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Review

Carotenoid biosynthesis in microorganisms and plants

Gerhard SANDMANN

Botanisches Institut, FB Biologie, J. W. Goethe Universität, Frankfurt, Germany

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The chemical structures of α- and β-carotene were elucidated about 60 years ago. Then in the middle of this century, ^{14}C-labelling experiments revealed the origin of the carbon atoms of the C_{40} skeleton. This knowledge led consequently to the establishment of the biosynthetic pathway of carotenoids by 1970 (see [1] for a compilation of various aspects of carotenoids). The following two decades were the period in which cell-free systems were developed for investigations on the reaction sequence and for enzymic studies. An excellent historical survey about this period was given by Goodwin [2]. The most comprehensive and most detailed review article was written by Spurgeon and Porter [3] covering all relevant aspects of carotenoid biosynthesis. The information presented there was up-dated in the following years in several subsequent articles (e.g. [4, 5]).

Molecular genetics was introduced by Marrs and coworkers [6, 7] and culminated in the sequencing of the first genes encoding carotenogenic enzymes in 1989 from *Rhodobacter* [8]. This development opened new perspectives for the work on the carotenoid biosynthetic pathway introducing new experimental approaches. Very soon it was evident that the availability of carotenogenic genes had a strong impact on the biochemical investigations and considerable progress was made in the analysis of catalytic reactions and in the purification and characterization of carotenogenic enzymes. The beginning of this development has already been reviewed [9].

The present review will focus on the enormous progress made recently which became possible by a combination of biochemical techniques with molecular genetics. The survey starts with a chapter on *in vitro* carotenogenic systems and describes then the different cloning techniques successfully employed for new carotenogenic genes. Finally, this article will focus on the biochemistry and molecular genetics of the biosynthetic reactions involved in carotenoid formation.

CAROTENOGENIC *IN VITRO* SYSTEMS

The first step towards the biochemical investigation of the carotenoid biosynthetic pathway was the development of *in vitro* systems. Carotenogenic organisms of choice can be found among heterotrophic bacteria and fungi where some species possess this biosynthetic capacity or among photosynthetic prokaryotes and eukaryotes. In the photosynthetic lower and higher plants carotenogenesis is obligatory for their photosynthetic activity. The most universal carotenoid biosynthetic pathway is the sequence leading to the formation of β-carotene which can be found in all the groups mentioned above. The initial reaction yielding phytoene, the first carotene of the pathway, is the condensation of two molecules of geranylgeranyl diphosphate (GG*PP*) as an intermediate (Fig. 1). The further conversion to β-carotene involves four desaturation and two cyclization steps. The formation of the double bonds proceeds in succession, alternately to the left and to the right of the central phytoene chromophore as indicated. Finally, both ends of the maximally desaturated lycopene molecule are cyclized yielding β-carotene with two β-ionone rings. In green algae and higher plants α-carotene carrying a β- and an ε-ionone ring is formed simultaneously from lycopene. Xanthophylls are derived from α- and β-carotene by introduction of oxygen groups. The carotenoid biosynthesis pathway in *Rhodobacter* branches off at the stage of neurosporene [10]. It will be dealt with in an extra chapter.

For carotenogenic assays active preparations and radioactive labelled substrates are needed. Many important intermediates of specific carotenogenic reactions are not commercially available as radioactive compounds. In most cases the sources were of biological origin and resulted from feeding

Correspondence to Prof. G. Sandmann, Botanisches Institut, J. W. Goethe Universität, P. O. Box 111932, D-60054 Frankfurt/M., Germany

Abbreviations. DMA*PP*, dimethylallyl diphosphate; F*PP*, farnesyl diphosphate; G*PP*, geranyl diphosphate; GG*PP*, geranylgeranyl diphosphate; I*PP*, isopentenyl diphosphate; MVA, mevalonic acid; PP*PP*, prephytoene diphosphate.

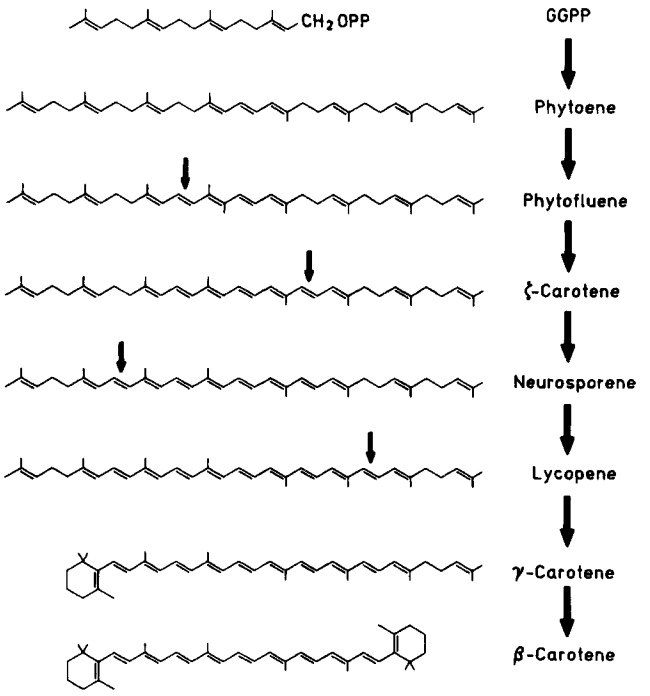


Fig. 1. Biosynthetic formation of cyclic α- and β-carotene from geranylgeranyl diphosphate (GG*PP*).

commercially available [^{14}C]mevalonic acid (MVA) to carotenogenic organisms or from conversion of this compound as well as [^{14}C]isopentenyl diphosphate (I*PP*) by a suitable enzymically active preparation. Cultures of the fungus *Phycomyces* [11] or of *Flavobacterium* [12] were used to synthesize β-[^{14}C]carotene or [^{14}C]zeaxanthin, respectively, from radioactive labelled MVA. Both organisms are also suitable to synthesize both end products *in vitro*.

The *Phycomyces in vitro* system was first introduced by Lee and Chichester [13] and was optimized in the following years [14]. Many carotenogenic *in vitro* systems do not pass the stage of phytoene but cell-free preparations especially from fungi show very good conversion of MVA all along the pathway to β-carotene. Besides *Phycomyces*, *in vitro* systems from other fungi like *Gibberella* (= *Fusarium*) [15] and *Aspergillus* [16] exhibited good carotenogenic activity, whereas with the fungus *Neurospora crassa* reasonable incorporation of radioactivity from [^{14}C]I*PP* in fully desaturated lycopene and further on to γ-carotene was achieved only recently [17]. Several mutants of *Phycomyces* are available which accumulate either phytoene or lycopene [18] and which are also active *in vitro* [19], those strains were employed to synthesize both carotenes radioactive labelled for use as substrates in cell-free assays of corresponding enzymes [20, 21]. In a similar way a GG*PP*-accumulating mutant of *Fusarium* was used as a source of radioactively labelled GG*PP* [22]. The *in vitro* system developed from the *Flavobacterium* should be mentioned as the only other non-fungal one which is able to convert [^{14}C]MVA to the end product of the biosynthetic chain which is zeaxanthin [23].

Good higher-plant carotenogenic systems were obtained from chromoplasts rather than from chloroplasts. The earliest enzymic system was developed from preparations of ripe tomato fruits [24, 25]. It carries out the desaturation of [^{14}C]phytoene to lycopene and cyclic carotenes. The carotenogenic potential of tomato chromoplasts was again demonstrated recently with fruit from transgenic plants [26].

Extensive studies have been carried out with chromoplasts from petals of daffodil (*Narcissus pseudonarcissus*) flowers [27] and red peppers (*Capsicum annuum*) plastids [28]. Both the pepper and the daffodil chromoplasts can utilize [^{14}C]I*PP* as substrate and convert it via phytoene and colored acyclic carotenes into β-carotene [29, 30]. The latter *in vitro* system was also capable of converting xanthophylls. Radioactivity from antheraxanthin and violaxanthin was incorporated into capsaxanthin and capsorubin.

Although carotenoids are very abundant in photosynthetic membranes, development of *in vitro* systems with chloroplasts was found to be very difficult. Incorporation of [^{14}C]MVA into phytoene has already been achieved with isolated chloroplasts in 1969 [31], but since then the chloroplast system was not very much improved in a way that phytoene was efficiently desaturated. Promising cell-free systems for study of carotenogenesis in organisms with oxygenic photosynthesis are thylakoid membranes from the unicellular cyanobacterium *Synechocystis* (= *Aphanocapsa*) and *Synechococcus* (= *Anacystis*) [32, 33]. The earliest substrate which is chanelled into the carotenogenic pathway *in vitro* is [^{14}C]GG*PP*, but [^{14}C]phytoene is also efficiently converted. In this case, radioactivity of the latter substrate was followed through the pools of phytofluene, lycopene, β-carotene, further on to β-cryptoxanthin and other xanthophylls like zeaxanthin [20, 34].

When the lipophilic carotenes or xanthophylls were used as substrates for further conversion, the problem of solubilization and application to the aqueous reaction mixture arises. One way of introducing carotenes was by evaporation of an acetone solution, dispersion in detergents and mixing with the reaction mixture [35]. Direct addition of carotenes dissolved in acetone was only successful in the daffodil *in vitro* system [36]. A very successful approach was the development of a coupled *in vitro* system [20]. Cell extracts from the fungus *Phycomyces* generated either phytoene, lycopene, or β-carotene from [^{14}C]MVA. These intermediates were efficiently converted by carotenogenic membranes (e.g. from *Synechocystis*) in which the subsequent reaction steps were investigated. The two simultaneously operating *in vitro* systems, one generating the substrate and the other carrying out the investigated enzymic reaction, allow for a very good substrate accessibility. The versatility of this coupled system was demonstrated recently with membranes isolated from *Escherichia coli* cells which were transformed with different carotenogenic genes from *Erwinia herbicola*. For the assays of three distinct enzymes, phytoene desaturase, lycopene cyclase, and β-carotene hydroxylase resulting from heterologous expression, different *Phycomyces* mutants generated the desired substrates *in vitro* from [^{14}C]MVA in the coupled system [37].

Finally, the purification of the reaction products and detection of accumulated radioactivity should be addressed. For the early *in vitro* systems alumina column chromatography and thin-layer chromatography (TLC) was used to achieve constant specific radioactivity of the reaction products. In general, a combination of both procedures [14] or the use of two subsequent TLC systems with different separation characteristics [38] were sufficient. Then radioactivity was determined by liquid scintillation counting. Since the introduction of high-pressure liquid chromatography (HPLC) as a standard procedure for the separation of carotenoids (see [39] for review) separation is much easier and more refined. Very recently, several HPLC systems were developed specifically for all the different enzymic reactions from GG*PP* synthesis

to hydroxylation of β-carotene [40]. Radioactivity was determined continuously on-line from the eluent. The chromatographic conditions were appropriate for separation of the substrates from the products formed and to achieve radiochemical purity of these carotenoids.

The *in vitro* systems described in this section were used to characterize the enzymic reaction to some extent at a time when none of these enzymes was purified. Moreover, cell-free reactions were essential to develop the appropriate assays needed to monitor the activity during purification of carotenogenic enzymes. Recent progress in this field will be dealt with in detail in the following chapters.

