RESEARCH PAPER

Biosynthesis of fucoxanthin and diadinoxanthin and function of initial pathway genes in *Phaeodactylum tricornutum*

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Abstract

The biosynthesis pathway to diadinoxanthin and fucoxanthin was elucidated in *Phaeodactylum tricornutum* by a combined approach involving metabolite analysis identification of gene function. For the initial steps leading to β-carotene, putative genes were selected from the genomic database and the function of several of them identified by genetic pathway complementation in *Escherichia coli*. They included genes encoding a phytoene synthase, a phytoene desaturase, a ζ-carotene desaturase, and a lycopene β-cyclase. Intermediates of the pathway beyond β-carotene, present in trace amounts, were separated by TLC and identified as violaxanthin and neoxanthin in the enriched fraction. Neoxanthin is a branching point for the synthesis of both diadinoxanthin and fucoxanthin and the mechanisms for their formation were proposed. A single isomerization of one of the allenic double bounds in neoxanthin yields diadinoxanhin. Two reactions, hydroxylation at C8 in combination with a keto-enol tautomerization and acetylation of the 3′-HO group results in the formation of fucoxanthin.

Key words: Carotenogenic genes, complementation, diadinoxanthin, fucoxanthin, genetic pathway, neoxanthin, *Phaeodactylum tricornutum*.

Introduction

Algae are able to synthesize very diverse carotenoid structures for their photosynthetic apparatus. Nevertheless, the initial steps of the biosynthesis pathway are common to all of them. Some algal groups contain ε-ring derivatives that are typical for chlorophyceae and rhodophyceae (Goodwin, 1980; Takaichi, 2011), relating their carotenoid composition to higher plants. Other algal groups, especially those evolved from secondary endosymbiosis, contain very unique carotenoids. They include allenic peridinin and fucoxanthin as well as acetylenic diadinoxanthin (Takaichi, 2011). The latter are the major carotenoids in phaeophyceae and bacillariophyceae (diatoms) (Hager and Stransky, 1970; Haugan and Liaaen-Jensen, 1994). Apart from peridinine, fucoxanthin is the most abundant algal carotenoid. It is located in fucoxanthin–chlorophyll a complexes that resemble the light-harvesting

complexes of chlorophyceae and plants (Owens and Wold, 1986). Fucoxanthin is responsible for *in vivo* absorption at about 500–560 nm (Goodwin, 1980). In these pigment–protein complexes, fucoxanthin acts as an antenna and transfers exitation energy to chlorophyll (Papagiannakis *et al.*, 2005). In the case of the epoxide diadinoxanthin, its deepoxidation to diatoxanthin changes the function from a light-harvesting to a photoprotective carotenoid (Lohr and Wilhelm, 1999).

The chemical structures of diadinoxanthin and fucoxanthin were elucidated decades ago (Johansen *et al.*, 1974; Englert *et al.*, 1990). Nevertheless, little is known about the final biosynthesis steps leading to the formation of both carotenoids. Different pathways have been proposed and discussed (Coesel *et al.*, 2008; Bertrand, 2010; Lohr, 2011; Takaichi, 2011) but

experimental details are missing to date. Both carotenoids are the end products of a branching pathway in diatoms (Jeffrey, 1961). Therefore, the model diatom *Phaeodactylum tricornutum* from which the genome sequence is available (Bowler *et al.*, 2008) is the perfect organism to investigate fucoxanthin and diadinoxanthin synthesis at the gene and the metabolite level.

This study followed a two-step strategy to elucidate the biosynthesis pathway to fucoxanthin and diadinoxanthin in *P. tricornutum*. For the initial part to β-carotene, genes were identified and expressed and the catalytic function of the enzymes elucidated. In order to establish the reaction sequences of the final steps leading to fucoxanthin and diadinoxanthin, intermediates of the pathway, present only in trace concentrations, were accumulated by TLC to concentrations that allowed their identification. This allowed the description of the entire carotenoid biosynthesis pathway in *P. tricornutum*, from the generation of phytoene to the formation of the end products fucoxanthin and diadinoxanthin.

Materials and methods

Strains and cultivation

P. tricornutum strain 646 (UTEX Culture Collection) was grown at 20 °C on a shaker with continuous illumination at 30 μE m^{-2} s^{-2} in ASP medium (Provasoli *et al.*, 1957) for 6 days. *Escherichia coli* DH5α was cultivated in LB medium with appropriate antibiotics according to Sambrook *et al.* (1989) and used for the cloning and the pathway complementation.

