Carotenoids Biosynthesis — a review

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Abstract

Plants synthesized an enormous variety of metabolites that can be classified into two groups based on their functions: primary metabolites, which participate in nutrition and essential metabolic processes within the plant, and secondary metabolites (also referred to as natural products), which influence ecological interactions between plants and their environment. The carotenoids pigments are secondary metabolites of isoprenoid origin. Despite their diversity of functions and structures, all isoprenoids derive from the common five-carbon (C_5) building units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). More complex isoprenoids are usually formed by «head-to-tail» or «head-to-head» addition of isoprene units. The most prevalent tetraterpenes (C_{40}) are carotenoids, which are pigments in many flowers and fruits. In this paper we discuss some aspects of carotenoid biosynthesis. The pathway involves a series of desaturations, cyclizations, hydroxylations, and epoxidations, commencing with the formation of phytoene. The pathway begins with the synthesis of IPP from the mevalonic acid (MVA) pathway and/or methylerythritol 4-phosphate (MEP) pathway.

Resumo

As plantas sintetizam uma enorme variedade de metabolitos, que podem ser classificados em dois grupos, de acordo com as suas funções: metabolitos primários, que participam na nutrição e processos metabólicos essenciais no interior da própria planta, e metabolitos secundários (também referidos como produtos naturais), os quais influenciam as interacções ecológicas entre as plantas e o ambiente. Os carotenóides são metabolitos secundários derivados do isopreno. O isopentenil-pirofosfato (IPP) é a unidade básica para a biossíntese dos carotenóides. O esqueleto carbonado dos carotenóides é sintetizado por adição sucessiva das unidades em C_5 que vão formar geranil-geranilpirofosfato, intermediário em C_{20} que por condensação origina a estrutura em C_{40}. Recentemente assumia-se que todos os isoprenóides se sintetizavam a partir do acetil-CoA via ácido mevalónico. Estudos recentes mostraram que o percurso metabólico começa com a síntese do IPP via ácido mevalónico (MVA) e/ou via metileritritol 4-fosfato (MEP). Neste trabalho discutem-se os avanços no conhecimento destas diferentes vias metabólicas assim como as enzimas e

reacções envolvidas na biossíntese dos carotenóides a partir da unidade fundamental (IPP).

1
Introduction

Plants synthesize an enormous variety of metabolites that can be classified into two groups based on their function: primary metabolites, which participate in nutrition and essential metabolic processes within the plant, and secondary metabolites (natural products), which influence ecological interactions between plants and their environment (Croteau *et al*., 2000). Isoprenoids (also called terpenoids) play diverse functional roles in plants as hormones (gibberellins, abscisic acid), photosynthetic pigments (phytol, carotenoids), electron carriers (ubiquinone, plastoquinone), mediators of polysaccharide assembly (polyprenyl phosphates), and structural components of membranes (phytosterols). In addition to these universal physiological, metabolic, and structural, the highest variety of isoprenoids (commonly in the C_{10}, C_{15} and C_{20} families) is secondary metabolites that function in protecting plants against herbivores and pathogens, in attracting pollinators and seed-dispersing animals, and allelochemicals that influence competition among plant species (Croteau *et al*., 2000). Many compounds with important commercial value as flavors, pigments, polymers, fibers glues, waxes, drugs, or agrochemicals are secondary metabolites of isoprenoid origin (Rodríguez-Concepción and Boronat, 2002)

Carotenoids are naturally occurring pigments synthesized as hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) by plants and microorganisms. They consist of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule so that the two central methyl groups are in a 1,6-positional relationship and the remaining nonterminal methyl groups are in a 1,5 – positional relationship. All carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure, having a long central chain of conjugated double bonds, by (i) hydrogenation, (ii) dehydrogenation, (iii) cyclization, or (iv) oxidation or any combination of these processes. Their major function is in protection against oxidative damage by quenching photosensitizers, interacting with singlet oxygen (1) and scavenging peroxy radicals (2), thus preventing the accumulation of harmful oxygen species.

Carotenoids, the most diverse and widespread group of pigments found in nature, are synthesized *de novo* by all photosynthetic and many non-photosynthetic organisms. The carotenoids pigments are synthesized in the plastids of plants. In chloroplasts they accumulate primarily in the photosynthetic membranes in association with the light-harvesting and reaction center complexes. In the chromoplasts of ripening fruits and flower petals and in the chloroplasts or senescing leaves the carotenoids may be bound in membranes or in oil bodies or other structures within the stroma (Cunningham & Gantt, 1998).

The lipid-soluble carotenoid pigments are but an example of the plethora of chemical compounds that are produced by what are collectively known as the pathways of isoprenoids biosynthesis. The isoprenoids comprise the largest family of natural products: over 23 000 individual compounds were identified to data (Tarshis *et al*., 1996).

2
Formation of Isopentenyl Diphosphate

Despite their structural of functions and diversity, all isoprenoids derive from the common five-carbon (C_5) building unit isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), also called isoprene units. The IPP formation, first investigated in yeast and in mammalian liver tissue, had been described as the acetate/mevalonate pathway which was later accepted as ubiquitous in all living organisms (Spurgeon & Porter, 1981). Until recently, it was generally assumed that all isoprenoids were synthesized from acetil-CoA via the classical mevalonate pathway to the central precursor isopentenyl diphosphate. A few years ago, a totally different route, in which mevalonate, is not a precursor and where IPP is formed from glyceraldehydes 3-phosphate (GAP) and pyruvate was found in bacteria and green algae (Rohmer *et al*., 1993; Broers, 1994; Rohmer *et al*., 1996; Schwender *et al*., 1996), and in plants (Schwarz, 1994). This pathway was originally named non-mevalonate pathway or Rohmer pathway. After the identification of the first steps of the pathway, its name was changed to indicate the substrates (pyruvate/ glyceraldehyde 3-phosphate [G3P] pathway) or the first intermediate, deoxyxylulose (DX) 5-phosphate (DXP pathway). According Rodríguez-Concepción and Boronat (2002), it is becoming more accepted to name the pathway after what is currently considered its first committed precursor, methylerythritol 4-phosphate (MEP), following the same rule used to name the MVA pathway.

Pyruvate and glyceraldehyde are mainly obtained by glycolysis. In the Embden-Meyerhof-Parnas pathway, glucose is converted into fructose 1,6-diphosphate that is cleaved into

dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Figure 1). Labeling experiments, with [^{14}C]acetate and [^{14}C]mevalonate, had shown that in several non-green tissues of higher plants (Braithwaite & Goodwin, 1960) and in the alga *Euglena gracilis* (Steele & Gurin, 1960), the carotenoids were labeled in a pattern that seemed similar to the general isoprenoid labeling pattern found, for example, in squalene derivatives in other organisms. This was interpreted on the basis of the classical acetate/mevalonate pathway. Thus there was no reason to believe that the biosynthesis of isoprenoids in higher plants or algae occurred other than via the acetate/mevalonate pathway.

However, Schwender *et al*. (1996) found that the antibiotic mevinolin, a highly specific inhibitor of mevalonate and sterol biosynthesis, efficiently blocked sterol biosynthesis in higher plants but did not affect the formation of chlorophylls and carotenoids in plastid. According these authors the experiments suggested that mevinolin couldn't penetrate the chloroplast or, more likely that chloroplasts possessed a separate and different biosynthetic pathway for IPP formation, which efficiently was blocked by mevinolin.

This novel pathway leading to IPP formation, had been detected in several eubacteria by Rohmer *et al*. (1993). Incorporation of ^{13}C-labelled precursors into triterpenoids of the several bacteria showed that these species did not use the acetate/mevalonate pathway for the formation of isoprenoids, but instead use precursors derived from triose phosphate metabolism. Figure 1 represents glycolysis of [1-^{13}C]glucose (·the ^{13}C-label, which arises from feeding of [1-^{13}C]glucose to plant seedlings and cell cultures) and formation of IPP via two different pathways, according Lichtenthaler *et al*. (1997):

a) [3-^{13}C]glyceraldehyde 3-phosphate (GAP) and [3-^{13}C] pyruvate derive from [1-^{13}C] glucose via glycolysis, [2-^{13}C] acetyl-CoA derives from [3-^{13}C] pyruvate by the pyruvate dehydrogenase complex.
b) According to the classical acetate/mevalonate pathway, observed in sterol biosynthesis, IPP is built up from acetyl-CoA ([2-^{13}C] acetyl-CoA) by the following main reactions: (1) Two molecules of acetyl-Coenzyme A (Ac-CoA) form acetoacetyl-CoA (AcAc-CoA). (2) Addition of a third molecule of Ac-CoA yields hydroxymethylglutaryl-CoA (HMG-CoA). (3) (HMG-CoA) is reduced to mevalonic acid (MVA). (4 and 5) MVA is phosphorylated twice at C_5. (6) MVA-5-diphosphate yields IPP via a decarboxylation/elimination step.
c) The novel IPP-biosynthesis pathway is based on ^{13}C-incorporation. Studies were performed with bacteria (Rohmer *et al*., 1993; Rohmer *et al*., 1996) and the green alga *Scenesdemus obliquus* (Schwender *et al*., 1996). According to this pathway, also found for the formation of chloroplast isoprenoids, the addition of a C_2 precursor (derived from pyruvate, most likely by formation of hydroxyethyl thiamine (TPP=thiamine diphosphate) to a C_3 precursor (glyceraldehydes 3-phosphate GAP) yields a first C_5 intermediate, most likely D-1-deoxyxylulose 5-phosphate [(Broers, 1994, in Lichtenthaler *et al*. (1997)]. The carbon skeleton of this intermediate or another related C_5-derivative undergoes a rearrangement reaction that provides the branched carbon skeleton of IPP.