STRATEGIES FOR THE CLONING OF CAROTENOGENIC GENES

Genes of many biosynthetic pathways were cloned after biochemical investigations provided the tools like antisera for the screening of cDNA libraries or information on the gene via the protein sequence. However, when the first carotenogenic genes were cloned this help was not available. Therefore, other strategies had to be followed. It is not surprising that the first carotenogenic genes were cloned from bacteria. Besides the comparably simple genetics of prokaryotes, it was found that these genes were clustered in operons which facilitated the screening.

For *Rhodobacter capsulatus*, analysis of the genome revealed a tight cluster of several genes involved in carotenoid biosynthesis more than 15 years ago [6]. A photosynthetic gene cluster was mobilized from the *R. capsulatus* chromosome in a conjugative plasmid and identified by complementation after transfer in non-photosynthetic mutants [7]. The resulting pRPS404 contained as a 46-kb insert a photosynthetic gene cluster which included all the carotenoid (*crt*) genes. Functional mapping of the genes on pRS404 was accomplished with mutants in which the *crt* genes were inactivated and subsequent analysis of their accumulated intermediates of the carotenoid biosynthetic pathway [41, 42]. It was found that eight *crt* genes are closely clustered together in an 11-kb region whereas another gene, *crtJ*, is separated by about 12 kb. Nucleotide sequences of all the *R. capsulatus* *crt* genes except *crtJ* were determined and four distinct operons were detected [8]. Parallel work on *R. spheroides* led to the cloning of a cosmid carrying 60 kb of the central section of the photosynthetic region [43]. It contained a *crt* cluster on which six of the *crt* genes present in *R. capsulatus* were found in the same arrangement [44]. One of them, *crtD*, was recently sequenced [45].

The *crt* gene cluster from *Erwinia herbicola* was cloned randomly [46] at a time when the yellow pigments of this enterobacterium were not yet identified. Upon transformation of *E. coli* with large DNA fragments in a cosmid vector, yellow transformants were obtained. Plasmid pPL376 with a DNA insert of 15.6 kb contained all the information for synthesis of the yellow pigment. Subsequent investigations have shown that *E. herbicola* as well as *E. coli* carrying pPL376 were able to synthesize zeaxanthin and zeaxanthin glucosides via phytoene, lycopene, γ-carotene, and β-carotene [47]. The glucosides of this species [48] and of *E. uredovora* [49] have been characterized in detail. After transformation of *E. coli* with the *crt* cluster carrying deletion mutations in all genes followed by carotenoid analysis and enzymic assays of corresponding enzymes a total of six carotenoid biosynthesis genes could be mapped and functionally assigned [50, 51]. Simultaneously to this work with *E. herbicola*, Misawa et al. [52] cloned a carotenogenic gene cluster from *E. uredovora* in a similar way, analyzed the function of these genes, and sequenced them. The same work has recently been carried out with the *crt* genes from another *E. herbicola* strain, Eho13 [53]. Gene fusion experiments revealed that two regions of this cluster contain promoter activity, and two transcription start sites were identified by primer-extension analysis.

Phytoene desaturase from cyanobacteria, algae and higher plants is inhibited by bleaching herbicides like norflurazon [54]. A mutant of the unicellular cyanobacterium *Synechococcus* with a phytoene desaturase resistant against this herbicide [55] was used to clone the *pds* gene coding for phytoene desaturase [56]. A library from mutant DNA was used to transfect wild-type *Synechococcus* which were then selected for herbicide resistance. The *pds* gene integrated into the genome of a resistant transformant was recovered by a special plasmid rescue technique. After the sequence of the cyanobacterial gene was available [57], *pds* was used to clone the same gene from soybean [58] and also the tomato gene by hybridization after a *pds* homolog was first isolated from *Dunaliella* [59]. Another *pds* gene was cloned from a norflurazon-resistant *Synechocystis* with principally the same technique described above [60]. In both cyanobacteria the genes encoding phytoene synthase *psy* were found downstream of *pds* [61, 62].

In *Neurospora crassa* the phytoene desaturase gene *albino(al)-1* was cloned not directly but by isolating first a well selectable gene with a map position in the genome nearby the *al-1* gene. In the following chromosome walking procedure the *hom* gene for homoserine requirement was used as a starting point to progress along the chromosome to *al-1* [63]. A different approach was followed to isolate another albino gene, *al-3*, from the same fungus. It was detected by its ability to complement an *al-3* mutation to the wild-type orange phenotype by reestablishment of carotenoid synthesis [64]. The sequence and the biochemical characterization after expression in *E. coli* confirmed that *al-3* codes for GG*PP* synthase [65, 66].

In contrast to this homologous complementation of a deletion mutation, in the same organism the gene *zds* encoding ζ-carotene desaturase was cloned by heterologous complementation in *E. coli* which lacks the potential to synthesize carotenoids. Cells of *E. coli* were first transformed with a plasmid which carried all genes necessary for the synthesis of the substrate ζ-carotene. Then this transformant was used to screen an *Anabaena* library. The positive clone was selected by its color change from yellow to red due to lycopene synthesis catalyzed by the product of the new gene [67]. The sequence was published recently [68]. From *Thermus thermophilus* the *crtB* was isolated as a gene for a rate-limiting carotenogenic enzyme by self-cloning using a 1−3-kDa library in a multicopy plasmid [69]. Screening of transformants was carried out by searching for colonies with a stronger pigmentation.

Only very recently, cDNAs of carotenogenic genes were isolated by use of a biochemical approach. A *Capsicum annuum* fruit cDNA library was screened with antisera raised against purified GG*PP* synthase as well as phytoene desaturase [70, 71]. Both cDNA clones were obtained in this way, expressed and sequenced. Several cDNA clones from a mRNA which accumulated during tomato fruit ripening were obtained after differential hybridization for genes specifically expressed during this process. One of them, pTOM5 [72],

Table 1. Cloned and sequenced genes involved in the carotenogenic pathway leading to cyclic carotenoids. Note that *psy* represents the bacterial/fungal type of phytoene synthase, while *crtI* represents the cyanobacterial/algal/higher plant type of phytoene desaturase. In [50] the *E. herbicola* genes *pds*, *crtY* and *crtX* were also assigned as *crtZ*, *crtH*, and *crtG*, respectively.

Genes	Enzyme	Source
crtE/al-3	GG*PP* synthase	*Rhodobacter capsulatus* [8], *Erwinia uredovora* [52], *E. herbicola* [53, 86], *Neurospora crassa* [65], *Capsicum annuum* [70], *Methanobacter thermoformicicum* [84]
crtB/psy	phytoene synthase	*R. capsulatus* [8], *E. uredovora* [52], *E. herbicola* [53, 86], *Synechococcus* [61], *Synechocystis* [62], tomato [72], *Thermus thermophilus* [69], *Capsicum* [100]
crtI/al-1 carC	phytoene desaturase	*R. capsulatus* [8], *E. uredovora* [52], *E. herbicola* [53, 86], *N. crassa* [64], *Myxococcus xanthus* [75]
pds	phytoene desaturase	*Synechococcus* [56], *Synechocystis* [60], soybean [58], tomato [59], *C. annuum* [71]
zds	ζ-carotene desaturase	*Anabaena* [68]
crtY	lycopene cyclase	*E. uredovora* [52], *E. herbicola* [53]
crtZ	β-carotene hydroxylase	*E. uredovora* [52]
crtX	zeaxanthin glucosilase	*E. uredovora* [52], *E. herbicola* [53]

was later identified to code for phytoene synthase by functional complementation, *in vitro* enzymic analysis and by an antisense RNA approach [26, 73]. It was used to clone and sequence the complete gene from tomato [74].

Some of the carotenogenic genes have been cloned directly by transposon tagging and subsequent rescue or localization with the transposon DNA. In the case of several structural and regulatory carotenoid genes of *Myxococcus xanthus*, transposon insertion was used for the first time for cloning which has already led to the sequence of phytoene desaturase [75]. This powerful strategy may be the method of choice for future work to identify especially higher-plant structural and also regulatory genes of the carotenoid biosynthetic pathway. A successful attempt was already made with the cloning of the gene *y1* from maize [76]. This gene is thought to be involved in the regulation of carotenogenesis. All the genes and cDNAs coding for any structural gene involved in the carotenogenic biosynthetic pathway which were sequenced and functionally identified to date are listed in Table 1. It should be mentioned that at the present time an attempt is being made by Dr J. Hirschberg (Jerusalem) to unify the naming of the cyanobacterial carotenoid genes in order to follow the nomenclature convention for bacteria. This means that the cyanobactarial genes may be renamed as *crt* genes. The following changes were proposed: *crtP* for *pds*, *crtQ* for *zds*, and *crtL* for *lyc*. In the present review the original names used to date in the literature will be used.

ENZYMOLOGY AND MOLECULAR GENETICS OF THE REACTIONS LEADING TO CYCLIC CAROTENOIDS

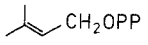
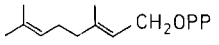
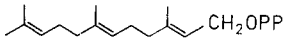
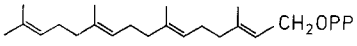
The entire carotenoid biosynthetic pathway is part of the terpenoid metabolism with formation of prenyl diphosphates as a common sequence for chain elongations. From the diffent prenyl diphosphates formed, specific routes branch off into various terpenoid end products. The question is: where does the carotenoid pathway divert? Clearly, the dimerization of GG*PP* leads to phytoene as the first carotene (Fig. 1). However, there are several indications that carotenoid biosynthesis relies on its independent synthesis of GG*PP*. For example, the *crtE* gene necessary for GG*PP* synthesis is part of the carotenogenic gene cluster in all bacteria investigated to date [8, 50, 52, 53]. Furthermore, during fruit ripening, which is accompanied by massive carotenoid formation in *Capsicum*, expression of the GG*PP* synthase gene is strongly enhanced [70]. Consequently, the start of the specific biosynthetic pathway of carotenoids can be considered to occur with the synthesis of GG*PP*. Therefore, the following chapter will start with a comparison of the properties of the different GG*PP* synthases.

Synthesis of GG*PP*

GG*PP* is synthesized by 1′-4 condensation(s) between isopentenyl diphosphate (I*PP*) molecules and an allylic diphosphate. As in all other prenyl transferase reactions, the mechanism of this head-to-tail joining is basically the same. The carbon bond is formed between the C4 of I*PP* and the C1 of the allylic cosubstrate. Simultaneously, inorganic diphosphate is liberated from the allylic partner. This condensation reaction involves an allylic carbonium anion as an intermediate which then attacks the 3,4-double bond of I*PP*. Further details on this mechanism and general information on prenyl transferases are given in a review by Poulter and Rilling [77].