Cloning of carotenogenic genes, cDNAs, and use of plasmids

RNA was isolated from *P. tricornutum* with the RNA Kit from JenaAnalytik and digested with TurboDNase and then cDNA was synthesized with Fermentas M MuLV reverse transcriptase. From it, *psy* cDNA was amplified by PCR with the primers Psy-EcoR1-forward (5′-GAATTCGATGAAAGTTTCGACAAAGCTCTG-3′) and Psy-HindIII-reverse (5′-AAGCTTTCATACTTGATCCAATTGGACC-3′). Genomic DNA was isolated after addition of 50 mM EDTA with the Promega Nuclei Lysis Buffer followed by protein precipitation. This preparation was used for carotenogenic gene amplification by PCR with the following primer pairs: *pds1* with Pds1-Acc65I-forward (5′-GAGCGGTACCAATGATGTTTCACTATAAGACAG-3′) and Pds1-HindIII-reverse (5′-GAGCAAGCTTCTAGGCTTCCACGAAT TGAC-3′), *zds* with Zds-BamHI-forward (5′-GAGCGGATC CATGAGGTTGTTGTTTGCTTC-3′) and Zds-HindIII-reverse (5′-GAG CAAGCTTCTACGCAACGACTTCTTTC-3′). Genes *pds2* and *lcy* were cloned with concurrent elimination of an intron by overlapping PCR with primer sets Pds2-Acc65I-forward1 (5′-GA GCGGTACCAATGAAGCTTGTGTTCTCGG-3′), Pds2-reverse1 (5′-CATGTCGAAAGCGCTTGGTTTCATCGGAC-3′), Pds2-forward2 (5′-CCAAGCGCTTTCGACATGGCCAGAAA-3′), and Pds2-PstI-reverse2 (5′-GAGCCTGCAGCTACACAACGACGGCC C-3′), or Lcyb-Acc65I-forward1 (5′-GAGCGGTACCAATGGTG CAAAGGTGGAAT-3′), Lcyb-forward2 (5′-GGTGGATCGTTTGA CATTCCAATGGAGG-3′), Lcyb-reverse1 (5′-GAATGTCAAACGAT CCACCAAAGTAACAG-3′) and Lcyb-PstI-reverse2 (5′-GAGCCT GCAGCTACTGGAAACCTTCCAC-3′). The PCR products were ligated into the corresponding restriction site of pUC18 (Yanisch-Perron *et al*., 1985) and pUC8-1 (Hanna *et al.*, 1984) yielding the plasmids for complementation in *E. coli*. Genes of interest from *P. tricornutum* are indicated by their genome ID number and their EEC accession number.

Pathway complementation in E. coli

The plasmids with the carotenogenic genes were co-transformed in *E. coli* together with a second compatible plasmid which provided the necessary carotenoid background, pACCRT-EB for phytoene, pACCRT-EBI for lycopene (Misawa *et al.*, 1995), and pACCRT-EBP for ζ-carotene (Breitenbach and Sandmann, 2005). After growth, carotenoids were extracted and analysed by HPLC.

Carotenoid analysis

Freeze-dried cells of *E. coli* or *P. tricornutum* were extracted with methanol by heating at 60 °C for 15 min. For *E. coli*, the extract was partitioned against 10% ether in petrol and the upper phase with the carotenoids collected. In case of *P. tricornutum*, water (5%) was added to the methanol extract which was partitioned against petrol. The lower phase was collected and further partitioned against 50% ether in petrol. Saturated NaCl solution was added for phases separated and transfer of the pigments into the upper phase. This was collected, evaporated in a stream of nitrogen, and redissolved prior to use. Only for enrichment, carotenoid extracts were fractionated and concentrated by TLC on activated silica plates developed with 30% acetone in hexane containing 0.2% ammonia. The band with an R$_f$ value of 0.35 above the fucoxanthin band was collected and extracted with acetone. HPLC of carotenoid extracts was carried out on a 25 cm Nucleosil C18 3 μm column (Machery & Nagel, Düren, Germany) with acetonitrile/methanol/2-propanol (85:10:5) as the mobile phase, at 32 °C for the *E. coli* carotenoids and at 10 °C for the carotenoids from *P. tricornutum*. Spectra of individual peaks were recorded online with a Kontron diode array detector 440. Standards for co-chromatography were isolated from spinach, corn seeds, *Fucus* species, and from *Amphidinium carterae*. Carotenes were generated by heterologous expression of appropriate genes in *E. coli* (Sandmann, 2002) and additionally identified by their typical spectra.

