophyll by reCCS. reCCS was incubated with antheraxanthin as described in the experimental section and products were analyzed by HPLC and on-line diode array detection. Peaks refer to 1, 5,6-diepikarpoxanthin; 2, capsanthin; 3, antheraxanthin. B, Violaxanthin conversion into κ-cyclic and 3,5,6-trihydroxy xanthophyll by reCCS. reCCS was incubated with violaxanthin and products were analyzed as described in A. Peaks refer to 4, putative 5,6-diepipilaxanthin; 5, capsorubin; 6, capsanthin-5,6-epoxide; 7, violaxanthin.
Figure 7. Molecular diversity of carotenoids generated by CCS. A, Flexible stabilization of incipient carbocation generated by CCS. B, Possible pathways of carotenoids produced by CCS during the formation of κ-cyclic carotenoids.