GG*PP* synthases catalyzing this reaction have been shown to belong to a family of enzymes with different allylic substrate and chain length specificity. Several types of this enzymes were isolated from carotenogenic and non-carotenogenic bacteria, fungi, plants and animal tissue, e. g. from tomato [78], *Capsicum* [79], *Phycomyces* [80], and *Sinapis* [81], as well as from yeast [82] and rat liver [83]. Some of these enzymes exhibit either affinities for several prenyl diphosphates or are monospecific for farnesyl diphosphate like the GG*PP* synthase from *Phycomyces* [80] and from rat [83]. In contrast, the enzymes from yeast [81] and from *Methanobacterium thermoformicium* [84] show no preference and convert the C_5 dimethylallyl diphospate (DMA*PP*), C_{10} geranyl diphosphate (G*PP*), and C_{15} farnesyl diphosphate (F*PP*) equally well [82]. GG*PP* synthase was purified from *Capsicum* chromoplasts and characterized in detail [79]. It has a dimeric structure with two 37-kDa subunits. As indicated by the K_m values, all prenyl diphosphates from C_5 to C_{15} are equally good substrates. Therefore, DMA*PP* may be considered to be the genuine substrate for GG*PP* synthase in higher-plant plastids. The same results were obtained with purified synthases from *M. thermoformicium* [84] and in case of *N. crassa* from complementation experiment with the *al-3* gene from this fungus [65]. An *E. coli* strain containing a heat-labile F*PP* synthase was used as the recipient host. By raising the temperature to 42 °C, *in vitro* formation of F*PP* was strongly inhibited but synthesis of GG*PP* from I*PP* was

Table 2. Substrate specificity and prenyltransferase steps of various geranylgeranyl diphosphate synthases. Arrows indicate the reaction sequence.

Substrate	Action by GG*PP* synthase from		
	Capsicum [79] yeast [82] *Methanobacterium* [84, 94]	*Erwinia [93]* *Micrococcus* [93]	rat [83] *Phycomyces* [80]
 DMAPP	yes	no	no
 GPP	yes	yes	no
 FPP	yes	yes	yes
 GGPP	yes	yes	yes

unaffected. All the different substrate specificities of the GG*PP*-synthesizing enzyme are listed in Table 2.

There was some uncertainity on the nature of the gene encoding GG*PP* synthase for some time. Mutants in which carotenogenic genes were inactivated leading to inhibition of the formation of any carotenoid were investigated for *in vitro* GG*PP* synthesis [85, 86]. The results obtained and the conclusions drawn from these experiments were misleading. The main problem with this approach was that a mutation of the GG*PP* synthase gene can only be selected if it is leaky. Especially in *Neurospora* tight mutations are lethal [64]. Consquently, the residual GG*PP* synthase activity was measured which led to incorrect interpretations. After the availability of appropriate genes, complementation studies in *E. coli* and *in vitro* analysis of expressed *crtE* and *al-3* genes clearly demonstrated that these very similar genes code for GG*PP* synthase [51, 66]. This assignment of *crtE* was confirmed in another publication [87]. Genes encoding GG*PP* synthase have been cloned and sequenced from *R. capsulatus* [8], three *Erwinia* species [52, 53, 88], *N. crassa* [65], and *C. annuum* [70]. The *N. crassa* GG*PP* synthase gene reveals three domains highly conserved in the *Capsicum* enzyme and other prenyl transferases. The relative position of the conserved regions are the same in these enzymes and are localized at similar distances. There is some evidence that these regions in the polypeptide represent the catalytic sites of prenyl transferases [65]. Comparison between the GG*PP* synthase gene from *Erwinia uredovora* and the others showed an identity of the peptide sequences around 30%. It should also be mentioned that the expressed product of *ORF323* from *Cyanophora paradoxa* which exhibits some similarity to *crt*E [89] shows no GG*PP* synthase activity and fails to complement *crtE* (Sandmann, unpublished observation). Most likely, *ORF323* codes for a prenyl transferase which synthesizes a product with a much longer chain length than C_{20}. As in some other microorganisms, the carotenogenic pathway is regulated by light in *N. crassa* [90]. Increased activities in illuminated cultures were found for three enzymes: phytoene desaturase, phytoene synthase, and also GG*PP* synthase [17]. In the case of the latter enzyme, this photoregulation occurs at the transcriptional level and via instability of its mRNA [91].

The *crtE* gene from *E. uredovora* [51] and the cDNA from *Capsicum* [70] were used for heterologous expression and demonstration of GG*PP* synthase activity in *E. coli*. After purification of the *Erwinia* enzyme, reaction rates and K_m values indicated that G*PP* as well as F*PP* were the genuine allylic substrates for this GG*PP* synthase but not DMA*PP* [92]. The enzymes from *Micrococcus* [93] exhibited the same exclusive preference for the C_{10} and C_{15} substrates as does the *Erwinia* GG*PP* synthase. In contrast, the enzyme from *Methanobacterium thermoautotrophicum* is a prenyl transferase specific also for DMA*PP* but with the highest affinity for F*PP* [94, 95]. Independent of the allylic substrates employed, GG*PP* was the only product of the enzymic reactions in all these bacterial GG*PP* synthases.

Phytoene synthesis

The colorless carotene phytoene is a C_{40} hydrocarbon with only three conjugated double bonds. In most cases, it is formed as the 15-*cis* isomer [96]. Although it is found only in trace amounts in organisms with a functional carotenogenic pathway, this carotene accumulated in mutants or in the presence of inhibitors which affect its conversion. Enzymic studies with the purified phytoene synthase from *Capsicum* [97] as well as *E. coli* transformed with the corresponding gene [51] demonstrated that a single gene product catalyzes the condensation of GG*PP* to phytoene with prephytoene diphosphate (PP*PP*) as an intermediate (Fig. 2). Based on the analogy to the formation of presqualene diphosphate, another cyclopropylcarbinyl diphosphate, and its conversion, a general mechanism for the synthesis of phytoene has been proposed [98, 99]. Two molecules of GG*PP* are joined by a 1′-2,3 condensation reaction with the loss of one hydrogen together with the diphosphate group from C-1′ of the same

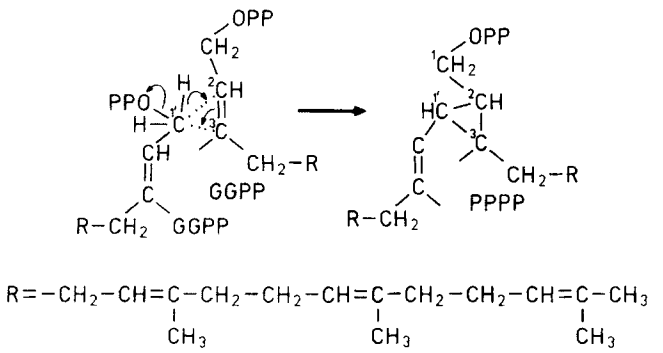


Fig. 2. Mechanism of prephytoene diphosphate (PPPP) formation as an intermediate step of phytoene synthesis.

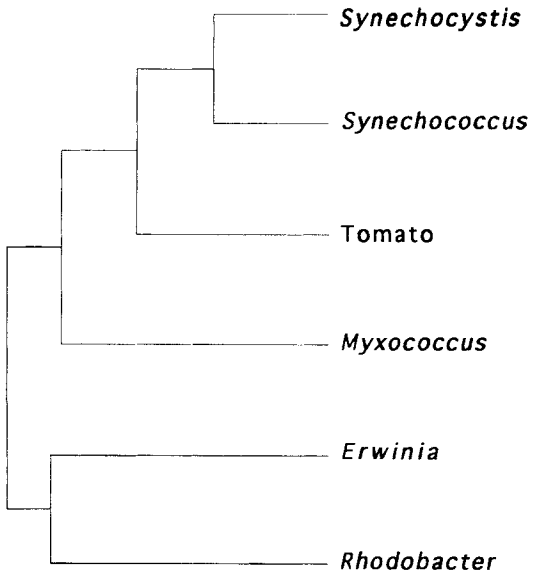


Fig. 3. Similarity dendogram for phytoene synthases based on their protein sequences.

GGPP molecule. Conversion of the resulting PPPP involves the cleavage of its C-1 diphosphate group and the formation of a cyclopropinylcarbinyl cation which then undergoes a 1'-1 rearrangement via a cyclobutyl cation. Depending on the stereochemistry of the subsequent hydrogen abstraction, 15-*cis* or all-*trans* phytoene is finally formed. The different intermediate steps in phytoene synthesis are illustrated in [3].

In vitro studies involving phytoene synthesis were carried out with crude preparations from different sources [5]. The results, however, were rather ambiguous. The only phytoene synthase purified to date originated from *Capsicum* chromoplasts [97]. The main purification step was affinity chromatography using an analogue of GGPP. The protein obtained had a molecular mass of 47.5 kDa and can convert either GGPP or PPPP as substrates with high affinity. The enzyme is dependent on Mn^{2+} and is inhibited by inorganic phosphate. Immunochemical localization of phytoene synthase revealed its presence in the stroma of chromoplasts as well as chloroplasts [79].

There is a group of genes which all show considerable similarity to *crtB* first described from *Rhodobacter* [8]. These are *crtB* from two *Erwinia* species [52, 88], and *Thermus thermophilus* [69] *psy* from two cyanobacteria [61, 62] and the cDNAs pTom5 from tomato [74] and *Capsicum* [100]. Assignment of the function of all these genes to code for phytoene synthase was performed by different approaches, recently. The *crtB* gene from *E. uredovora* was introduced into *Agrobacterium tumefaciens* [51]. The resulting transformants showed phytoene synthase activity. Enzyme extracts converted GGPP into phytoene. This result was further supported by demonstration of 15-*cis* phytoene accumulation in *E. coli* harboring the *crt*E gene for GGPP synthase together with *crtB*. Furthermore, coexpression of *crtE* with the homologous *psy* gene from *Synechococcus* also resulted in phytoene formation in *E. coli* [61]. A different approach was followed to identify the function of the homologous pTOM5 [72] *in situ*. Transgenic tomato plants with a pTOM5 antisense gene construct were used to down-regulate translation of its gene product [101]. Tomato fruit with a yellow phenotype were obtained containing reduced levels of pTOM5 RNA and also of lycopene. *In vitro* analysis of isolated plastids from these fruit demonstrated that conversion of GGPP to phytoene was impaired [26]. All these results are consistent with earlier observations that, in a *crtB* mutant of *R. capsulatus*, *in vitro* conversion of IPP is blocked at the level of GGPP [86]. Due to the problems with *in vitro* analysis of *crt*E mutants performed in parallel, as discussed in the previous chapter, a wrong conclusion was drawn for the function of *crtB*.

Significant similarity among the *Ewinia* and *Rhodobacter crtB* gene products is in the range of 30% identity [52, 88]. A similar value is found between the bacterial and the *Synechococcus* enzymes [61]. The latter protein resembles very much the tomato phytoene synthase with 59% identity. The alignments in Fig. 3 show the protein relationship of the different phytoene synthases, demonstrating that the enzymes from organisms with oxygenic photosynthesis are close together. Surprisingly, the phytoene synthase from *Myxococcus* (EMBL Accession no. Z21955) has a better link to this group than to the proteins from the other bacteria. Comparison of deduced amino acids from all phytoene synthase genes reveals three highly conserved boxes. The one most downstream of these conserved regions contains a sequence of 30 charged amino acids which also shows similarity to various prenyl transferases which catalyze condensation reaction with allylic diphosphates as substrates. Therefore, it was proposed that this region in phytoene synthase is involved in binding of the substrate GGPP [61]. Comparison of the sequences of eukaryotic and prokaryotic genes revealed that, for example, the tomato cDNA codes for a larger polypeptide than the genes from the prokaryotic organisms. This extension consists of more than 100 amino acids. Mendelian inheritance of various mutations affecting carotenogenesis in higher-plant plastids indicated that carotenogenic genes are nuclear encoded and synthesized on 80S ribosomes [102]. Therefore, the N-terminal leader sequence can be regarded as a signal peptide for import into the plastid. Import of the expressed phytoene synthase from tomato into chloroplasts was accompanied by processing of the polypeptide to a shorter protein [73].