Screening of P. tricornutum *genomic database and comparison to carotenogenic genes*

P. tricornutum genome sequence data were obtained from the DOE Joint Genome Institute website (http://genome.jgi-psf.org/Phatr2). A database search was carried out with the similarity search tool BLASTP version 2.2.10 (Altschul *et al.*, 1997) with known carotenoid pathway gene sequences from other algae. Resulting genes are referred to in this manuscript with Pt for *P. tricornutum* followed by the genome sequence number from the above database. Phylogenetic analysis of amino acid sequences were performed with the program CLUSTAL X (Thompson *et al.*, 1997) and the alignments were visualized with TreeView.

Results

Screening of the whole genomic sequence database of *P. tricornutum* revealed several candidate genes for the specific carotenoid biosynthesis pathway. Up to 12 candidate cDNAs with putative carotenogenic involvement were obtained by multiple sequence alignment and cloned in frame into the expression vector pUC18. The resulting plasmids were all individually transformed into *E. coli* together with a second plasmid which provides the ability to synthesize the substrates for the expressed carotenogenic products of the *P. tricornutum* genes. Subsequent formation of the reaction products identifies the function of the genes (Steiger *et al.*, 2005). Fig. 1 shows the HPLC analysis of carotenoid substrates and products from positive function tests. The product of gene Pt56881 (EEC49474.1) was identified as a phytoene synthase (Pds), since trace A exhibited the formation of a phytoene peak at 33.2 min with absorbance maxima at 275, 285, and 297 nm. Complementation of gene Pt45735 (EEC48362.1) in a phytoene background resulted in the formation of three ζ-carotene isomers at 24.0 (all-*trans*), 25.4 (9,9′-*dicis*), and 26.2 min (9′-*cis*, Breitenbach and Sandmann, 2005) (Fig. 1B). The spectral absorbance maxima for the central isomer were at 380, 401, and 426 nm. The spectra for the other
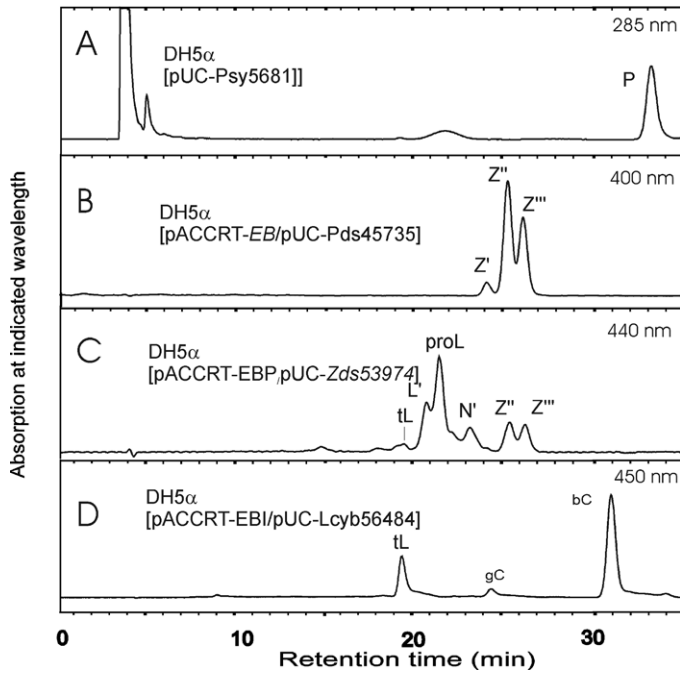
Fig. 1. HPLC separation of carotenoids from *Escherichia coli* with different carotenoid backgrounds co-transformed with plasmids containing carotenogenic genes from *Phaeodactylum tricornutum*, as follows: (A) phytoene synthase gene Pt56881; (B) phytoene background and a phytoene desaturase gene Pt45735; (C) ζ-carotene background with a ζ-carotene desaturase gene Pt53794; (D) lycopene background with a lycopene β-cyclase gene Pt56484. Peak names: bC, β-carotene; gC, γ-carotene; L', non-specified lycopene *cis* isomer; N, neurosporene; P, phytoene; proL, prolycopene (7,9,7′,9′-tetra-*cis* lycopene); tL, all-*trans* lycopene; Z, ζ-carotene.