According to ^{13}C-labeling experiments Lichtenthaler *et al*. (1997) have concluded that the cytoplasmic sterols are formed in all three higher plants via the acetate/mevalonate pathway, whereas the plastidic isoprenoids are synthesized via a new non-mevalonate IPP pathway. In this new pathway, IPP is formed from pyruvate and glyceraldehydes 3-phosphate (Rohmer *et al*., 1996) yielding, after condensation, 1-deoxyxylulose-5-phosphate, which is most likely the first C_5 in the alternative IPP biosynthesis pathway (Figure 1). A transposition (Rohmer *et al*., 1993) yields, finally, the branched isoprenic skeleton from the straight-chain deoxypentulose framework (Figure 1). The ^{13}C-labeling experiments done by Lichtenthaler *et al*. (1997) suggest that this mevalonate-independent route is not restricted to bacteria and green algae, but also possesses a wide distribution in plastids of higher plants.

According Lichtenthaler *et al*. (1997) the plastid-derived isoprenoids of plants, including carotenoids and the prenyl side chains of chlorophyll and plastoquinone, as well as isoprene (Zeidler *et al*., 1997), monoterpenes (Eisenreich *et al*., 1997) and diterpenes (Eisenreich *et al*., 1996; Schwarz, 1994) are synthesized via the pyruvate/ GAP route to IPP.

Rohmer *et al*., 1996) working with *E. coli* mutants defective in enzymes of the triose phosphate metabolism suggested that the first reaction of the novel pathway involved the head-to-head condensation of (hydroxyethyl) thiamin derived from pyruvate with the C_1 aldehyde group of G3P to yield DXP. Three independent approaches led to the identification of the first gene of the MEP pathway, encoding DXP synthase (Sprenger *et al*., 1997; Lois *et al*., 1998; Lange *et al*., 1998)

Lois *et al*.(1998) have described the cloning of a gene encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS) from *Escherichia coli*, but they did not give any sequence information. Lange *et al*. (1998) have cloned and characterized this gene. They described the cloning, heterologous expression, and transcriptional regulation of the gene encoding DXPS from peppermint. According Lange *et al*. (1998)

the cloning of DXS from peppermint provides direct evidence for the presence in plants of the plastidial mevalonate-independent pathway, which operates in parallel with the classical, cytosolic mevalonate pathway to IPP to produce a very broad range of isoprenoid compounds. The mevalonate-independent pathway offers a novel approach to transgenic manipulation of plant isoprenoid biosynthesis, and because this new pathway is present in bacteria and plants but not animals, it provides a unique target for the design of highly specific antibiotics and herbicides. They proposed also the mechanism of DXS (Figure 2). The addition of hydroxyethyl TPP, formed by decarboxylation of pyruvate, to C_1 of GAP and subsequent loss of TPP yields 1-deoxy-D-xylulose 5-phosphate, which ultimately gives rise to IPP. The circled P denotes the phosphate moiety (Lange *et al*., 1998)

Kuzuyama *et al*. (1998) and Takahashi *et al*. (1998) have identified the bacterial gene encoding DXP reductoisomerase (DXR), the enzyme that converts DXP into MEP.

Rohdich *et al*. (2000) showed, in experiments with the recombinant enzymes from *E. coli* and tomato (*Lycopersicon esculentum*), that *E. coli ygbp* encoded a CDP-ME synthase (CMS) that produced CDP-ME from MEP and CTP (Figure 3). Herz *et al*. (2000) and Lüttgen *et al*. (2000) studied that the recombinant enzyme encoded by the *E. coli ychB* gene was a CDP-ME kinase (CMK) that catalyzes the ATP– dependent phosphorylation of CDP-ME to CDP ME 2-phosphate (CDP-MEP). This compound was then converted into ME 2,4-cyclodiphosphate (ME-cPP) by the enzyme ME-cPP synthase (MCS), encoded by the E. coli *ygbB* gene. Charon *et al*. (2000) and Rodríguez-Concepción *et al*. (2000) have demonstrated that the MEP pathway branched at some point after MEP leading to the separate synthesis of IPP and DMAPP. Hecht *et al*. (2001), Seemann *et al*. (2002a), Seemann *et al*. (2002b) and Wolff *et al*. (2002) showed that the *gcpE* gene product encoded an enzyme (hydroxymethylbutenyl 4-diphosphate [HMBPP] synthase [HDS]) that catalyzes the formation of HMBPP from ME-cPP (Figure 3). Rohdich *et al*. (2002) showed that *lytB* encode an enzyme (IDS) that directly coverts HMBPP into a 5:1 mixture of IPP and DMAPP. The branching is an important difference (Figure 1 and Figure 3) with the MVA pathway, in which IPP and DMAPP are generated sequentially, the latter arising from the former in a reaction catalyzed by IPP isomerase (Rodríguez-Concepción & Boronat (2002).

3
Assembly of the C_{40} Backbone

3.1. IPP isomerase

IPP is the fundamental C_5 biosynthetic unit from which the carotenoids, and indeed all terpenoids, are constructed. However, an isomerization of IPP into dimethylallyl diphosphate (DMAPP) must occur before chain elongation can begin.

Figure 1
Glycolysis of [1-^{13}C] glucose and formation of isopentenyl diphosphate (IPP) via two different pathways (Lichtenthaler et al., 1997).

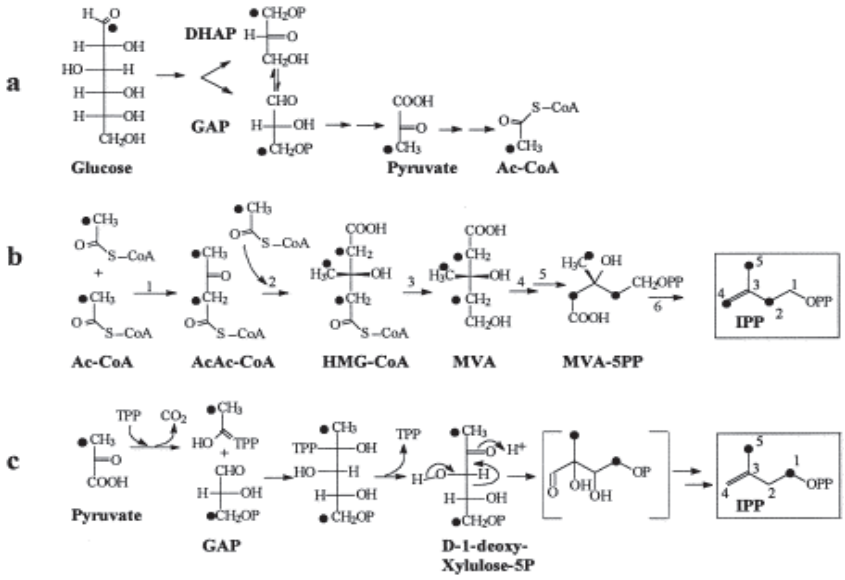


Figure 2
Proposed mechanism of DXS (Lange et al., 1998)

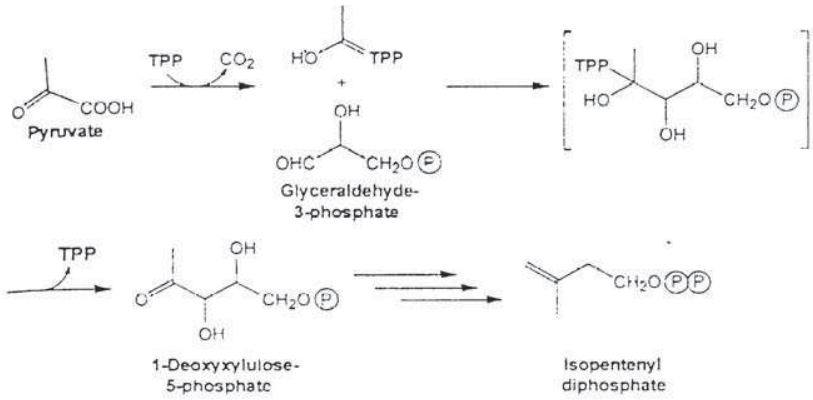
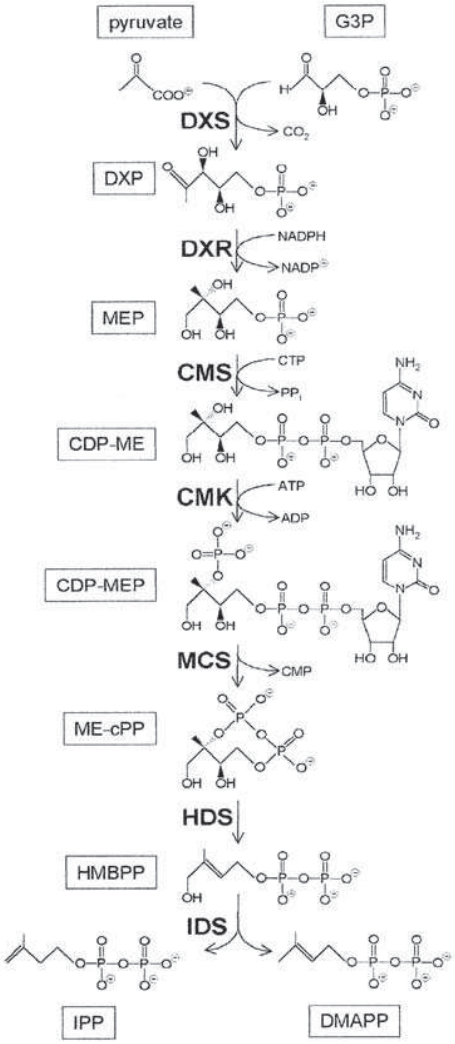


Figure 3
The MEP pathway (Rodríguez-Concepción & Boronat, 2002).