Recent work has concentrated on the organ-specific regulation of phytoene synthesis in tomato. Analysis of the pTOM5 antisense plants showed that the pTOM5 gene product is involved in carotene biosynthesis only of flowers and ripening fruit but not of green plastids of fruit and leaves [26]. After the discovery of two different loci on the tomato genome with sequence similarity to pTOM5 [103], two different genomic sequences were cloned, GTOM5 and clone

F [72], which were attributed to the genes *psy1* and *psy2*, respectively [104]. Both genomic clones differ in the number of exons related to the pTOM5 sequence and in the promoter sequence. The gene *psy1* was expressed during tomato fruit ripening [72]. Using reverse transcriptase PCR, *psy2* expression could be detected mainly in green tissue [104]. Transcription of the *psy1* gene increased more than 20-fold during fruit ripening and more than 10-fold during flower development [105]. Complementation of carotene synthesis in the yellow flesh tomato mutant affected at the r locus on chromosome 3 was achieved by over-expression of pTOM5 [106]. This indicates that the r mutation is caused by lesions of the *psy1* phytoene synthase gene which is expressed during fruit ripening. In light-grown seedlings the transcript levels of both phytoene synthase genes were severalfold higher than in etiolated tissue [104].

Desaturation sequence of phytoene to lycopene

Four desaturation steps are performed in the conversion of phytoene to the maximally desaturated lycopene (Fig. 1). For many years there was a debate on how many enzymes are involved. One opinion was that two distinct enzymes are necessary for the four-step desaturation process to lycopene. The first enzyme was thought to utilize each half of the symmetrical phytoene molecule as a substrate and to yield ζ-carotene with phytofluene as an intermediate. The second desaturase was supposed to carry out a similar reaction with ζ-carotene to form lycopene via neurosporene [5]. The occurrence of mutants of maize and tomato [102] and *Scenedesmus obliquus* [107] in which ζ-carotene is accumulated, as well as inhibitor studies with herbicides that specifically inactivate phytoene desaturation, and others that predominantly inhibit ζ-carotene desaturation [54], supported this view. Genetic complementation studies with *Phycomyces* mutants defective in either the phytoene desaturase or the lycopene cyclase gene gave the first indication that, in this fungus, ζ-carotene desaturation, like phytoene desaturation, is carried out by a single enzyme, which is the product of the phytoene desaturase gene [108].

Since genes encoding phytoene desaturases from different organisms are available, they were employed to characterize the desaturase reaction and to solve the problem of how many enzymes catalyze the desaturation sequence. An extensive study was undertaken to accumulate the products of phytoene desaturases by functional complementation of this enzyme from different organisms in *E. coli* which basically is non-carotenogenic. For this purpose, *E. coli* was co-transformed with two different plasmids. One carried the genes *crtB* and *crtE* which are necessary for the synthesis of the substrate phytoene [51], and a second plasmid carrying one of the phytoene desaturase genes. The resulting *E. coli* transformants were pigmented and HPLC analysis of accumulated carotenes was performed [109]. It was shown that the reaction product of the *Synechococcus* desaturase is ζ-carotene. Three different isomers of this carotene with two double bonds additional to phytoene were detected of which all-*trans* ζ-carotene was the minor one. In contrast, the *Rhodobacter* enzyme introduced three double bonds yielding mainly all-*trans* neurosporene together with two *cis* isomers. In the case of the *Erwinia* enzyme, all-*trans* lycopene with four newly introduced double bonds was the major product, but also a *cis* lycopene isomer and small amouts of bisdehydrolycopene, which resembles a phytoene molecule with six additional double bonds, were detected. Very similar results were obtained in cell-free reactions in which phytoene was converted by membranes isolated from *E. coli* transformants carrying phytoene desaturase genes from different organisms [110]. Complementation of the phytoene desaturase gene from tomato resulted in accumulation of ζ-carotene in *E. coli* [59], as observed in the case of the cyanobacterium, whereas the phytoene desaturase from the fungus *Neurospora* synthesized lycopene after complementation of its gene in *Rhodobacter* [111] resembling the *Erwinia* enzyme. Obviously, there is a functional diversity of these three different types of phytoene desaturases which is reflected by the number of desaturation steps carried out. Their properties are summarized in Table 3.

Mechanism

Information on the mechanism of phytoene desaturation is available from studies with photosynthetic membranes from *Synechococcus* (= *Anacystis*) and chromoplasts from daffodil petals. In general, two alternative ways are conceivable: a dehydrogenase/electron transferase mechanism or a dehydrogenase/oxygenase mechanism involving hydroxylation and subsequent water elimination. In experiments with membranes from *Synechococcus*, addition of either NAD or NADP enhanced phytoene desaturation, whereas NAD(P)H did not affect this reaction [33]. Phytoene desaturation is also oxygen-dependent when complex membrane preparations are the source of the enzyme [5]. However, studies on cofactor requirement of the purified phytoene desaturases demonstrated that either NAD(P) or FAD, depending on the species, are sufficient to catalyze the reaction [112, 113].

All these results support a mechanism involving a dehydrogenation reaction for both types of phytoene desaturases with an oxidized dinucleotide as electron and hydrogen mediator, as previously proposed [2]. For *Synechococcus* an NAD(P)-dependence was reported for the subsequent desaturation of ζ-carotene [33]. All four double bonds originate from *trans* eliminations of the hydrogens [23]. From the substrate specificity of the *crtI* and the *pds* phytoene desaturase, as well as the ζ-carotene desaturase, a model on the different regions of the substrates which are recognized by the catalytic sites of the enzymes can be proposed. They are marked in Fig. 4. For the *crtI*-type phytoene desaturase, to interact with the enzyme, the substrate may need a double bond at one side and a dienoic group at the other side of the position where the double bond is formed. This structural requirement is found in phytoene and in ζ-carotene as well as in their unsymmetrical products. If, for the binding to the *pds* phytoene desaturase, a trienoic region of the substrate is required, the *cis* configuration of the central double bond as well as the position of the methyl groups in phytoene is completely different to the corresponding region in ζ-carotene. This can account for the fact that ζ-carotene is not a substrate for this enzyme. The specificity of ζ-carotene desaturase for ζ-carotene and neurosporene only can be explained by the assumption that this enzyme recognizes an even longer conjugated system, e.g. a heptaenic structure adjacent to the position where the double bond is inserted.

For some time, the identification of hydroxyphytoene in the presence of herbicidal inhibitors has been taken as a possible indication of a hydroxylation step involved in desaturation [114]. However, the assignments of the positions of the hydroxy groups in several monohydroxy phytoene derivatives revealed that they were located at C1, C5, C6, C9 and C10 and not at C11 or C12, where the introduction of the

Table 3. Properties of three different functional types of phytoene desaturases. P = phytoene, NFZ = norflurazon, DPA = diphenylamine.

Source	Catalytic reaction	No. of desaturation steps	Cofactor requirement	Sequences similarity to	Inhibitor
Synechococcus	P → ζ-carotene	2	NAD/NADP	–	NFZ etc.
Rhodobacter	P → neurosporene	3	FAD	*Erwinia*	DPA
Erwinia	P → lycopene (and bisdehydrolycopene)	4 6	FAD	*Rhodobacter*	DPA

double bond occurs [115]. It is now evident that all identified hydroxyphytoenes are photooxidation products of the accumulated phytoene (G. Sandmann, unpublished results).

In daffodil chromoplast membranes, the desaturation mechanism is different to the systems investigated to date. Desaturation of either phytoene or ζ-carotene is independent of $NAD(P)^+$ [116]. Instead, O_2 seems to be the final acceptor for hydrogen, and an oxidoreductase is postulated as a redox mediator. Different quinones in the oxidized form can substitute for O_2 in daffodil chromoplasts [117].

Isomerization

Carotenoids are generally synthesized as all-*trans* isomers or at least as a mixture in which the all-*trans* form is dominant. As pointed out in the previous chapter, phytoene is produced as the 15-*cis* isomer by most phytoene synthases and is accumulated in this form [51, 118]. As the conversion of 15-*cis* phytoene has directly been shown *in vitro* with *Synechococcus* membranes [34] and with daffodil chromoplasts [36], isomerization has to occur either before, during, or between the desaturation reactions. Molecular genetics of the gene clusters of *E. uredovora* and *E. herbicola* revealed that an isomerase is not involved because such a gene is absent. Either a plasmid with only the phytoene desaturase gene from *Erwinia*, in addition to all genes necessary for synthesis of 15-*cis* phytoene [52], or a deletion mutation in which the same sequence of genes as above is operative [50] allows for the formation of mainly all-*trans* lycopene. Studies with the purified enzyme from *Erwinia* revealed that the *cis* to *trans* isomerization within the reaction sequence occurred at the level of phytoene and showed the same dependency on FAD as the desaturation reaction [112]. In contrast, the reaction product of the two-step phytoene desaturase from organisms with oxygenic photosynthesis is predominantly ζ-carotene in a *cis* configuration [109]. The same isomers were also formed *in vitro* with the purified phytoene desaturase from *Synechococcus* [113]. The combination of cyanobacterial phytoene and ζ-carotene desaturase genes is sufficient for the conversion of 15-*cis* phytoene to predominantly all-*trans* lycopene [67]. The necessary *cis* to *trans* isomerization occurs at the ζ-carotene molecule.

As an individual isomerase does not exist, isomerization of the 15-*cis* bond to *trans* has to be associated with the desaturation reactions. All carotenes are highly lipophilic and water-insoluble. Therefore, it can be assumed that all steps from phytoene synthesis to cyclization of lycopene take place at a membrane-integrated multienzyme complex, which works as a tight assembly line [2]. As speculated by Goodwin [2], it might well be that phytoene is modified to a strained molecule during interaction with the catalytic site. Then, configuration of the central double bond is determined during detachment from the assumed enzyme complex, either after cyclization, in the course of interaction with specific inhibitors, or in mutants that lack one of the enzyme components. In organisms which possess the *crtI*-type of phytoene desaturase this enzyme is involved in the isomerization. In species with the *pds* type, the isomerization step must be associated with the catalytic activity of the individual ζ-carotene desaturase operative only in these organism.

In a special type of mutation, ζ-carotene is accumulated as poly-*cis* isomer accompanied by other poly-*cis* acyclic carotenes. This was found in the tomato variety Tangella [119] and *Scenedesmus* C-6D [120]. Carotenoid analysis indicates that the mutation responsible is located at the phytoene desaturase level [121]. The functional modification of the mutated phytoene desaturase is responsible for a simultaneous isomerization of the C9 and C9' *trans* bond of the precursors phytoene and phytofluene to the *cis* configuration during the synthesis of regular *trans* bonds at C11 and C11' [2]. Then the resulting poly-*cis* isomer of ζ-carotene is fur-

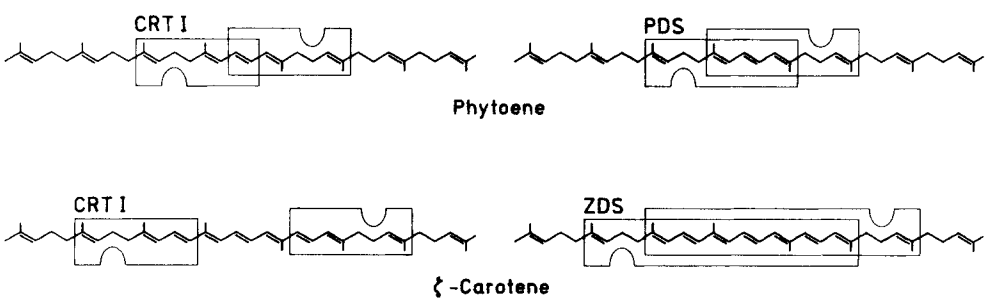


Fig. 4. Proposed recognition sites for substrates of *crtI*- and *pds*-type phytoene desaturases and ζ-carotene desaturase.