isomers were very similar. Thus, Pt45735 encoded a phytoene desaturase. Introduction of gene Pt53974 (EEC51148.1) in a ζ-carotene background (trace C) generated a neurosporene isomer at 23.2 min with absorbance maxima at 414, 440, and 467 nm in addition to tetra-*cis* prolycopene (21.8 min, absorbance at 416, 439, and 469 nm), another lycopene *cis* isomer (20.8 min, maxima at 445, 472, and 503 nm) which was most like 5-*cis*, and small amounts of all-*trans* lycopene (19.5 min, absorbance at 446, 472, and 504 nm). Thus, the product carotenoids identified Pt53974 as a ζ-carotene desaturase (Zds) gene. In an all-*trans* lycopene-forming *E. coli*, the *P. tricornutum* gene Pt56484 (EEC51075.1) mediated the cyclization via γ-carotene (trace D, 24.4 min, absorbance at 435, 461, and 491 nm) to ζ-carotene (31.0 min, maxima at 425 (shoulder), 450, and 478 nm). Thus, Pt56484 was identified as a lycopene cyclase (Lcy) gene.

Several putative carotenogenic genes did not reveal their assumed function by complementation in *E. coli*. They included phytoene desaturase-like gene Pt55102 (EEC43911.1), four prolycopene isomerase-like genes Pt45243 (EEC49067.1), Pt9210 (EEC51326.1), Pt54842 (EEC45983.1), and Pt51868 (EEC48817.1), and three zeaxanthin epoxidase-like genes Pt45845 (EEC48429.1), Pt56488 (EEC51398.1), and Pt56492 (EEC50032.1).