DMAPP this then adds two further IPP units to produce successively farnesyl diphosphate (FPP), the C_{15} precursor of sterols and triterpenes and the C_{20} geranylgeranyl diphosphate (GGPP).

Britton (1988) refers that the reaction catalysed by IPP isomerase is reversible, the proportions at equilibrium of IPP and DMAPP being approximately 1:9. The olefinic protons of IPP are stereochemically distinct; conversion of IPP into DMAPP destroys this stereospecificity. Isomerisation of DMAPP can then give any one of three species of IPP, including an equal mixture of the two-labeled species (xiv) and (xv) (Figure 4).

DMAPP is the initial, activated substrate for formation of long chain polyisoprenoid compounds such as GGPP. The formation of DMAPP from IPP is a reversible reaction that is catalyzed by the enzyme IPP isomerase (EC 5.3.3.2). This soluble enzyme has been isolated from pepper (Dogbo & Camara, 1987) and daffodil (Lützow & Beyer, 1988), but more detailed information on enzyme structure, cofactors, and reaction mechanisms is available from studies of the related yeast and mammalian enzymes (Reardon & Abeles, 1986; Street *et al*., 1994; Hahn *et al*. 1996).

Blanc & Pichersky (1995) have identified a plant cDNA for IPP isomerase in *Clarkia brewerii* (*Ipi1*). Blanc *et al*. (1996) have reported a second *C. brewerii* gene (*Ipi*2). The Arabidopsis *Ipp*2 (GenBank accession number U49259) predicts a polypeptide of 284 amino acids (32 607 mol. wt.) with an N-terminal extension, relative to the mammalian and bacterial enzymes, that has been suggested to target this enzyme to the chloroplast (Blanc & Pichersky (1995). The Arabidopsis *Ipp1* (U47324) predicts a polypeptide of 233 amino acids (27 110 mol. wt.) and lacks the N-terminal extension of *Ipp*2, thereby is suggesting a cytosolic location (Cunningham & Gantt, 1998).

Both plastid and cytosolic locations for IPP isomerase are amply supported by evidence (Dogbo & Camara, 1987; Kleinig, 1989; Bach, 1995). A peroxisomal location for a human enzyme has been reported (Paton *et al*. 1997), and a mitochondrial location for a plant has been reported (Lützow & Beyer, 1988).

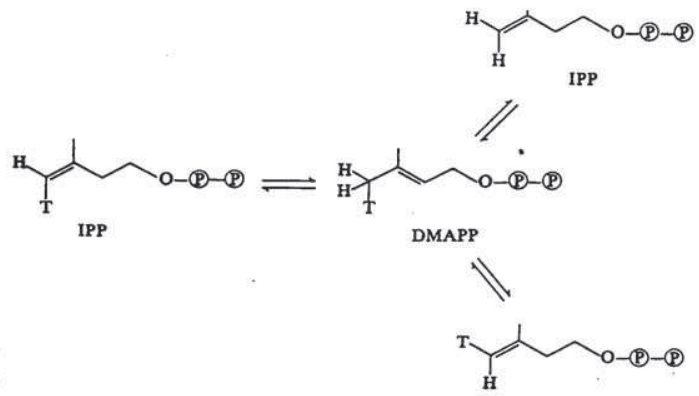
According Cunningham & Gantt (1998) the localization in several different cell compartments ordinarily implies the existence of specific genes or multiple transcripts to produce polypeptides targeted to these compartments. As yet, no more than two different cDNAs or genes have been identified for any plant.

A reversible isomerization reaction as catalyzed by the IPP isomerase would seem to be an unlikely candidate for a controlling or regulatory step in isoprenoid biosynthesis. However, it has been found that the activity of this enzyme in *E. coli* is limiting for isoprenoid production as indicated by the accumulation of carotenoids in strains engineered to produce these pigments (Cunningham & Gantt, 1998). Introduction of any of a number of different plant, algal, or yeast IPP isomerase cDNAs, or of additional copies of the *E. coli* gene for this enzyme, enhances the accumulation of carotenoid pigments by several-fold (Kajiwara *et al*., 1997; Sun *et al*., 1996; Sun *et al*., 1998). These observations raise the possibility that IPP isomerase activity might also limit biosynthesis of carotenoids and other isoprenoids in plants.

3.2. GGPP Synthase

The isoprenoid biosynthetic pathway is built around a family of diphosphate esters of linear alcohols that contain increasing numbers of isoprene units. Beginning with the C_5

Figure 4
Loss of stereospecificity of hydrogen labeling at C4 of IPP due to isomerization to DMAPP (Britton, 1988).

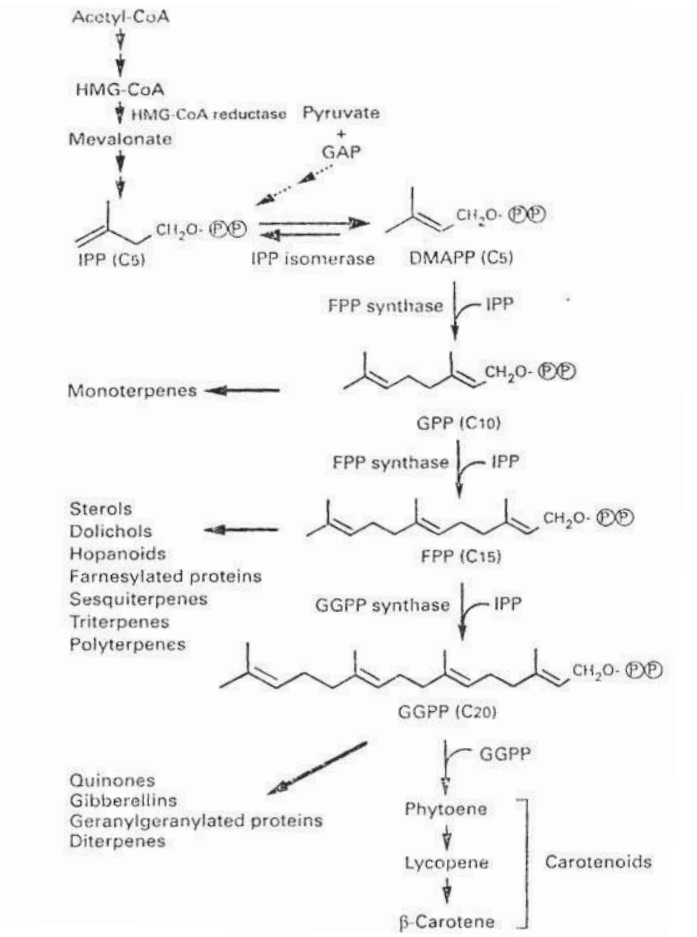


molecule dimethylallyl diphosphate (DMAPP), a series of C_{10} (geranyl diphosphate, GPP), C_{15} (farnesyl diphosphate, FPP), C_{20} (geranylgeranyl diphosphate, GGPP) and higher molecular weight isoprenoid diphosphate are formed by the 1′-4 addition of IPP to the growing chain (Figure 5). These compounds are the substrates for biosynthesis of all isoprenoid metabolites, including monoterpenes, sesquiterpenes, diterpenes, sterols, carotenoids, ubiquinones, dolichols and prenylated proteins (Tarshis *et al*., 1996). The 1′-4 condensation reactions are catalyzed by a family of prenyltransferases, the IPP synthases, which are highly selective for the chain lengths and double bond stereochemistry of both substrates and products. For example, *Saccharomyces cereviseae* contains at least four distinct enzymes for chain elongation. FPP synthase (FPPS) converts DMAPP to FPP for synthesis of sterols, farnesylated proteins, heme a, and higher chain length isoprenoid diphosphates (Anderson *et al*., 1989). The yeast GGPP synthase converts FPP to GGPP for synthesis of geranylgeranylated proteins (Jiang *et al*., 1995).

The 20-carbon GGPP, which serves as the immediate precursor for carotenoids biosynthesis, is formed by the sequential and linear addition of three molecules of IPP to one molecule of DMAPP (Figs. 5 and 6). The enzyme that catalyses these reactions, the GGPP synthase (GGPPS; EC 2.5.1.29), is one member of a closely related family of prenyltransferase enzymes that are distinguished by the length of the final product (Ogura *et al*., 1997). A molecular understanding of the basis for the determination of chain length by these prenyltransferases has emerged from the construction and functional analysis of site directed mutants (Tarshis *et al*. 1996) and from the selection and analysis, after random mutagenesis, of enzymes with altered function (Scolnik & Bartley, 1993).

Tarshis *et al*. (1994) studied avian FPPS which catalyses the sequential chain elongations of DMAPP to GPP and GPP to

Figure 5
Formation of geranylgeranyl diphosphate (GGPP) from isopentenyl diphosphate (Kajiwara *et al*., 1997).



FPP. The enzyme is a homodimer, and the sub-units each contain a single site for the C_5 to C_{15} elongation.

Although these enzymes catalyze similar condensation reactions, they do not catalyze the condensation beyond the limit of the chain length of product determined by their own specificity. Why does the condensation stop at the step that is determined by each enzyme? It is not easy to understand the mechanisms that force each prenyltransferase to yield its intrinsic product.