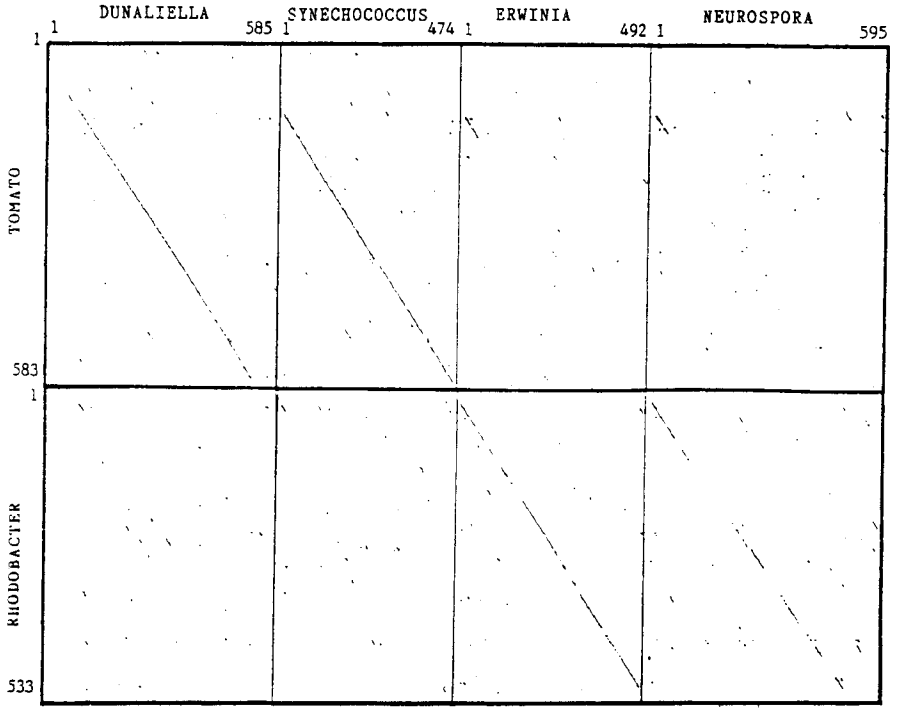


Fig. 5. Similarity plots of deduced amino acid sequences of phytoene desaturases from different organisms [59].

ther desaturated to prolycopene less effectively than the corresponding all-*trans* isomer [121].

Genes and phylogenetic relationship

As summarized in Table 1, genes of the *crtI*-type phytoene desaturase have been cloned and sequenced from *Rhodobacter capsulatus* [8, 122], *Ewinia uredovora* [52] and *E. herbicola* [53, 86], *Myxococcus xanthus* [75], and *Neurospora crassa* [63]; *pds*-type phytoene desaturase genes were cloned from the cyanobacteria *Synechococcus* [56] and *Synechocystis* [60], from the green alga *Dunaliella bardawil* [123] as well as from higher-plant species like soybean [58], tomato [59], and *Capsicum annuum* [71]. A gene cloned by hybridization of *Synechocystis* DNA with the *Rhodobacter crtI* gene [124] did not complement the desaturation reaction [109] and is therefore not a phytoene desaturase gene.

Similarity plots of Fig. 5 compare the amino acid sequence deduced from the tomato *pds* gene with the sequence from a green alga or a cyanobacterium [59]. A remarkably high similarity is observed as indicated by the dotted lines. All the known *pds*-type phytoene desaturases share an amino acid identity of more than 65%. In contrast, similarity to bacterial and fungal phytoene desaturases is negligible. Nevertheless, the fungal and bacterial phytoene desaturases are highly conserved, forming a second related group. The *Rhodobacter* enzyme, although different to the *Erwinia* and *Neurospora* enzyme with respect to its reaction product, is similar to them and shows an identity to the *E. uredovora* and *Neurospora* peptide sequences of 41% and 31%, respectively. All the results demonstrate that, during the course of evolution, two completely different and unrelated phytoene desaturase proteins were acquired. The alignment of phytoene desaturases in Fig. 6 shows both independent groups of *crtI*-related and *pds*-related genes. In the first one the cyanobacterial genes were much more related to each other than to the higher-plant genes. Among *crtI*, the bacterial genes group closer together but are rather distant to the *Neurospora* gene.

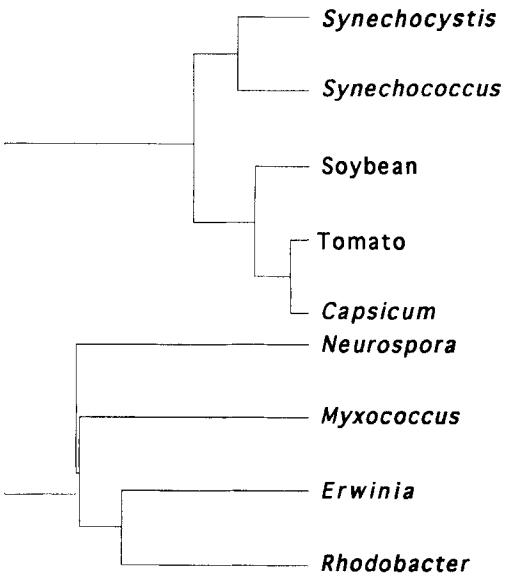


Fig. 6. Similarity dendograms for both groups of phytoene desaturases based on their protein sequences.

In spite of the different phylogenetic origin of the two types of phytoene desaturases, both proteins possess the motifs similar to a $\beta\alpha\beta$ dinucleotide-binding site near the N terminus. It is still a matter of speculation whether this region of the protein is involved in the interaction with the soluble cofactor [59] or can be regarded as a binding site for a prosthetic group like FAD [68]. In the higher-plant phytoene desaturases, this region of the protein resembles more a composite sequence that is ambiguous in its specificity for either NAD or NADP [59]. This view corresponds to the cofactor requirement of isolated *pds*-type phytoene desaturases [113].

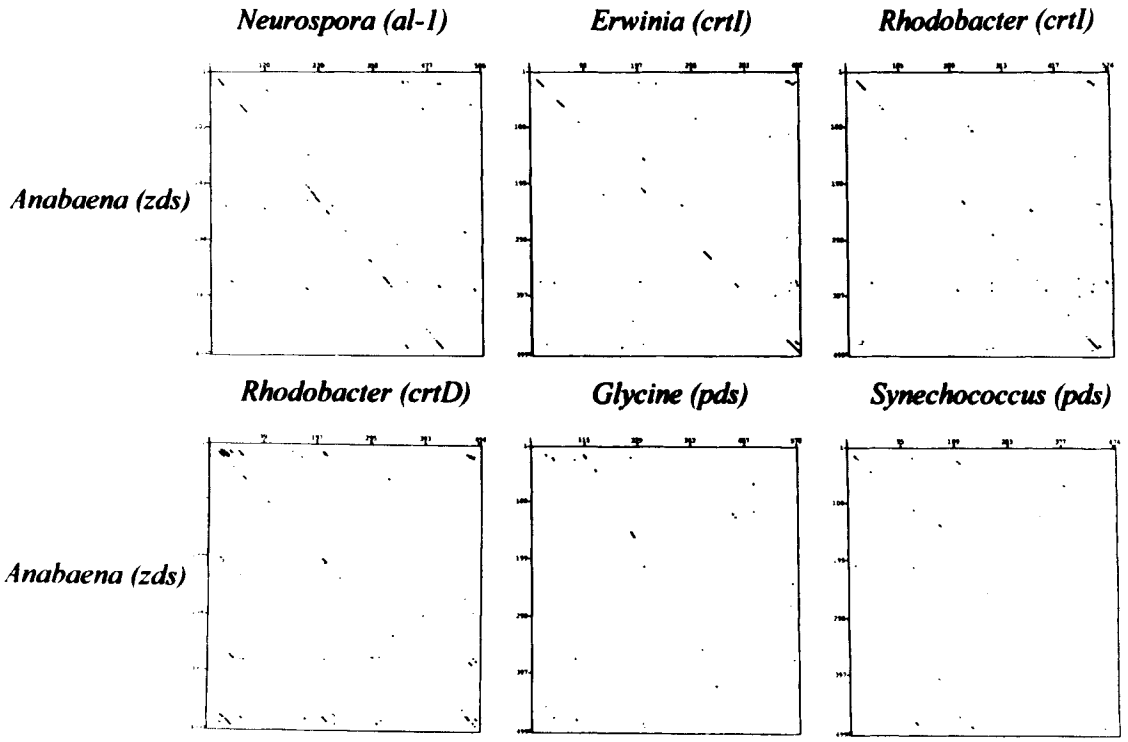


Fig. 7. Protein similarity plots of ζ-carotene desaturase (*zds*) in comparison to various carotenoid desaturases.

In desaturases from heterotrophic organisms or bacteria with anoxygenic photosynthesis another conserved region could be found close to the C terminus. For this region a role as an active site in the desaturation reaction was proposed [8]. However, this region can not be found in phytoene desaturases from photosynthetic organisms like cyanobacteria, green algae, and plants. In the latter a different conserved region was identified instead, which is absent in the *crtI*-type phytoene desaturases. For the tomato *pds* the genomic sequence has been cloned. It covers 9 kb and contains 11 introns [120, 125].

All available data indicate that the two protein types of phytoene desaturases do not have a common ancestor and that they were acquired through convergent evolution [21]. A consequence of the replacement of the four-step desaturase *crtI* by a two-step desaturase *pds* in the course of evolution is the necessity for the presence of a ζ-carotene desaturase in all *pds*-containing organisms [67]. The gene *zds* encoding the proposed ζ-carotene desaturase was cloned by heterologous complementation in *E. coli*. The sequence was obtained recently [68].

Fig. 7 presents the amino acid comparison of ζ-carotene desaturase with carotenoid desaturases from different species using protein similarity plots. The best similarity was found with the *crtI* phytoene desaturase genes from bacteria and fungi and a methoxyneurosporene desaturase gene *crtD* [8]from *Rhodobacter*. The highest similarity was 29% identity for the *Neurospora* phytoene desaturase. In contrast, the similarity between the ζ-carotene desaturase gene and the *pds* phytoene desaturase genes from cyanobacteria and higher plants was comparably weak. It can be seen from the plots (Fig. 7) that the amino acid similarity of ζ-carotene desaturase to the latter is mainly limited to the N terminus of the polypeptides. As indicated by quantitative similarity calculations, the following ranking of the structural conservation of ζ-carotene desaturase to other desaturases could be established: *al-1*, *Neurospora* > *crtI*, *Erwinia* > *crtD*, *Rhodobacter* > *crtI*, *Rhodobacter* ≫ *pds*, *Glycine* > *pds*, *Synechococcus*. Based on these results, it can be proposed that the newly appearing ζ-carotene desaturase gene was phylogenetically derived from the *crtI* phytoene desaturase gene of bacteria and fungi [68]. This specialization occurred parallel to the evolution of oxygenic photosynthesis.