The genetic relationship of the carotenogenic genes from *P. tricornutum* is exemplified in the phylogenetic tree for closely related Pds and Zds (Fig. 2). The deduced amino-acid sequences for both functionally assigned genes, including the non-functional *pds*-like gene Pt55102, were compared to other algal genes. The *P. tricornutum* Pds and Zds were most closely related to those from the other diatom, *Thalassiosira pseudonana*. Also, they were more closely related to those from both the red algae *Galdieria sulfuraria* and *Cyanidionschizon merolae* as well as
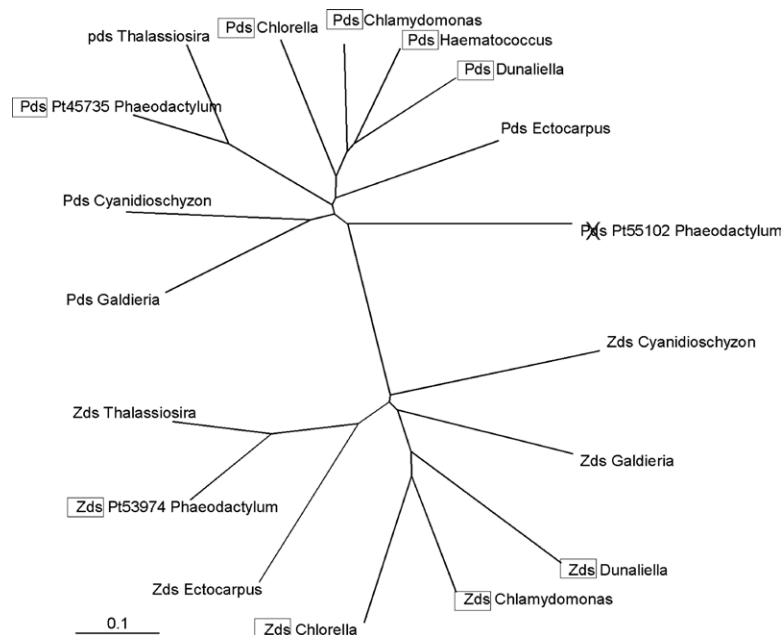
Fig. 2. Phylogenetic tree of the related Pds and Zds proteins. It includes the gene products from *Phaeodactylum tricornutum* including the non-active *P. tricornutum* Pds (indicated by X) together with the gene products from another diatom (*Thalassiosira pseudodona*), two red algae (*Galdieria sulfuraria* and *Cyanidionschizon merolae*), a brown alga (*Ectocarpus siliculosus*), and several green algae. The algal genes that have been functionally assigned are boxed. Bar, 0.1 substitutions per amino acid.
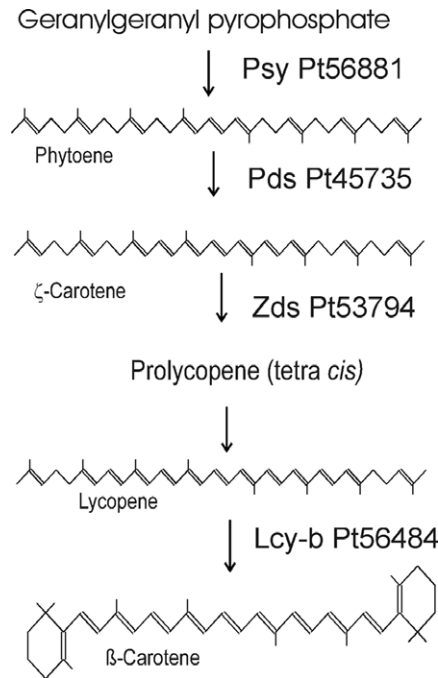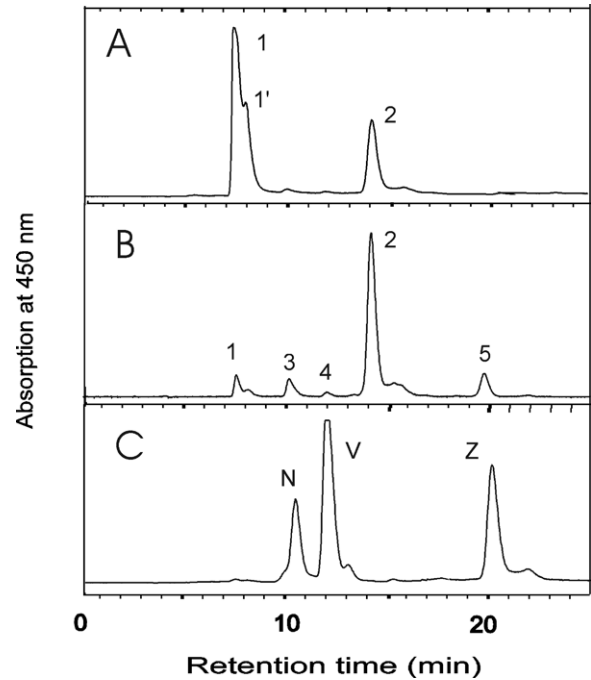
Fig. 3. The carotenoid biosynthesis pathway in *Phaeodactylum tricornutum* from the synthesis of phytoene to β-carotene. The functionally assigned carotenogenic genes from *P. tricornutum* are arranged with their numbers from the genome sequence next to the reaction which is catalysed by their products. Lcy-b, lycopene β-cyclase; Pds, phytoene desaturaser; Psy, phytoene synthase; Zds, ζ-carotene desaturase. A gene for the isomerization of prolycopene to all-*trans* lycopene was not found.


Fig. 4. Identification of carotenoids from *Phaeodactylum tricornutum* by HPLC in extracts (A) or enriched fraction (B) together with reference carotenoids (C). Peaks: 1, fucoxanthin; 1′, a fucoxanthin *cis* isomer; 2, diadinoxanthin; 3, neoxanthin (N); 4, violaxanthin (V); 5, zeaxanthin (Z).

to the brown algae *Ectocarpus siliculosus* than to those from green algae.

By the identification of the function of the initial carotenogenic genes, the pathway from phytoene to β-carotene could be established (Fig. 3). None of these carotenes nor any intermediate beyond β-carotene could be identified in standard carotenoid extracts of *P. tricornutum*. Fig. 4A shows the presence of fucoxanthin (peak 1, all-*trans* isomers; peak 1′, *cis* isomers) and diadinoxanthin (peak 2) in the HPLC diagram. When the extracts were fractionated by TLC and the carotenoids enriched, a faint yellow band separated close to the diadinoxanthin band. The carotenoids therein concentrated about 40-fold over the total extracted were analysed (Fig. 4B). In addition to traces of fucoxanthin together with some diadinoxanthin, three peaks appeared: peak 3 at 10.6 min (absorbance maxima at 420, 444, and 472 nm), peak 4 at 12.1 min (absorbance maxima at 420, 445, and 473 nm), and peak 5 at 20.2 min (absorbance maxima at 429, 452, and 478 nm). For identification, a mixture of isolated neoxanthin from spinach and a maize seed carotenoid extract was used (Fig. 4C). The following co-chromatography was obtained: neoxanthin with peak 3, violaxanthin with peak 4, and zeaxanthin with peak 5. All spectra corresponded. A TLC fraction running close to the solvent front on TLC contained β-carotene (data not shown).