During the past few years the amino acid sequences of FPP synthases (Clarke *et al*., 1987; Anderson *et al*.; 1989; Fujisaki *et al*., 1990; Wilkin *et al*.; 1990; Koyama *et al*., 1993) and GGPP synthases (Misawa *et al*.; 1990; Carattoli *et al*.; 1991; Cheniclet, *et al*., 1992; Math *et al*.; 1992; Chen *et al*., 1994; Ohnuma *et al*., 1994) have been determined. In FPP synthase, several groups (Marrero *et al*., 1992; Joly & Edwards, 1993; Asai *et al*.; 1994; Koyama *et al*., 1994; Song & Poulter, 1994) have carried out site-directed mutagenesis studies with special attention to the two aspartate-rich domains. These studies have indicated that the aspartate-rich domains are essential for catalytic activity. It is suggested that the aspartate residues bind the diphosphate moieties of IPP and allylic substrate through a magnesium bridge.

Ohnuma *et al*. (1996) with their work tried to obtain information about amino acid residues that are related to chain-length determination. Their results indicated that the binding of allylic substrate to prenyltransferase causes a conformational change that affects the affinity of IPP. Moreover, during the consecutive reaction of prenyltransferase, a series of conformational changes might occur, and the changes might be essential for the prenyltransferase reaction.

Kuntz *et al*. (1992) have shown that the gene which codifies GGPPS is expressed in all tissues in which carotenoids biosynthesis occurs. They have shown also that the expression of the GGPPS gene increased in ripening fruits in parallel in carotenoids biosynthesis during chloroplast to chromoplast differentiation in fruits. The deduced amino acid sequence of pepper GGPPS shows regions of homology to bacterial and fungal prenyltransferases, including conserved diaspartic (DD) and diarginine (RR) residues. The conserved DD residues are involved in catalytic function because treatment of the *E. coli*-produced protein with a carboimide reagent that reacts with free carboxyl groups inhibited GGPPS activity (Kuntz *et al*., 1992). Alignment of the deduced pepper GGPPS sequence with the bacterial and fungal counterparts indicates that the plant protein contains a 60-residue amino terminal extension, likely to correspond a transient peptide for plastid localisation.

According Tarshis *et al*. (1996) an analysis of the x-ray structure of avian FPPS suggested that the ultimate length of the polyisoprenoid chain obtained during successive condensations of the growing allylic substrate with IPP is governed by the size of a hydrophobic pocket in the interior of the enzyme.

A GGPP synthase has been isolated as a soluble and functional homodimer from the chromoplasts of pepper (Dogbo & Camara, 1987), and the corresponding cDNA has been identified and sequenced (Kuntz *et al*., 1992). Immunolocalization experiments confirmed a predominant localization in the chromoplast for GGPP synthase in pepper fiuits (Kuntz *et al*., 1992). The enzyme was not, however, distributed homogeneously throughout the stroma; rather, it appeared to be concentrated in discrete locations, in particular at the developing stroma globuli where carotenoid accumulation is thought to occur (Cheniclet *et al*., 1992).

In Arabidopsis, five different cDNA or genomic clones that predict polypeptides with substantial sequence similarity to the pepper GGPP synthase have been identified (Scolnik & Bartley, 1994; Scolnik & Bartley, 1995; Scolnik & Bartley, 1996; Zhu *et al*., 1997). One of the products of one of cDNA, GGPSI, encodes a 371-amino acid polypeptide (40206 mol. wt.) with an N-terminal extension of 76 amino acids, relative to bacterial enzymes, that has been suggested to target this enzyme to the chloroplast (Scolnik & Bartley, 1994). However, the specific roles and subcellular locations of the various Arabidopsis GGPP synthases have not been ascertained, whether there might be a specific isoform of GGPP synthase in the plastid that is dedicated to carotenogenesis is also unknown. The presence of GGPP synthase genes in bacterial carotenogenic gene clusters underscores the importance of this enzyme in carotenoid biosynthesis (Cunningham & Gantt, 1998).

3.3. Phytoene Synthase

The formation of the symmetrical 40-carbon phytoene (7,8,11,12,7',8',11',12'-octahydro-ψ,ψ-carotene) from two molecules of GGPP (Figure 6) is the first reaction specific to the pathway of carotenoid biosynthesis. The biosynthesis of phytoene from GGPP is a two-step reaction catalyzed by the enzyme phytoene synthase (PSY; EC 2.5.1.32). The sequence of reactions is common to all terpenoids, including other plastid terpenoids (chlorophylls, plastoquinones, tocopherols, phylloquinones, and polyterpenes). However the two steps in which prephytoene diphosphate (PPPP) and phytoene are formed, are catalyzed by the first carotenoids-specific enzymes

in the pathway. The reaction has two steps: (1) the coupling of two molecules of geranylgeranyl diphosphate to yield prephytoene diphosphate and (2) the conversion of prephytoene diphosphate to phytoene. The identification of the enzyme components catalyzing these two steps is crucial for the understanding of the molecular mechanism of the carotenoids biosynthesis and of its regulation; a particularly interesting question is whether the two activities reside on two different enzymes or on the same enzyme (Cunningham & Gantt, 1998).

Using *Capsicum* chromoplasts stroma, Dogbo *et al* (1988) have isolated and characterized a bifunctional enzyme that catalyze the synthesis of phytoene. They showed that a single monomeric protein (mol. wt. 47500) catalyzes the dimerization of geranylgeranyl diphosphate into prephytoene diphosphate and the conversion of the latter into phytoene. The two reactions followed conventional Michaelis-Menten kinetics, with Km values of 0,30mM and 0,27mM, respectively, for GGPP and PPPP. The activity of the enzyme depends strictly upon the presence of Mn^{2+}. This selectivity may be one of the factors regulating the competition with potentially rival enzymes converting GGPP into other plastid terpenoids. The two-enzymatic reactions were inhibited by inorganic pyrophosphate and by the arginine-specific reagent hydroxyphenylglyoxal. In no instance were the two reactions kinetically uncoupled. These properties strongly suggested, to the authors, that the same enzyme catalyses the two consecutive reactions and they proposed the name phytoene synthase.

Scolnik & Bartley (1994b) studying the nucleotide sequence of an Arabidopsis cDNA for PSY have predicted a polypeptide of 423 amino acids with a mol. wt. of 47611, but according Karvouni *et al*. (1995) the mature size will be probably about 40 kDa. Bartley *et al*. (1992) have concluded that this enzyme may normally be loosely if not tightly associated with chloroplast or chromoplast membrane. A specific requirement for galactolipid was demonstrated for catalytic activity of the PSY of *Narcissus pseudonarcissus* (daffodil) chromoplasts (Schledz *et al*., 1996). A membrane association of PSY is expected because of the need to deliver the lipid-soluble phytoene to the membranes of the chloroplast where phytoene and subsequent intermediates and end products of the pathway are localised (Cunningham & Gantt, 1998).

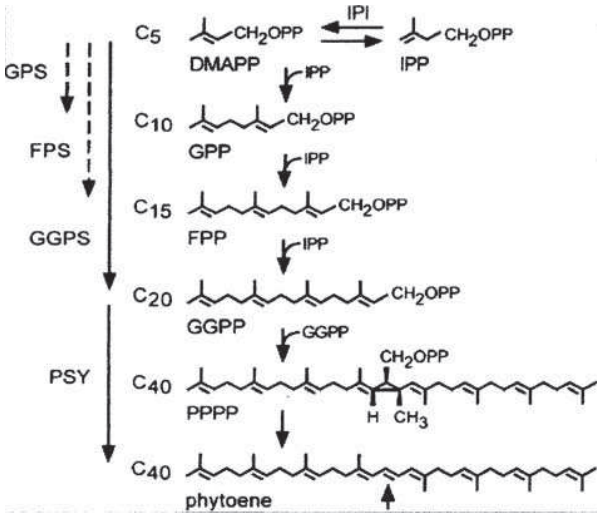
4
Desaturation and Cyclization

4.1. The Desaturases

Phytoene undergoes a series of four desaturation reactions (Figure 7) that result in the formation of first phytofluene (7,8,11,12,7',8'-hexahydro-Ψ,Ψ-carotene) and then, in turn, ξ-carotene (7,8,7',8'-tetrahydro-Ψ,Ψ-carotene), neurosporene (7,8-dihydro-Ψ,Ψ-carotene), and lycopene (Ψ,Ψ-carotene). These desaturation reactions serve to lengthen the conjugated series of carbon-carbon double bonds that consti-

Figure 6

The C_{40} carotenoid phytoene is derived by a head-to-head condensation of two molecules of the C_{20} geranylgeranyl pyrophosphate (GGPP), which itself is assembled from three molecules of the C_5 isopentenyl diphosphate (IPP) and one molecule of its isomer, dimethylallyl diphosphate (DMAPP). FPP (farnesyl diphosphate), GPP (geranyl diphosphate), PPPP (prephytoene diphosphate) (Cunningham & Gantt, 1998).



tutes the chromophore in carotenoid pigments, and thereby transform the colourless phytoene into the pink-coloured lycopene (Cunningham & Gantt, 1998).

At each stage, two hydrogen atoms are removed by trans-elimination from adjacent positions to introduce a new double bond and extend the conjugated polyene chromophore by two double bond (McDermott *et al*., 1973).

The four sequential desaturations undergone by phytoene are catalysed by two related enzymes in plants: phytoene desaturase (PDS) and ξ-carotene desaturase (ZDS). Armstrong (1994) and Sandmann (1994) have described that bacteria and fungi achieve the same result with a single gene product (CRTI).