Enzyme purification

Purification of phytoene desaturase from plant tissue is very difficult to achieve. Nevertheless, this enzyme was isolated from *Capsicum* chromoplasts and purified in an active state [71]. It had a size of 56 kDa and binding of FAD to the protein was suggested. Phytoene desaturases from *Erwinia* and *Synechococcus* were purified after overexpression of their genes in *E. coli*. For expression of phytoene desaturase, a plasmid was constructed by cloning the entire coding region of the *crtI* gene from *Erwinia* behind the *lacZ* promoter of pUC18 resulting in a reading frame for the full polypeptide with additional nine amino acids at the N terminus [112]. In *E. coli* transformants this plasmid mediated the expression of phytoene desaturase to a final concentration of about 10% of total cellular protein. Under these conditions, the recombinant protein is sequestered in inclusion bodies where it can be solubilized by urea treatment. Subsequent purification to homogeneity involved urea treatment which left the resulting enzyme poorly active. However, it was possible to regain its activity by removal of the denaturant and dilution of the sample in the presence of dithiothreitol which allows refolding of the enzyme. The *Erwinia* phytoene desaturase has a molecular mass of 56.2 kDa and catalyzed, as expected, the conversion of 15-*cis* phytoene to *trans* lycopene and also to some extent to bisdehydrolycopene. FAD was involved as cofactor in the desaturation reaction of this bacterial type of phytoene desaturase.

The same strategy was followed in the purification of the phytoene desaturase from *Synechococcus* [113] and several milligrams of the homogenous enzyme were also obtained. This 53-kDa membrane protein could be reactivated after lipid replenishment. Inhibition was observed by several bleaching herbicides [126]. The cofactors for this cyanobacterial/algal/plant-type phytoene desaturase were either NAD or NADP whilst FAD was ineffective as electron acceptor. The dependence of both purified phytoene desaturases on oxidized dinucleotides as electron and hydrogen mediators confirms a dehydrogenase/electron-transferase mechanism for the desaturation reaction as proposed by Goodwin [2]. By comparison of the amino acid sequences from cyanobacterial and higher-plant phytoene desaturase genes, N-terminal protein extensions were found in the prokaryotic enzymes [58, 59, 123]. Chloroplast import studies with the *pds* gene product from soybean showed that the expressed protein is processed after import into a smaller mature form [58]. Purification and enzymological characterization of the ζ-carotene desaturase from *Anabaena* after expression of the *zds* gene in *E. coli* is in progress (Sandmann et al., unpublished results).

Localization

The desaturation of phytoene is the first reaction in the carotenogenic pathway in which a lipophilic substrate is converted. The function of the involved desaturase strongly indicates that phytoene desaturase is an intrinsic membrane protein [9]. Hydropathity analysis using the deduced amino acid sequence from the *Capsicum* enzymes reveals several hydrophobic domains which can be regarded as membrane-spanning regions of the protein [71]. The nature of phytoene desaturase as an integral protein of the thylakoid membrane could be confirmed by *in vitro* phytoene desaturation of purified thylakoids from *Synechocystis* and isolated chloroplasts from green Capsicum fruit [127] and from radish seedlings [128]. In daffodil chromoplasts the enzyme responsible for desaturation as well as cyclization are integral proteins of the intraplastidic membranes [30]. These enzymes can be solubilized by various detergents. They can be obtained either in an active state with mild detergents [129], or by more denaturating ones in an inactive form, which then can be reactivated by integration of the enzymes into lipid vesicles [130]. A recent immunogold localization study with an antiserum against the *pds*-type phytoene desaturase showed that in different higher plant chloroplasts the majority of this enzyme is localized within the thylakoid membranes and that its presence in the envelope is of minor significance [131]. This was demonstrated by *in situ* localization with an immunogold microscopy procedure, as well as by Western blotting of isolated envelope and thylakoid fractions. In photosynthetic prokaryotes like *Rhodobacter*, *Anabaena*, and *Synechocystis* where the photosynthetic membranes are not localized in distinct organelles, phytoene desaturase could be detected exclusively in the photosynthetic membranes by immunocytochemical localization [132].

Regulation of the pathway and modulation of activity

The activity of phytoene desaturase can be modulated by endogenous or external factors. As already demonstrated for the fungus *Phycomyces blakesleeanus* [5], *in vitro* studies with the cyanobacterium *Synechococcus* showed feed-back regulation by subsequent carotenes of the pathway [33]. The strongest inhibitory effect was observed with the fully desaturated lycopene containing 11 conjugated double bonds. A lower degree of desaturation or the presence of one or even two ionone rings made inhibition less effective. Under certain conditions, this feedback regulation of phytoene desaturase is also evident in intact cells, e.g. in *Scenedesmus* mutants blocked in ζ-carotene desaturation [107]. Not only ζ-carotene is accumulated in these strains as expected from the mutation, but also phytoene in a ratio of 2:1. Several *Synechococcus* mutants resistant against the phytoene desaturase inhibitor norflurazon were selected [55]. The activities of the mutated phytoene desaturases were negatively correlated to their degree of resistance. As a consequence, the lower phytoene desaturase activities led to decreased formation of carotenoids in intact cells. It was concluded from this result that, at least in cyanobacteria, phytoene desaturation may be the rate-limiting step of carotenogenesis.

Phytoene desaturase from organisms with oxygenic photosynthesis is inhibited by a great number of bleaching herbicides (see [54] for review). Enzyme-kinetic studies revealed the reversible non-competitive nature of these inhibitors [133]. ζ-Carotene desaturase is inhibited by other chemically different compounds. In contrast to inhibitors of phytoene desaturation which are very specific, ζ-carotene desaturase inhibitors also affect phytoene desaturation, although to a much lower degree [126].

Both types of phytoene desaturases are differentially inhibited by various inhibitors [126]. Due to the different polypeptide composition, the *crtI*-type is not sensitive to bleaching herbicides. This feature has been exploited to engeneer resistant cyanobacteria and higher plants. The *E. uredovora crtI* gene was inserted into the *Synechococcus* chromosome together with different promoters [134]. Depending on the strength of the promoters, different expression of the *Erwinia crtI* gene was determined by immunodetection of the *crtI* polypeptide which corresponded to the degree of resistance acquired against the herbicide norflurazon. The same strategy has been followed to obtain a resistant higher plant [135]. For this purpose a 5′ fusion of the *crtI* gene to the sequence of a transit peptide for import into the chloroplast was made. After transformation of tobacco, the chimeric gene product was targeted into and processed in the chloroplasts. There it was enzymologically active which gave raise to elevated norflurazon resistance.

Another way of obtaining resistance was by conventional mutagenesis of the endogenous phytoene desaturases of *Synechococcus* and *Synechocystis* and selection against bleaching herbicides [136, 137]. In most mutants different point mutations were found in the coding region which modified the phytoene desaturase to a less susceptible enzyme. This type of investigation may lead to a model of the inhibitor binding site. In a special mutation the *pds* promoter was modified and the enhanced expression of the original protein was responsible for resistance [136].

Developmental and physiological regulation

In tomato there is one single *pds* gene involved in the biosynthesis of carotenoids in leaves, flowers and fruit [123]. Furthermore, the increase of carotenoids during fruit ripening is accompanied by an increase of the *pds* transcript [59, 105]. Northern analysis showed an increased *pds* expression at high light conditions [123] and in the presence of an inhibitor of phytoene desaturase [105]. In *Neurospora crassa* the *crtI* phytoene desaturase is one of several enzymes which are

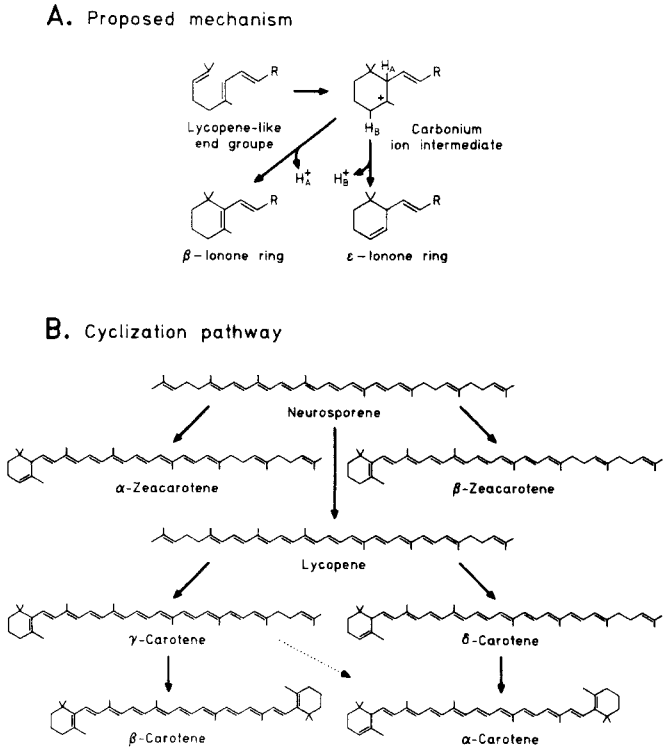


Fig. 8. General mechanism for the formation of β- and ε-ionone rings (A) and cyclization pathway in the reaction sequence leading to α- and β-carotene (B).

involved in photoregulation of carotenogenesis. This regulation occurs at the level of initiation of transcription [63]. In *Myxococcus xanthus* the *crtI*-type phytoene desaturase gene *carC* is up-regulated by light in a mechanism which operates when the cells reach the stationary phase [75]. This regulation is under the control of a *trans*-acting genetic element [138].

Lycopene cyclization

Lycopene is the regular substrate for cyclization reactions. Two types of ionone rings are formed: the β-ring as an end group of both sides of β-carotene and the ε-ring at one side of α-carotene in addition to a β-ring (Fig. 1). The mechanisms of cyclization which involves proton attack at C2 and C2′ of lycopene is substantially supported by D_2O-labelling experiments with *Flavobacterium* and a *Scenedesmus* mutant followed by determination of the position of the deuterated carbon at the β- or ε-ionone ring [139, 140]. The resulting carbonium ion intermediate is stabilized by loss of proton from either C1 or C4 to yield a β- or ε-ring, respectively. The mechanism is outlined in Fig. 8A. As indicated by gene analysis of *E. herbicola*, only one gene product is sufficient for introduction of the two β-ionone rings at both sides of lycopene to form β-carotene via γ-carotene [47, 50]. Lycopene cyclase recognizes each half of the substrate molecule lycopene separately. Therefore, it is able also to convert neurosporene in which one side already carries the conjugated system of lycopene-yielding β-zeacarotene (Fig. 8B). This has been shown by genetic complementation studies with *E. coli* [106]. Furthermore, β-zeacarotene can been found in cultures treated with moderate doses of ζ-carotene desaturase inhibitors [141].

Formation of the β- or ε-ring is under different gene control. Introduction of the b gene into red (= lycopene-accumulating) tomato resulted in the production of β-carotene at the expense of lycopene, whereas the *Del* gene is responsible for formation of ε-rings [102]. Consequently, we can assume that formation of β-carotene is catalyzed by a single enzyme, lycopene cyclase β. In the case of α-carotene, two different enzymes, lycopene cyclase β and lycopene cyclase ε, are involved.