All identified carotenoid intermediates were placed into the carotenoid biosynthesis pathway of *P. tricornutum* from β-carotene to fucoxanthin and diadinoxanthin (Fig. 5). After

hydroxylation at positions 3 and 3′ of β-carotene leading to the formation of zeaxanthin, both ionone rings are epoxidized at positions 5,6 and 5′,6′, yielding violaxanthin. The last common pathway step is the opening of one 5,6-epoxy ring and the rearrangement to allenic double bonds at position 6 of the β-ionone ring according to Goodwin (1980), yielding neoxanthin. Neoxanthin is the branch point for the formation of either fucoxanthin or diadinoxanthin. The latter is formed in a single reaction by formation of an acetylenic bond from the allenic double bonds by elimination of the 5-HO group as water (Goodwin, 1980). The formation of fucoxanthin from neoxanthin involves a ketolation reaction at C8 and acetylation of the hydroxy group at C3′.

Discussion

By gene cloning and expression, the carotenoid pathway in *P. tricornutum* could be reconstituted from phytoene synthesis to β-carotene with the exception of a prolycopene cyclase gene (Fig. 3). Since the genes for the following steps were not available or putative candidates not functional, intermediate enrichment and identification was used to establish the final pathway steps to both end products fucoxanthin and diadinoxanthin. Violaxanthin, one of the intermediates found (Fig. 4), was previously regarded as a precursor of fucoxanthin in *P. tricornutum* (Lohr and Wilhelm, 2001). The current sutdy found a close biosynthesis link between the allenic double bonds and acetylenic
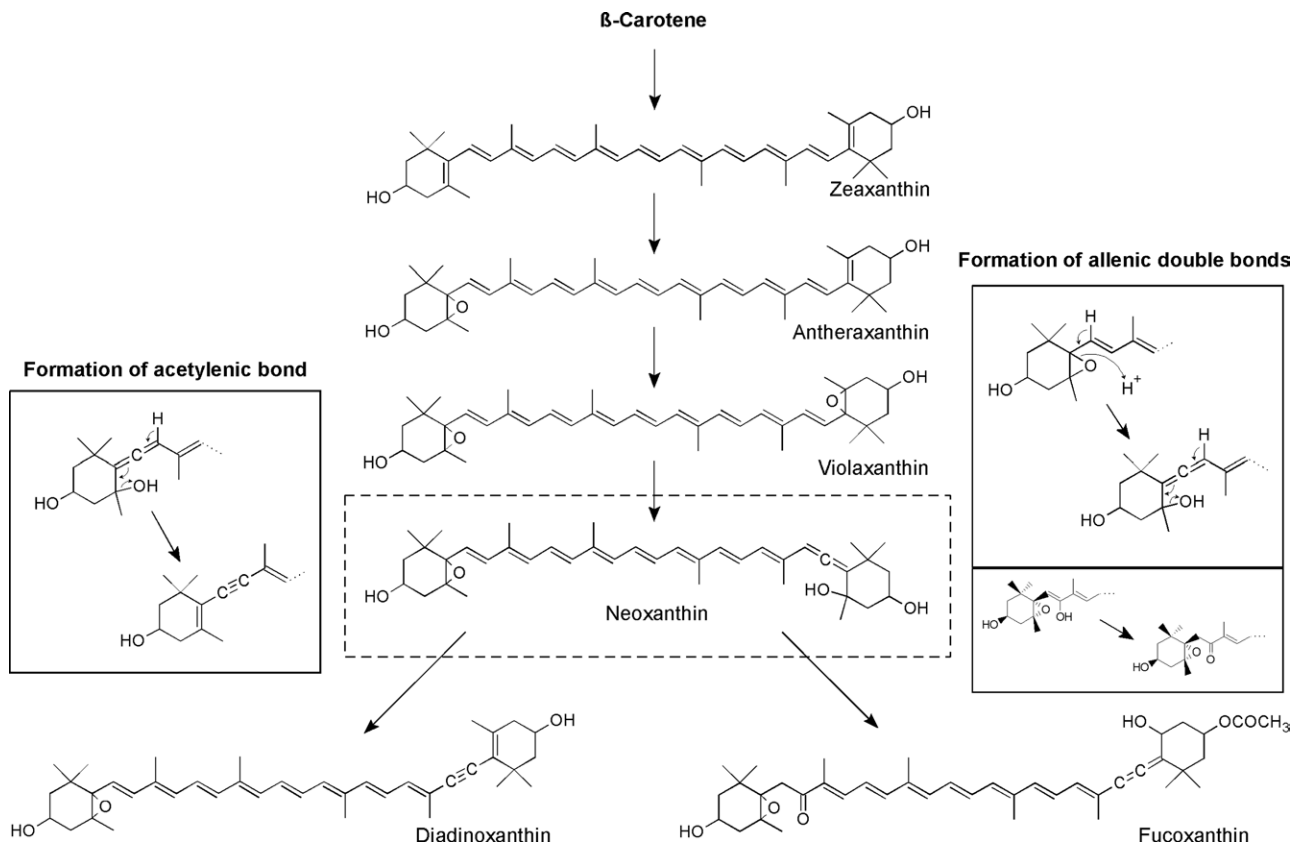
Fig. 5. Proposed carotenoid biosynthesis pathway in *Phaeodactylum tricornutum* from β-carotene to the end products diadinoxanthin and fucoxanthin. Neoxanthin as the branch point is boxes. The mechanisms for the formation of an acetylenic bond, of allenic double bonds and the formation of the C8 keto group is indicated.