Plant and cyanobacterial PDS are unusually well conserved in amino acid sequence. Scolnik & Bartley (1993) have studied PDS from Arabidopsis concluding that is a polypeptide of 566 amino acids (61964 mol. wt.).

A cDNA encoding a ZDS was identified by functional analysis in *E. coli* of a pepper cDNA that predicts a plant enzyme mediating zeta carotene desaturation, which is a polypeptide distantly resembling the known plant PDS (Albrecht *et al*., 1995). The pepper ZDS is about equidistant from plant and cyanobacterial PDS in predicted amino acid sequence comparisons (33-35% identities). An Arabidopsis homologue of the pepper ZDS was described by Scolnik & Bartley (1996), which is a polypeptide of 558 amino acids (60 532 mol. wt.).

Hugueney *et al*. (1995) have characterized a flavoprotein which catalyses the synthesis of phytofluene and zeta carotene in *Capsicum* chromoplasts.

Bramley (1985) has demonstrated that the desaturases are membrane-associated in plants although the predicted amino acid sequences are not particularly hydrophobic overall. From a titration of the amount of detergent required to release PDS from daffodil chromoplast membranes, it was concluded that PDS is not an integral membrane protein (Schledz *et al*., 1996).

According Britton (1979) the presence of the oxidised coenzymes FAD or NADP (or both) was essential, though their direct involvement in the desaturation reactions has not been proved. He suggested the involvement of the some kind of simple electron transport system related to cytochrome P450 in the desaturations. The oxidized coenzymes could be required to maintain the electron transport components in the required oxidation state.

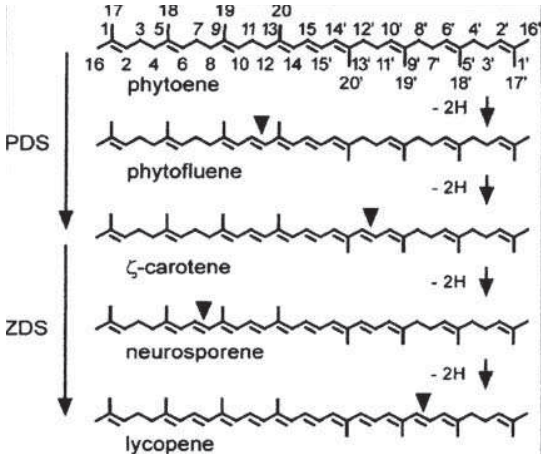
Schledz *et al*. (1996) observed for the daffodil PDS, that FAD must be bound before or at the time of membrane integration, otherwise the membrane-associated enzyme will not be active. Enzyme assays of the flavinylated membrane-associated enzyme do not require additional FAD, supporting a role for tightly bound FAD as cofactor and implicating a membrane-associated electron acceptor. Mayer *et al*. (1990) and Nievelstein *et al*. (1995) demonstrated the involvement of quinones as electron acceptors for the desaturase reactions.

4.2-The Cyclases

The carotenoids in the photosynthetic apparatus of plants are bicyclic compounds, most commonly with two β or modi-

Figure 7

A series of four consecutive desaturation reactions at the 11-12, 11'-12', 7-8, and 7'-8' positions extend the conjugated series of double bonds that constitutes the chromophore in carotenoid pigments. Double bonds introduced by the desaturation reactions are indicated by inverted triangles. Conventional numbering of the carbon atoms is shown for phytoene (Cunningham & Gantt, 1998).



fied β rings (Figure 8). The cyclization reaction would be the same when acyclic intermediates are lycopene or neurosporene. β-, γ– and ε-end groups are formed by proton loss from alternative positions in the same transient carbonium ion intermediate (Britton, 1988). The proposed mechanism for cyclization involves initial proton attack at C_2 of the acyclic precursor. The incoming hydrogen atom would be retained at C_2 of the cyclic carotenoids formed (Figure 8).

A single gene product, the lycopene β-cyclase (LCYB), catalyzes the formation of the bicyclic β-carotene (Figure 9) from the linear, symmetrical lycopene in plants and cyanobacteria (Cunningham *et al*., 1994; Cunningham *et al*., 1996; Hugueney *et al*., 1995; Pecker *et al*., 1996). Some authors (Cunningham *et al*., 1994; Cunningham *et al*., 1996; Pecker *et al*., 1996) thought that desaturation of the 7-8 carbon-carbon bond were a prerequisite for the cyclization reaction. However, in a subsequent report on a pepper LCYB it was found that the bicyclic compound 7,8-dihydro-β,β-carotene was produced in *E. coli* when neurosporene (7,8,-dihydro-ψ,ψ-carotene) was provided as the substrate (Tarshis *et al*., 1996). According Cunningham & Gantt (1998) the desaturation at the 7-8 position, while not an absolute requirement for cyclization, may play a role in substrate recognition and/or binding. An association of the cyclases in the form of a homodimer could explain cyclization at both ends of neurosporene and the lack of cyclization for ξ-carotene. Neurosporene, recognised and bound at one end by virtue of desaturation of the 7-8 carbons at that end, would, at the same time, be constrained at the other end of the molecule in close proximity to and in the proper orientation for cyclization by a second cyclase subunit.

Lutein, the predominant carotenoid in the photosynthetic tissues of many plants and algae, has one β ring and one ε ring. Carotenoids with two ε rings are uncommon in plants and algae (Goodwin, 1980). The ε ring differs from the β ring only in the position of the double bond within the cyclohexene ring (Figures 8 and 9). Cunningham *et al*. (1996) have identified a

Figure 8
Mechanism of the formation of β-, γ– and ε– rings from a common precursor (Britton, 1988)

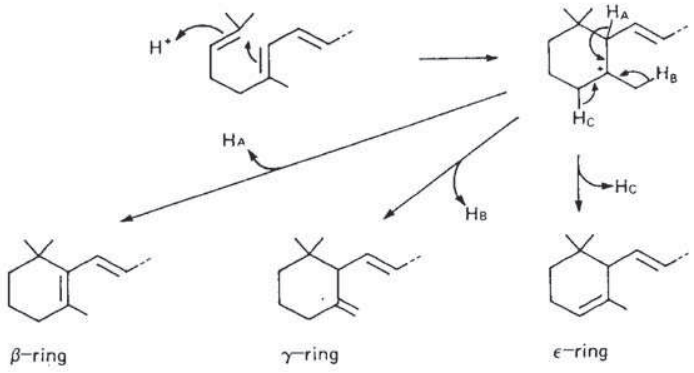
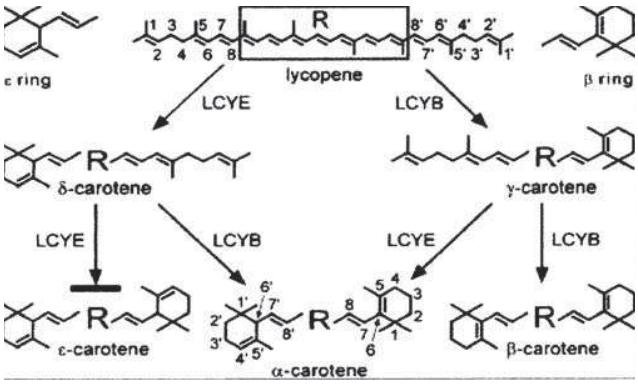


Figure 9
Cyclization of lycopene is a branch point in carotenoid biosynthesis. β-Carotene, with two β rings, is an essential end product and serves as the precursor for several other carotenoids that are commonly found in the photosynthetic apparatus of plants. α-Carotene, with one β and one ε ring, is the immediate precursor of lutein, the predominant carotenoid pigment in the photosynthetic membranes of many green plants (Cunningham & Gantt, 1998).



cDNA encoding the enzyme that catalyses formation of the ε ring in Arabidopsis; it was identified by making use of the pink-to-yellow color change that accompanies lycopene cyclization in *E. coli*. The lycopene ε-cyclase (LCYE) of Arabidopsis is a homologue of the β-cyclase, and related, single-copy genes encode both enzymes. The ε-cyclase adds only one ring to the symmetrical lycopene, forming the monocyclic δ-carotene (e,y-carotene). Cunningham *et al*. (1996) have showed that when combined, the β-and ε-cyclases convert lycopene to α-carotene (β, ε-carotene), a carotenoid with one β and one ε ring that serves as the precursor for formation of lutein.

The inability of the ε-cyclase of Arabidopsis to add more than one ε ring to lycopene has been suggested to provide a mechanism for control of cyclic carotenoid formation (Cunningham *et al*., 1996). The apportioning of substrate into the pathways leading to β,ε-carotenoids (e.g. the abundant lutein) and to β,β-carotenoids (e,g, β-carotene, zeaxanthin, and violaxanthin) could be determined quite simply by the relative amounts and/or activities of the ε– and β-cyclase enzymes.

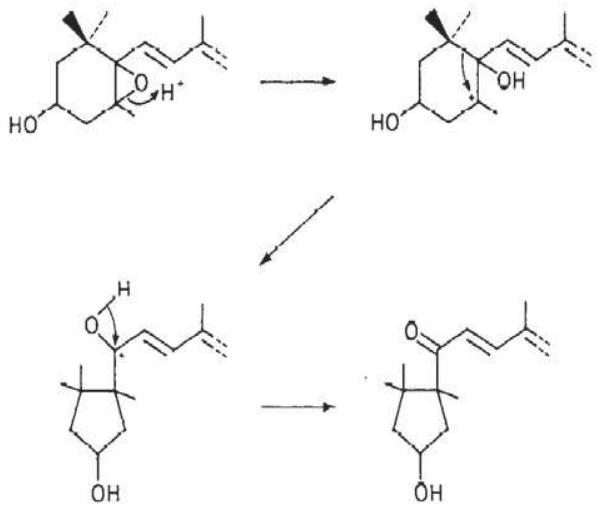
Carotenoids with two ε rings are not commonly found in plants. Lettuce is one of the rare examples of plants known to accumulate substantial amounts of a carotenoid with two ε– rings: lactucaxanthin (ε,ε-carotene-3,3'-diol; Siefermann-Harms *et al*., 1981). The ε-cyclase of romaine lettuce has been found to be a dose homologue of the Arabidopsis ε –cyclase (ca 80% identity for the amino acid sequences).