Since the work of Kushawa et al. [142] only a few *in vitro* studies on lycopene cyclization have been carried out. Lycopene cyclase was solubilized from *Capsicum* chromoplasts [143]. Osmotically shocked chromoplasts were acetone-treated, and the resulting powder was extracted with buffer. This extract was capable of converting [^{3}H]lycopene into β-carotene. Cofactors were not required but the reaction was sensitive to sulfhydryl reagents. Furthermore, lycopene cyclization was inhibited by tertiary amines. Especially 2-(4-chlorophenylthio)-triethylamine · HCl has been used for several years as an inhibitor for lycopene cyclization *in vivo* or *in vitro* [54]. The *E. herbicola* gene was overexpressed in *E. coli* and a 43-kDa protein band was obtained which was enzymically inactive [144]. By modification of the expression conditions, it was possible to solubilize by detergent an active lycopene cyclase fraction from the membranes and to purify this enzyme in an active state to homogeneity (Sandmann et al., unpublished results).

In contrast to other plants, the lycopene isomer formed *in vitro* by daffodil chromoplasts under aerobic conditions is the poly-*cis* prolycopene (7,7′,9,9′-tetra-*cis*) and the cell-free biosynthetic chain is stopped at this stage [116]. Prolycopene cannot be converted by lycopene cyclase as demonstrated for the *Scenedesmus* mutant C-6D with a poly-*cis* biosynthetic pathway in the dark [120]. In this unique mutant, prolycopene and other poly-*cis* precursors are isomerized to the all-*trans* isomer by illumination and only then conversion to α- and β-carotene is possible [121, 145]. Lycopene cyclization to β-carotene in the daffodil cell-free system was only effective under strict anaerobic conditions [116]. As the substrate for this reaction is in this case the poly-*cis* prolycopene and β-carotene is formed in the all-*trans* configuration, isomerization must occur simultaneously to the ring formation. Both reactions could be distinguished by use of the inhibitor 2-(4-chlorophenylthio)-triethylamine · HCl and it was shown that NADPH plays an important role in the isomerization step [146]. This complex mechanism is obviously not the general model for lycopene cyclization in other plants and bacteria. For example, lycopene cyclization is mediated in *Erwinia* by only the *crtY* gene [50], and *in vitro* reactions demonstrate that the all-*trans* isomer is used as a substrate without addition of NADPH under aerobic conditions [37]. Furthermore, the *lcy* gene product, which is assumed to resemble the higher-plant type, mediates the cyclization of all-*trans* lycopene to β-carotene [147]. The converted all-*trans* substrate is the direct product formed in the plant-type desaturation sequence [67].

The only sequence data on genes of a β-ring-forming lycopene cyclase is available from *E. uredovora* [52] and *E. herbicola* [53] *crtY* (which was also named *crtZ* in [50] in parallel but describes the same gene). Both genes code for a 43-kDa polypeptide. The overall similarity between the two genes or the corresponding enzymes is more than 80%. Recently, the first lycopene cyclase gene *lcy* was cloned from a photosynthetic organism [147]. Similar to the cloning of *pds* from *Synechococcus*, herbicide resistance against an inhibi-

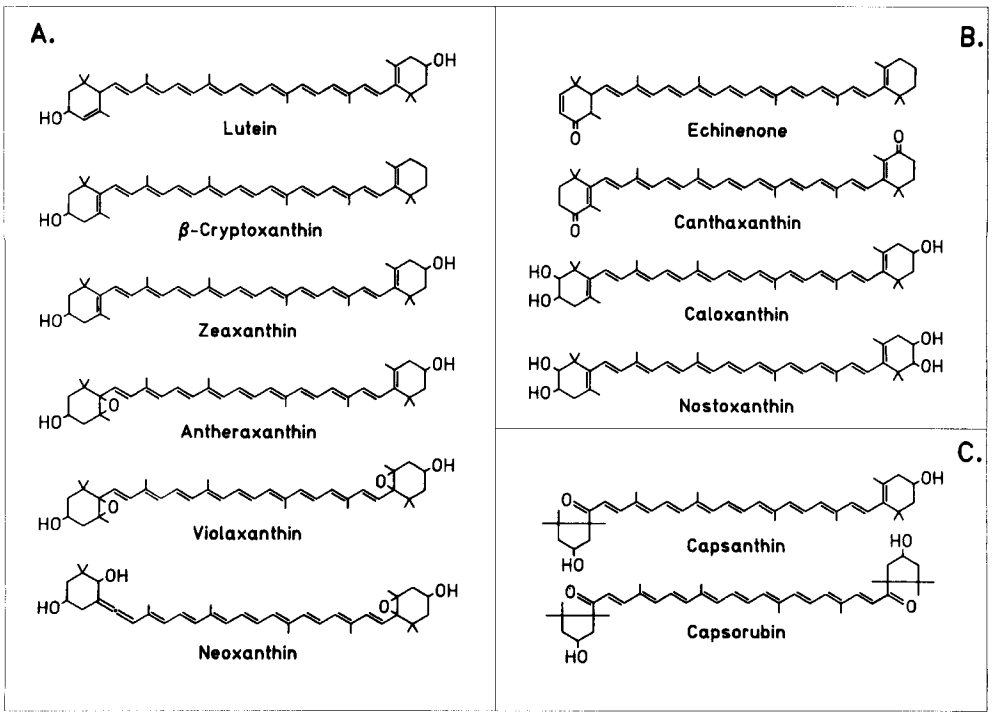


Fig. 9. Chemical structures of carotenoids derived from α- and β-carotene found in chloroplasts (A), special hydroxy and oxo derivatives produced by cyanobacteria (B), and secondary carotenoids of red pepper fruit (C).

tor, MPTA, and genetic complementation of this resistance in the wild type was the technique employed. The sequence data indicate that there is almost no similarity to the *Erwinia crtY* genes (J. Hirschberg, personal communication).

Formation of xanthophylls derived from cyclic carotenoids

Xanthophylls are enzymically formed oxidation products of α- and β-carotene (Fig. 9). The most common oxygen groups found in xanthophylls are hydroxy at C3, oxo at C4 and epoxy at the 5,6 position of the ionone ring. Lutein, the 3,3′-dihydroxy α-carotene, is formed only by algae and higher plants which have the potential to form α-carotene with its ε-ring. Zeaxanthin, the corresponding 3,3′-dihydroxy-β-carotene, is synthesized by some heterotrophic bacteria and all organisms with oxygenic photosynthesis. The 4-mono and and 4,4′-dioxo β-carotene derivatives echinenone and canthaxanthin are characteristic for cyanobacteria and some heterotrophic bacteria but can also be found as secondary carotenoids, e.g. in green algae growing under various stress conditions. As far as is known, all the oxygen groups originate from molecular oxygen [148, 149].

Hydroxy carotenoids

Comparably little is known about the enzymology of xanthophyll formation. The cell-free reaction studied in most detail is the hydroxylation of β-carotene to β-cryptoxanthin (= 3-hydroxy-β-carotene) performed by *Synechocystis* membranes [34]. This catalytic step shows the typical characteristics of a monooxygenase reaction. The cell-free conversion is stimulated by NADPH, dependent on oxygen, and is sensitive to CO as well as to different plant-specific monooxygenase inhibitors. This *in vitro* reaction mainly led to the monohydroxylation product. In some cyanobacterial species e.g., *Synechococcus* and *Nostoc*, further hydroxy groups are introduced at C2 and C2′ in addition to the ones at positions 3 and 3′ leading to the 2,3,3′-triol caloxanthin and the 2,2′,3,3′-tetrol nostoxanthin [150, 151].

The only gene for an enzyme involved in the introduction of an oxygen function into a cyclic carotene was cloned from *Erwinia* species. The gene *crtZ* [52], which was also named *crtH* in a simultaneous publication [50], encodes β-carotene hydroxylase. From studies of deletion mutations of the *Erwinia* hydroxylase gene it is known that one enzyme catalyzes the formation of both 3-hydroxy-β-carotene and the 3,3′-dihydroxy derivative zeaxanthin from β-carotene [50, 52]. *In vitro* β-carotene hydroxylation was carried out with membrane preparations of *E. coli* in which the corresponding *Erwinia* gene was expressed [37]. The catalyzed reaction involved a two-step formation of zeaxanthin with monohydroxy β-carotene (= β-cryptoxanthin) as a detectable intermediate. The hydroxylation steps were independent of NADPH but needed the presence of 2-oxoglutarate together with Fe_2SO_4 and ascorbate. This indicates that, unlike the enzyme from *Synechocystis* [34], the *Erwinia* β-carotene hydroxylase catalyzes a dioxygenase reaction. The *Erwinia* β-carotene hydroxylase was overexpressed as a 22-kDa protein [144]. Although this is a membrane-integrated protein, enzymic activity was only found with a cytoplasmic fraction. No further purification or characterization of this β-carotene hydroxylase was achieved. Zeaxanthin is glucosylated in *Erwinia* and the gene of the responsible glucosyltransferase *crtX* [52], which equals *crtH* [50], was cloned. Using this gene from *E. herbicola*, a 45-kDa protein was overexpressed in *E. coli* and the enzyme purified [152]. Enzymic characterization revealed its affinity for the substrates zeaxanthin and UDP-glucose.

Oxo carotenoids

Very little is known about the reaction responsible for the formation of oxo groups at C4 of β-carotene. *In vitro* conversion of β-carotene to the 4-oxo derivative echinenone was studied with membranes of the cyanobacterium *Synechocystis* [34]. This is a NADPH-dependent process which is inhibited by KCN. Evidence that the formation of the C4 oxo group proceeds via a combination of hydroxylation and dehydrogenation steps comes from inhibitor studies with the alga *Dictyococcus* and with *Brevibacterium* [153]. Inhibition of the synthesis of echinenone and the 4,4′-dioxo derivative canthaxanthin resulted in the accumulation of the corresponding 4-hydroxy compounds.

Epoxy carotenoids

Starting from zeaxanthin, antheraxanthin with one epoxy group and the diepoxy-violaxanthin are formed by introduction of these epoxy groups into the 5,6 and 5′,6′ position. These epoxidation reactions could be demonstrated *in vitro* by isolated chromoplasts from *Capsicum* [154]. Recently, different abscisic-acid-deficient (*aba*) mutants were isolated from *Arabidopsis thaliana* [155, 156]. All mutations in this *aba* locus resulted in a strong decrease of the levels of all epoxy carotenoids, indicating that the β-carotene epoxidase gene is affected.

Epoxidation of zeaxanthin to violaxanthin is part of a reversible cycle operative in plant chloroplasts. Under light stress, deepoxidation of violaxanthin occurs via antheraxanthin to zeaxanthin which is thought in some way to protect the chloroplast against high light conditions. This light/dark regulation of the reversible epoxidation/deepoxidation process has been reviewed recently [157]. Epoxidation activity, as indicated by the zeaxathin to violaxanthin conversion, was found in a chloroplast envelope preparation [158].

In chloroplasts from algae and higher plants the epoxycarotenoid neoxanthin with an allenic end group is present. It has been assumed that this allenic group originates from proton abstraction from C7 neighbouring the 5,6-epoxy-5,6-dihydro-β-rings of violaxanthin followed by rearrangement of the epoxy group to 5-hydroxy [3]. Cell-free reactions demonstrating these epoxidation steps were possible with homogenates of *Amphidinium carteri* [159].