bond, which is formed through conversion of neoxanthin to diadinoxanthin. Its pathway through zeaxanthin and neoxanthin coincides with *in vitro* labelling experiments for diadinoxanthin in *Amphidinium carterae* (Swift *et al.*, 1982). As required from their interconversion, neoxanthin and fucoxanthin share the same absolute configuration at C3 and C3′ as 3′S, 5′R, and 6′R (Bernhard *et al.*, 1976). Neoxanthin is not only a common precursor but also the branch point where the pathway diverges (Fig. 5). In the pathway to violaxanthin, all modifications are symmetrical, i.e. both ends of the carotenoid molecule are modified in the same ways. Asymmetrical modifications resulting in the formation of asymmetrical products start with the formation of allenic double bonds at only one side of the carotenoid molecule forming neoxanthin. This reaction proceeds via a proton-catalysed opening of the 5,6-epoxy ring and proton abstraction from C7 leading to a new double bond at C6 (Fig. 5). The pathway to neoxanthin is very common in most algal groups including green algae and higher plants (Goodwin, 1980). The specific formation of diadinoxanthin is catalysed by only a single reaction. It is a proton-catalysed reaction of the allenic double bonds to an acetylenic bond with elimination of the 5'-HO group as water. Formation of fucoxanthin from neoxanthin involves two modification steps. Apart from the acetylation of the 3′-HO group, a single hydroxylation step at C8 together with the tautomerization of the C7 double bond yields the C8 keto group.

In the phylogenetic tree, the functionally identified *P. tricornutum* Pds and Zds show a close relationship to a brown algal and to two red algal gene products (Fig. 2). This reflects the common systematic grouping of diatoms and brown algae into the heterokontophyta and also the origin of this group. They evolved from a secondary endosymbiosis by the integration of a red alga into a flagellate (Delwiche and Palmer, 1997). Due to the secondary symbiosis, the chloroplast of diatoms is surrounded by two double membranes. Analysis of the carotenogenic genes from *P. tricornutum* with the SignalP 4.0 program indicated a signal sequence in all functionally characterized genes for protein transfer across the outer double membrane into the periplastic compartment (data not shown).

Genetic pathway complementation is a powerful tool to identify functions of gene products. However, there are two limitations when a whole pathway is assessed. No orthologues may be found in the databases as for *P. tricornutum* β-carotene hydroxylase. Another may be a limited performance in *E. coli*. When, for example, three zeaxanthin epoxydase genes give no active product, it is important to test these genes in another complementation system or to try to elucidate their function directly in *P. tricornutum* by gene inactivation. Nevertheless, the combined genetic and analytical approach was successful to reveal the whole pathway to diadinoxanthin and fucoxanthin. This knowledge will facilitate targeted carotenogenic pathway engineering in *P. tricornutum* e.g. for increase of the synthesis of fucoxanthin, a ketocarotenoid with anti-tumorigenic activity (Das *et al.*, 2005) or the use of *P. tricornutum* as a platform for the synthesis of other bioactive carotenoids.

Acknowledgements

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