The signature pigments of pepper, capsanthin and capsorubin, contain an unusual cyclopentane ring (the κ ring-Figure10) that is formed from the 3-hydroxy-5,6-epoxy-β rings found in violaxanthin and antheraxanthin. The capsanthin-capsorubin synthase enzyme (CCS) has been purified, and a cDNA encoding it has been identified and sequenced by Bouvier *et al*. (1994). The pepper CCS (498 amino acids and 56659 mol. wt.) closely resembles β-cyclases in its predicted amino acid sequence (Bouvier *et al*., 1994), and the gene product has more recently been shown to also possess a β-cyclase activity (Hugueney *et al*., 1995; Pecker *et al*., 1996). The pepper CCS was purified by Bouvier *et al*. (1994) from detergent-solubilized chromoplast membranes to yield a pale-yellow, intensely fluorescent polypeptide of approximately 60 kDa, as estimated by gel filtration.

The ε- and β-cyclases and CCS all have an amino acid sequence signature that are conserved in enzymes that bind dinucleotides such as FAD and NADP (Cunningham *et al*., 1996.

The predicted amino acid sequences of the Arabidopsis β-cyclase (501 amino acids and 56174 mol. wt.) and ε-cyclase (524 amino acids; 58512 mol. wt.) are as much as 100 amino acids longer at the N-terminus than the homologous *Synechococcus* enzyme (411 amino acids and 46085 mol. wt.) when the sequences are aligned (Cunningham *et al*., 1996). Highly conserved but distinctly different sequence regions in the β– and ε-cyclases suggest that the mature plant ε– and β-cyclases may retain 30-50 amino acids of N-terminal sequence upstream of the position coincident with the start of the cyanobacterial β-cyclase. This would yield polypeptides of slightly greater than 50 kDa (Cunningham & Gantt, 1998)

Figure 10
Proposed mechanism for formation of the κ-ring in capsanthin and capsorubin (Britton, 1988).



5
Xanthophyll Formation

5.1. The Hydroxylases

Xanthophylls or oxygenated carotenoids comprise most of the carotenoid pigment in the thylakoid membranes of plants. The dihydroxy carotenoid zeaxanthin (β,β-carotene-3,3'-diol) is thought to play a central role in the nonradiative dissipation of light energy under conditions of excessive photon capture by the photosynthetic light-harvesting apparatus (Demmig-Adams & Adams, 1993).

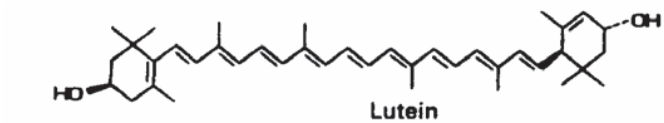
Hydroxylation at the number three carbon of each ring of the hydrocarbons β-carotene and α-carotene (Figure 9) will produce the well-known xanthophyll pigments zeaxanthin (β,β-carotene-3,3'-diol-Figure 13) and lutein (β,ε-carotene-3,3'-diol), respectively (Figure 11). Apart from the hydroxylation at C_1, which take place as early as the phytoene stage, oxygen functions and other structural modifications in carotenoids are believed to be introduced at the end of the biosynthetic sequences (Britton, 1988). Hydroxylation at C_3 of the β-ring (Figure 12) (e.g. in zeaxanthin and lutein) normally proceeds by direct replacement of the hydrogen precursor by OH (Britton, 1976).

Because the chirality of the hydroxyl on the e-ring is opposite that of the hydroxyl on the b ring of lutein (Britton, 1990), it is thought that different enzymes catalyze these reactions. Genetic evidence (Pogson *et al*., 1996) and functional analysis of an Arabidopsis β-hydroxylase enzyme (Block *et al*., 1983) support the existence of separate hydroxylases specific for the β– and ε-rings.

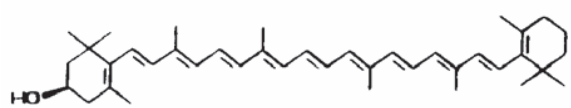
Zeaxanthin is formed from β-carotene (β,β-carotene) by hydroxylation (Figure 13). Zeaxanthin, in turn, serves as the substrate for biosynthesis of many other important xanthophylls. Sun *et al*. (1996) have identified an *A. thaliana* cDNA encoding the enzyme β-carotene hydroxylase, by functional complementation in *E. coli*. The product of this cDNA adds hydroxyl groups to both β rings of the symmetrical β-carotene (β,β-carotene) to form zeaxanthin and converts the monocyclic β-zeacarotene (7′,8′-dihydro-β,ψ-carotene) to hydroxy-ß-zeacarotene (7′,8′-dihydro-β,ψ-carotene-3-ol). The ε rings of δ-carotene (ε,ψ-carotene) and α-zeacarotene (7′,8′-dihydro-ε,ψ-carotene) are poor substrates for the enzyme. According Pogson *et al*. (1996) the inefficient hydroxylation of ε-rings in heterologous *E. coli* assay system is consistent with genetic evidence that implies the existence of a separate ε-hydroxylase. The chirality of the hydroxyl group in the ε-ring of lutein (β-ε-carotene-3,3'-diol) extracted from *Calendula officinalis* is opposite to that of the hydroxyl group in the b ring of this same compound which implies a separate ε– hydroxylase.

According Sun *et al*. (1996) the Arabidopsis β-hydroxylase cDNA predicts a polypeptide of 310 amino acids (34000 mol. wt. and a pI of 9,4). The identity between the predicted sequences of the A. *thaliana* and the bacterial hydroxylases ranges from 31-37% with more than one-fourth of the identically conserved residues being histidines. The β-carotene hydroxylase of A. *thaliana* is presumed to be located in the thylakoid membranes of the chloroplasts in this plant. They found that the sequence of the predicted enzyme includes an N-terminal region that is not found in the bacterial enzymes. They truncated the A. *thaliana* cDNA to examine whether and how much of this N-terminal extension was essential to enzyme function. The product of this truncated cDNA efficiently converted β-carotene to zeaxanthin (92-93% of the total carotenoid) in cells of *E. coli*. As in the «full– length» cDNA (which lacks the first 16 amino acids) a small amount of β-cryptoxanthin (7-8% of the total) was also detected (Figure 12). They made a second truncation removing the portion of the cDNA encoding the first 129 amino acids of the A. *thaliana* β-carotene hydroxylase. The product of this construct does hydroxylated most of the β-carotene produced in cells of *E. coli*. However, the major product (75-77% of the total) was β-cryptoxanthin (one ring available for hydroxylation) 16-18%

Figure.11
Chemical structures of some of the xanthophylls



Lutein



β-Cryptoxanthin

of zeaxanthin and 7% of β-carotene (two rings available for hydroxylation), indicating a marked preference of the truncated enzyme for β-ring of β-carotene. They concluded that the β-rings of β-carotene are hydroxylated with greater efficiency than the not yet hydroxylated β-ring of β-cryptoxanthin.

It was speculated that a portion of the cleaved N-terminal region might be involved in formation of enzyme homodimers (Sun *et al*., 1996), though other plausible explanations (e.g, accessibility of the second ring) could certainly be offered (Cunningham & Gantt, 1998). Fraser *et al*. (1997) studied the biosynthesis of astaxanthin in *vitro* and observed the requirement of oxygen and the catalytic effect of iron for two of bacterial β-hydroxylases, to produce astaxanthin. The authors noted the resemblance of a series of conserved histidine motifs (all of which are also present in the Arabidopsis enzyme) to those of enzymes containing nonheme iron. The arrangement of the histidine motifs and their position with respect to the predicted transmembrane helices are notable in their resemblance to the arrangement and positioning of similar motifs found in a structurally related group of oxygen-dependent, di-iron-containing membrane-integral enzymes (Shanklin *et al*., 1994; Shanklin *et al*., 1997; Shanklin & Cahoon, 1998). Members of this large and diverse group of di-iron oxygenases, which includes the membrane-associated fatty acid desaturases and various hydroxylases and oxidases such as the β-C-4-oxygenase or ketolase described in the following section, share an ability to attack unactivated carbons (Shanklin & Cahoon, 1998). They thereby provide an alternative class of enzymes with an ability to carry out the type of reactions usually associated with cytochromes P450

Figure 12

Stereochemistry of hydroxylation at C_3 in the β– and ε-rings (Britton, 1988).