Another derivative of violaxanthin is the oxo-carotenoid capsorubin with two cyclopentane rings found together with capsanthin carrying one cyclopentane ring as the major pigments in chromoplasts of ripe *Capsicum* fruit [29]. Cyclopentane rings are formed by proton attack of the epoxy groups at the β-ionone ring in antheraxanthin or violaxanthin, respectively, and pinacolic rearrangement by which the epoxy group is transformed into an oxo group. The kinetics of [^{14}C]*IPP* incorporation into carotenoids by isolated *Capsicum* chromoplasts support this sequence [154].

FORMATION OF ACYCLIC XANTHOPHYLLS IN *RHODOBACTER*

In *Rhodobacter* species like *R. sphaeroides* and *R. capsulatus* the major carotenoid of the photosynthetic membrane is spheroidene under strict anaerobic growth conditions [153]. In the presence of oxygen, it is converted into spheroidenone. The initial part of the biosynthetic pathway in *Rhodobacter*, including the desaturation of phytoene to neurosporene, is common to the reaction sequence leading to the formation of cyclic carotenoids as described in the previous chapter. However, desaturation stops at the stage of neurosporene which is converted to 1-hydroxyneurosporene, the first oxygen-containing carotenoid in this pathway of acyclic xanthophylls. This introduction of a hydroxy group occurs without the participation of molecular oxygen as a hydration reaction by addition of water (Fig. 10).

The next two reactions are introduction of a double bond into 1-hydroxyneurosporene at position 3,4 or methylation of the hydroxy group. These steps can proceed in an alternative sequence leading either to demethylspheroidene or methoxyneurosporene as intermediate and then to spheroidene. The latter can further be metabolized to hydroxyspheroidene. An oxygen-dependent formation of an oxo group at C2 is possible for all intermediates of this pathway which contain the 3,4-double bond. Details on this pathway came mainly from analysis of accumulated carotenoids in different mutants of *R. capsulatus* [41] and from inhibitor and deuterium-labelling studies [160]. The latter work revealed the mechanism of the hydration reaction of positions 1,2 and 1′,2′. After inhibition of both reactions by nicotine and removal of the inhibitor in the presence of deuterium oxide, deuterium was incorporated into C2 and C2′. This addition of water to the terminal double bonds is considered to be analogous to the first stage of the cyclization reaction which also starts with a proton attack at C2 and which is blocked by the same inhibitors, nicotine and 2-(4-chlorophenylthio)-triethylamine · HCl [4].

As already outlined, the *crt* cluster from *R. capsulatus* has been completely sequenced [8]. Eight of the nine identified *crt* genes are located on an 11-kb DNA fragment as indicated in Fig. 11. In addition, the gene *crtJ* identified by transposon mutagenesis [42] is 12 kb away from this cluster downstream of *crtA*. A minimum of four promoters seem to be present in this cluster based on the different orientations of these genes. Two promoters in front of *crtI* and *crtD* could be identified by their similarities to the *E. coli* σ^{70} promoter [8]. Other *Rhodobacter*-specific promoters could not be recognized due to the limited knowledge about those promoters. Studies with transposons indicated the existence of a *crtEF* operon but the results did not support the existence of a *crtDC* [8] or a *crtIB* operon [86]. However, cotranscription of *crtI* and *crtB* was proposed [161]. Therefore, at least three or four promoters of an unknown type can be expected in the *crt* cluster from *R. capsulatus*.

The sequence of *crtA* as presented in [8] has been revised recently [161]. Redetermination of the nucleotide sequence revealed that *crtA* encodes a much shorter polypeptide of only 241 amino acids. The extended sequence previously related to *crtA* is in addition part of an open reading frame with great similarity to *bchI* [162, 164].

The functions of the genes *crtE*, *crtB*, and *crtI* which are homologous to those of other species were discussed in the previous chapters (see also Fig. 1). Four other genes involved in the biosynthesis of cyclic xanthophylls by *R. capsulatus* have been functionally identified long before they were cloned [41]. They include *crtA* which is responsible for an enyzme catalyzing the introduction of an oxo group at C2 of all 3,4-desaturated intermediates carrying a hydroxy or methoxy group at C1. The genes *crtC* and/or *crtK* are connected to 1,2-hydration, *crtD* with 3,4-dehydrogenation, and *crtF* with *O*-methylation (Table 4). Based on interposon insertions into the carotenogenic gene cluster followed by pigment analysis, a gene *crtG* was proposed to be located between *crtC* and *crtD* [41]. But the sequence of the *crt* cluster

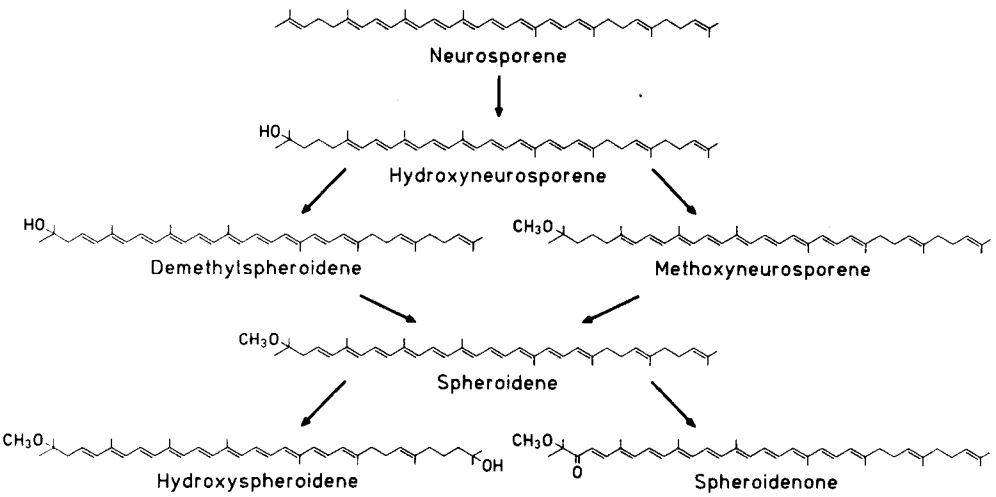


Fig. 10. Pathway for the formation of acyclic xanthophylls from neurosporene in *Rhodobacter*.

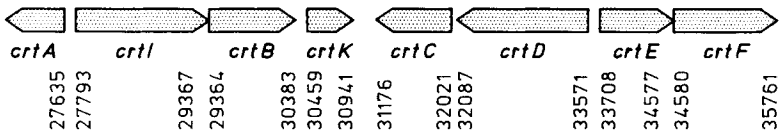


Fig. 11. The carotenogenic gene cluster of *Rhodobacter capsulatus*.

Table 4. Sequenced carotenoid genes of *Rhodobacter capsulatus* [8] involved in the biosynthesis of acyclic xanthophylls.

Genes	Encode enzymes
crtC,K	neurosporene 1,2-hydratase
crtD	1-hydroxyneurosporene 3,4-desaturase
crtF	1-hydroxyneurosporene methylase
crtA	spheroidene ketolase

demonstrates that a corresponding open reading frame did not exist [8]. Instead, a new gene, *crtK*, was located between *crtB* and *crtC*. Interposon insertions into the loci of both genes resulted in the accumulation of neurosporene [161]. Obviously, the *crtC* gene locus, mapped to have a minimum size of 1.2 kb and a maximum size of 2.2 kb, comprises the genes *crtC* with 0.85 kb and *crtK* with 0.48 kb. Definite functional assignment of both genes and elucidation of their role in 1,2-hydration awaits further investigations. Another gene with an obscure function is *crtJ* [42]. Lesions in this gene resulted in a *Rhodobacter* mutant devoid of carotenoids. *In vitro* studies demonstrated that this mutant is unable to convert [^{14}C]GG*PP* and channel the radioactivity into the carotenoid biosynthetic pathway [86]. It is most likely that *crtJ* has a regulatory function.

Rhodobacter is a facultative photoautotrophic bacterium. The synthesis of the intracytoplasmic membrane containing the photosynthetic apparatus, including the pigment-protein complexes, depends on light and oxygen conditions. Simultaneous decrease of oxygen together with the onset of illumination triggers the formation of carotenoids [10] and results in differential expression of the genes involved in their biosynthesis [163, 164]. The kinetics of mRNA accumulation during transition from aerobic respiration to anaerobic photosynthesis showed in parallel a transient increase of up to 12-fold for all genes of the *crt* cluster except for *crtB* and *crtI* which were unaffected. With transcriptional fusions of several *crt* genes to *lacZ*, oxygen regulation of promoters could be demonstrated accordingly [161, 165]. Obviously, regulation of the carotenoid biosynthetic pathway of *R. capsulatus* is under transcriptional control. The regulated enzymes are the initial GG*PP* synthase encoded by *crtB* and all other enzymes involved in formation and conversion of acyclic *Rhodobacter* xanthophylls. Very recently, a gene locus *pps* has been identified in *R. spheroides* which induces *trans* suppression of bacteriochlorophyll and carotenoid formation in both *R. spheroides* and *R. capsulatus* [166]. This gene is located about 11 kb downstream of *crtA*. This is the same region where *crtJ* was mapped. Perhaps both genes are identical in their nature and their function to control the biosynthesis of photosynthetic pigments in *Rhodobacter*.

CONCLUSION AND OUTLOOK

The latest developments demonstrated that molecular genetic approaches were of considerable help in understanding carotenoid biosynthesis. Cloning of genes involved also boosted the research on the biochemistry of this pathway. The tremendous progress was possible due to the availability of new tools like gene probes, overexpressed enzymes, and antisera. The future issue will be to further exploit these possibilities.

The efforts have so far focused mainly on genes involved in the sequence to carotenes and acyclic xanthophylls. Beyond this there are more new genes to be cloned, especially in the xanthophyll pathway of algae and higher plants, where hopefully progress will be made soon. But there remain also many open questions on the structural organization of the

genes already cloned. Evolutionary aspects based on gene similarities and diversities, for example between organisms with and without oxygenic photosynthesis, are still of considerable interest. Finally, the biotechnological production of carotenoids can now benefit from the availability of several genes. Besides fermentative mass production of carotenoids, new carotenoids can be obtained by combination of different genes from different organisms and are then available, for example, for biomedical application in cancer and tumor prevention. Another practical application is the construction of transgenic plants resistant to herbicides which inhibit carotenogenic enzymes.

A significant feature is the regulation of carotenoid biosynthesis both at the gene and the enzyme level. First results are now available on organ-specific expression of carotenoid genes in plants and ripening-specific expression in fruit which contain a high carotenoid content. Judging by the efforts put into this field, more progress can be expected soon. In the case of the fungus *Neurospora* and the bacterium *Myxococcus*, light regulation of carotenogenesis is currently under investigation. For a general understanding of reaction mechanisms and regulatory properties, there is a continuing need to purify carotenogenic enzymes from different parts of the biosynthetic pathway followed by identification of their active sites as well as their regulatory and inhibition sites.

Our current understanding of carotenoid biosynthesis has received a considerable stimulus in the last few years. It will be interesting to see what is going on in the near future. A lot of exciting research on many aspects of carotenoid biosynthesis lies ahead.

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