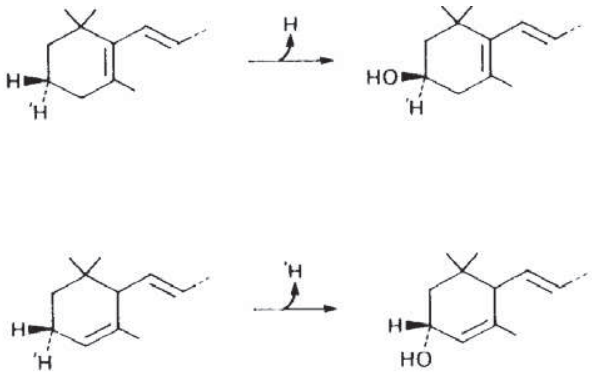
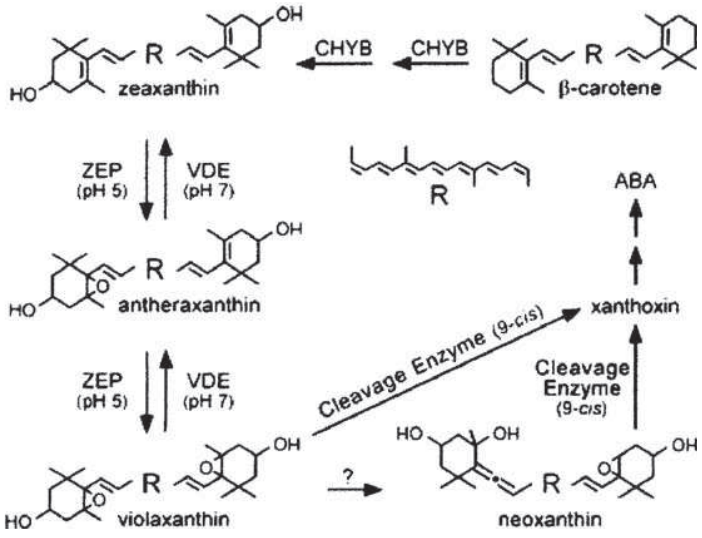


Figure 13

Formation of some common xanthophylls from β-carotene, and cleavage of 9-*cis* epoxycarotenoids to produce xanthoxin, the precursor of abscisic acid (ABA) (Cunningham & Gantt, 1998).



(Estabrook, 1996). Studies concerning the structure, function, cofactor requirements, and catalytic mechanisms of others in this class of enzymes should provide valuable insights into the function of the plant and bacterial β-ring hydroxylases and oxygenases (Cunningham & Gantt, 1998).

5.2. β-C-4-Oxygenase

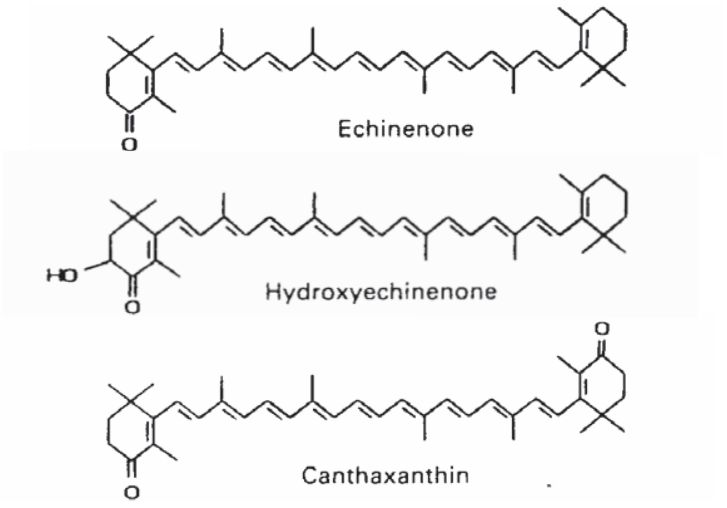
Addition of a keto group at the 4 position of one or both rings of the yellow β-carotene will produce the reddish-orange to red pigments (Figure 14) echinenone (β,β-carotene-4-one), canthaxanthin (β,β-carotene-4,4'-dione), hydroxyechinone (3-hydroxy-β,β-carotene-4-one) and astaxanthin. Lotan & Hirschberg (1995) studied a gene encoding β-C-4-oxygenase (a ketolase) that converts β-carotene to the ketocarotenoid canthaxanthin in *Haematococcus pluvialis*. Kajiwara *et al*. (1995) have isolated and identified a cDNA for astaxanthin biosynthesis from green alga *H. pluvialis*. The CrtO gene product of Lotan & Hirschberg (1995) and the *Bkt* gene product of Kajiwara *et al*. (1995) isolated from two different strains of *H. pluvialis* are very much alike (greater than 80% identity for the predicted amino acid sequences). Also Misawa *et al*. (1995a) and Misawa *et al*. (1995b) studied two bacterial ketolase enzymes, products of *crtW* gene of *Agrobacterium aurantiacum* and *Alcaligenes* sp. These polypeptides resemble those of the *H. pluvialis and Adonis palaestina*.

The *H. pluvialis* and bacterial ketolases (but not the *Synechocystis* ketolase) are, like the *Arabidopsis* and bacterial β-ring hydroxylases (and the *Adonis* ketolase), members of a large class of membrane-integral, di-iron oxygenase enzymes (Breitenbach *et al*., 1996). These authors and Fraser *et al*. (1997) observed a requirement for molecular oxygen and a stimulatory effect of iron for the *H. pluvialis Bkt* gene product and two bacterial *crtW* gene products. The 320-amino acid polypeptide (35989 mol. wt.) predicted by the *Bkt* of *H. pluvialis* has an N-terminal extension of 65 amino acids when aligned with the two bacterial CRTW enzymes (both 242 amino acids and ca. 27000 mol wt). The CrtW enzyme likewise has such an N-terminal extension. Truncation of the Bkt cDNA to yield a polypeptide lacking the first 32 amino acids did not eliminate enzyme activity in *E. coli*, but removal of 60 amino acids did result in loss of activity (Kajiwara *et al*., 1995a,b).

The end product ketocarotenoid in *H. pluvialis* and in many bacteria and fungi is the diketo, dihydroxy compound astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione). The initial report for the *crtO* gene product of *H. pluvialis* (Lotan & Hirschberg, 1995) indicated that the enzyme, expressed in *E. coli*, was unable to utilize the dihydroxy carotenoid zeaxanthin as a substrate. Therefore it was concluded by Lotan & Hirschberg (1995), that the route to astaxanthin proceeds with addition of the keto groups before hydroxylation. *Bkt* gene product, also expressed in *E. coli*, exhibited a similar preference for β rings lacking a 3-hydroxyl (Breitenbach *et al*., 1996). However, in *vitro* assay done by Fraser *et al*. (1997) with the *H. pluvialis* BKT showed only a moderate preference for β-carotene (no hydroxylation) over zeaxanthin (dihydroxy carotenoid).

A pathway (Figure 15) was proposed by Misawa *et al*. (1995b). The hydroxylation of β-carotene at positions 3 and 3′

Figure 14
Some ketocarotenoids (Britton, 1988)



on the β-ionone ring forming zeaxanthin via β-cryptoxanthin is mediate by the product of the gene designed *crtZ*, which has been isolated from *Erwinia* species (Misawa *et al*., 1990). The direct conversion of methylene to keto groups at positions 4 and 4′ on the β-ionone ring forming canthaxanthin via echinone are reactions performed by the gene product encoded by the *crtW* gene from marine bacteria (Misawa *et al*. 1995b) and *bkt* gene of *H. pluvialis* (Kajiwara *et al*., 1995).

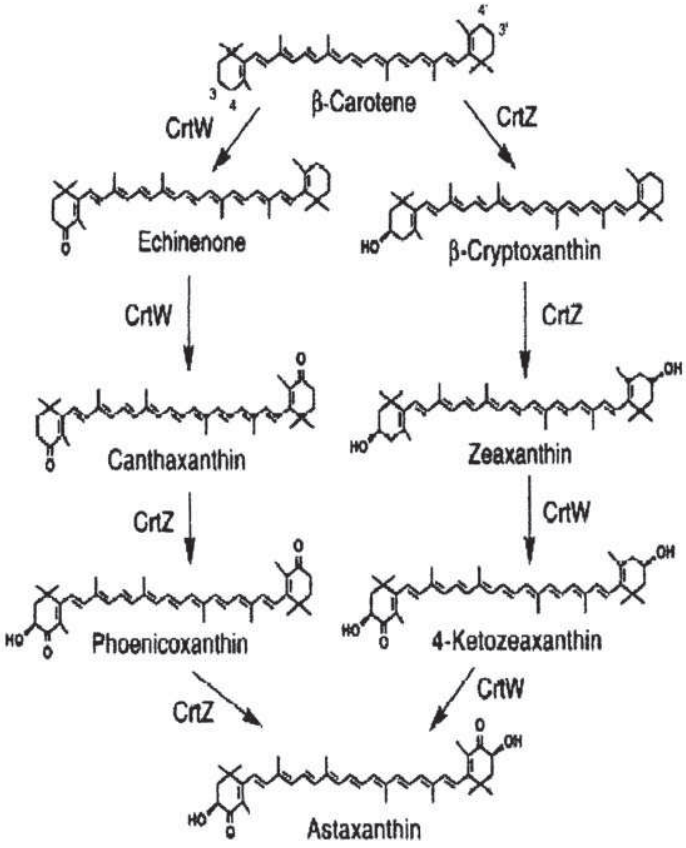
5.3. Epoxidase and de-epoxidase

Carotenoids serve as accessory pigments in the capture of photon energy (Cogdell & Frank, 1987) and efficiently quench the deleterious effects of triplet chlorophyll and singlet oxygen (Krinsky, 1979). Carotenoid epoxides, known to occur in plants and alga, display additional roles. First, the cyclic de-epoxidation of violaxanthin (Figure 16) and epoxidation of zeaxanthin represent key mechanisms in the adapting plants and green alga to high light intensity (Yamamoto, 1979; Demmig-Adams & Adams, 1992). Second xanthophyll epoxides serve as precursors of the plant hormone abscisic acid (Rock & Zeevart, 1991). Finally, when xanthophyll epoxides are converted to the keto-xanthophylls capsanthin and capsorubin, they yield the red colour of ripe pepper fruits that track the transformation of chloroplasts into chromoplast (Camara *et al*., 1995).

The epoxidation of zeaxanthin to form violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol) via antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-carotene-3,3'-diol) and de-epoxidation of violaxanthin to regenerate zeaxanthin comprise what is variously referred to as the xanthophyll, violaxanthin, or epoxide cycle (Demmig-Adams *et al*., 1996; Yamamoto, 1979). A cDNA encoding the zeaxanthin epoxidase (ZEP) or «β-cyclohexenyl xanthophyll epoxidase» in *Nicotiana plumbaginifolia* was identified by Marin *et al*. (1996).

Pepper (*Capsicum annuum*) β-cyclohexenyl xanthophyll epoxidase cDNA was cloned and the corresponding enzyme over expressed and purified from *E. coli* by Bouvier *et al*. (1996). The pepper epoxidase showed 88% identity to the amino acids

Figure 15
Postulated astaxanthin biosynthetic pathway deduced from in *vitro* studies (Misawa *et al*., 1995b).



sequence of *Nicotiana plumbaginifolia* (Marin *et el*., 1996). The molecular weight of the mature epoxidase was 65 kDa.

Bouvier *et al*. (1996) tested the ability of heterologously expressed and purified epoxidase to catalyse the epoxidation of zeaxanthin in the presence of 1mM NADPH and molecular oxygen. Under these minimal conditions, enzymatic formation of epoxy derivatives was observed. They showed two possible reasons: a) it was required a peroxygenase reaction; b) it was needed an additional electron transport system for the reaction to proceed. *In vitro* assay of a pepper ZEP these authors concluded that cyclohexenyl carotenoid epoxidase could be classified as a monooxygenase that catalyses the introduction of molecular oxygen in the presence of NADPH, ferredoxin and ferredoxin-like reductase. Since the catalytic mechanism of flavoprotein monooxygenase involves the formation of a flavin hydroperoxide enzyme intermediate, the resulting hydroperoxy-flavin is thus cleaved to incorporate one oxygene atom into zeaxanthin, while the others is reduced into water (Figure 17).

The pepper epoxidase acted specifically on the β-ring of xanthophylls such as β-cryptoxanthin, zeaxanthin, and antheraxanthin. The reaction mechanism proposed, by Bouvier *et al*. (1996), for epoxidation involves the formation of a transient carbocation. In examination the specificity of the purified epoxidase, they observed that α-carotene and lutein, which have β,ε-rings, were not epoxidized, concluding that the cloned epoxidase appears to be a β-cyclohexenyl epoxidase catalysing the reactions depicted in Figure 18.

The amino acid sequences of the pepper and tobacco ZEP are similar to that of a flavoprotein monooxygenase (salicylate-1-monooxygenase) and those of a number of other bacterial hydroxylases (Bouvier *et al*., 1996; Marin *et al*., 1996). The tobacco epoxidase is predicted to encode a polypeptide of 663 amino acids (72524 mol wt).

Some authors studied violaxanthin de-epoxidase (Bugos & Yamamoto, 1996 and Rockholm & Yamamoto, 1996) obtained from romaine lettuce. N-terminal sequencing of the purified lettuce enzyme indicates a cleavage site for transit peptide of the lettuce enzyme immediately after residue 125 to yield a mature polypeptide of 348 amino acids (39929 mol. wt.).

Figure 16
Violaxanthin (Britton, 1988)

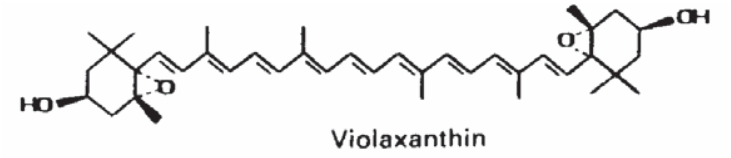


Fig 17
Redox cofactors involved in the monooxygenase activity of β-cyclohexenyl carotenoid epoxidase (Bouvier et al., 1996)

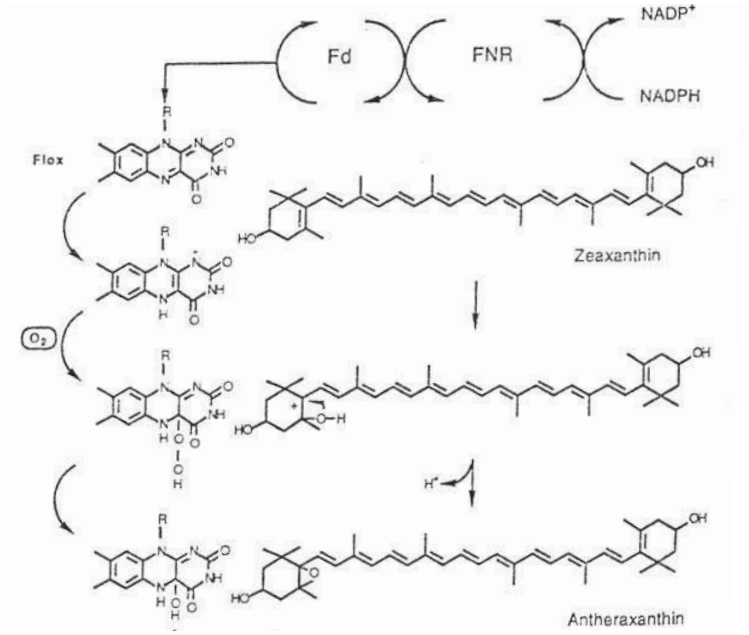
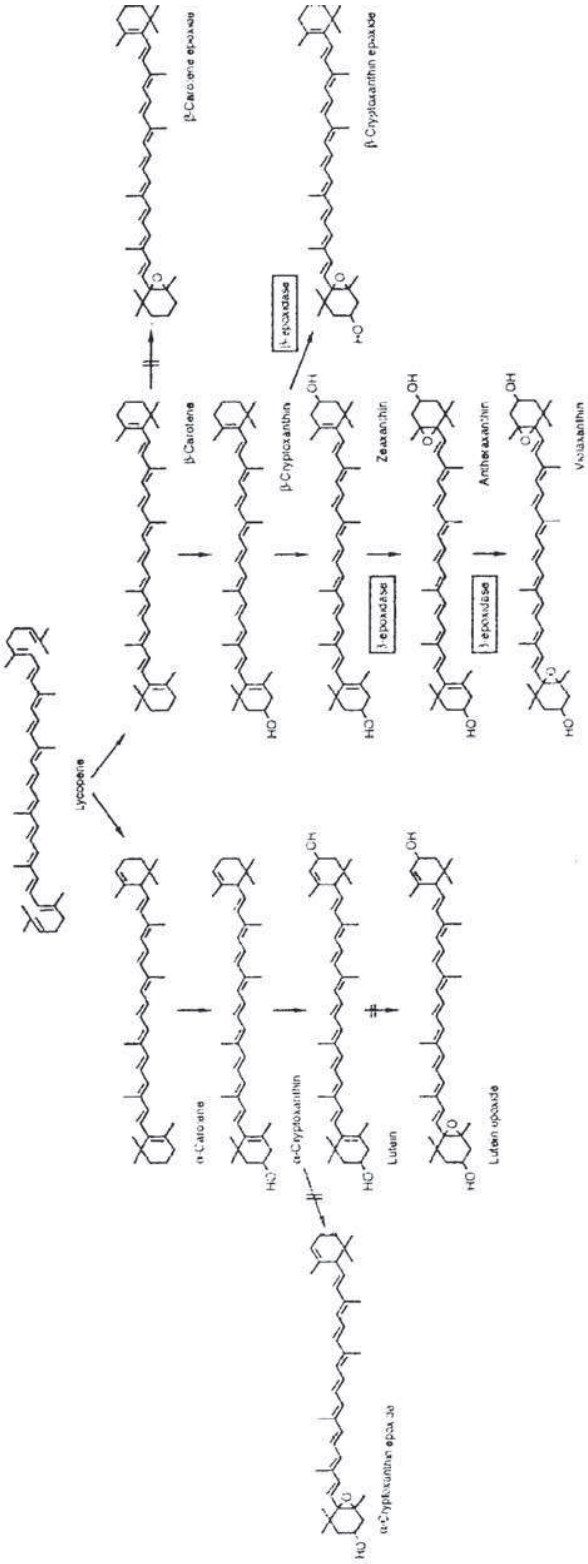


Figure 18
Enzymatic steps catalysed by Capsicum annuum β-cyclohexenyl epoxidase



5.4. Epoxycarotenoid cleavage enzyme

Zeevaart & Creelman (1988) studied the metabolism and physiology of abscisic acid (ABA). They considered that the epoxycarotenoids violaxanthin and neoxanthin (5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-caroten-3,5,3′-triol) are the precursors for biosynthesis of the plant growth regulator. Schwartz *et al*. (1997) characterized a maize enzyme that promotes the conversion of epoxycarotenoids to ABA. They studied the maize *vp14* gene, which encodes a protein that cleaves only the 9-*cis* geometrical isomers of violaxanthin and neoxanthin to yield cis-xanthoin (Figure 13). They proved that these enzymes *in vitro* assays require oxygen, iron and a detergent. The cDNA encoding the maize VP14 enzyme predicts a polypeptide of 604 amino acids (64 587 mol. wt.).

6
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