Molecular cloning and functional expression in *E. coli* of a novel plant enzyme mediating ζ-carotene desaturation

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Abstract **We have cloned a cDNA from the plant *Capsicum annuum* which encodes a novel enzyme mediating the dehydrogenation of ζ-carotene and neurosporene to lycopene when expressed in *E. coli* cells accumulating ζ-carotene or neurosporene. This enzyme is unable to dehydrogenate either phytoene or lycopene. The deduced amino acid sequence suggests that this cDNA encodes a polypeptide whose mature size is ca. 59 kDa and which is synthesized as a precursor with a NH_2-terminal extension resembling transit peptides for plastid targeting. Sequence comparison reveals 33–35% similarity with previously cloned plant or cyanobacterial phytoene desaturases. In contrast, only limited sequence similarity is found with a ζ-carotene desaturase from the cyanobacterium *Anabaena*.**

Key words: Capsicum annuum; Carotenoid; Lycopene; Neurosporene; Plastid; ζ-Carotene

1. Introduction

Carotenoids represent a major class of pigments widely distributed in nature. They are synthesized by photosynthetic organisms, ranging from bacteria to plants, and by certain non-photosynthetic bacteria and fungi [1]. Carotenoids are involved in the protection against photooxidative damage and contribute to the light-harvesting process in photosynthetic organisms [2]. In certain non-photosynthetic organs of plants, such as some ripening fruits, carotenoids accumulate in large amounts in chromoplasts [3] and are involved in the attraction of animals which will facilitate pollination or seed dispersal. In mammals, certain carotenoids such as β-carotene, are precursors of vitamin A, retinoic acid and retinal, and have been proposed to act as anti-cancer agents [4].

During carotenoid biosynthesis, the first carotenoid, phytoene, is formed by condensation of two molecules of geranylgeranyl pyrophosphate. The subsequent steps involve 4 dehydrogenation reactions to produce lycopene, a fully desaturated and linear carotene which contains a chromophore of 11 conjugated double bonds. These dehydrogenation reactions proceed via phytofluene, ζ-carotene and neurosporene. These reactions are catalyzed by a single enzyme, encoded by the gene *crtI*, in non-photosynthetic bacteria and fungi. In contrast, cyanobacteria and plants possess a phytoene desaturase (PDS) which catalyzes only the first two dehydrogenation reactions, producing ζ-carotene as an end product [1]. A second desaturase, catalyzing the conversion of ζ-carotene to lycopene via neurosporene, has been cloned to date only from the cyanobacterium *Anabaena* [5]. This enzyme, called ζ-carotene desaturase (ZDS), is more closely related to the *crtI*-encoded desaturases than to the PDS type of desaturase [6]. Unexpectedly, the *Anabaena zds* gene does not cross-hybridize with DNAs from other tested cyanobacteria. In addition, antisera against the ZDS protein showed no cross-reactivity (unpublished results). These results suggest either a poor sequence conservation for this gene, or that this gene is not widely distributed amongst cyanobacteria and plants.

Here we report cloning and characterization of a plant cDNA encoding an enzyme mediating the desaturation of ζ-carotene to lycopene via neurosporene. This enzyme is more closely related to PDS from cyanobacteria and plants than to *Anabaena* ZDS.

2. Materials and methods

2.1. Cloning of cDNAs

Synthesis of first strand cDNA in the presence of reverse transcriptase, oligo-dT primer and *Arabidopsis thaliana* total RNA, followed by PCR amplification (RT-PCR) was performed under standard conditions in a Crocodile II (Appligene) thermal cycler. The following oligonucleotides were used for PCR:

5′-end: GGGCAGAATTCATCTAAGGTGGG

3′-end: TCTGAGCTGTACTGTAACCACAGGTACTCCCTCGAG

The presence of *Eco*RI and *Xho*I cleavage sites allowed subcloning of the PCR fragment into the same sites in the pBluescript KS⁻ plasmid vector. A *C. annuum* cDNA library prepared in λgt11 from poly(A)⁺ RNA isolated from a fruit at an early ripening stage [7] was screened using a radiolabeled probe prepared by PCR amplification of the subcloned *A. thaliana* insert in the presence of [^{32}P]dCTP. Hybridizations and washes were performed in 2 × SSC at 55°C.

2.2. Sub-cloning and sequencing

Subcloning of DNA in pBluescriptKS⁻ was performed as described previously [7]. Sequencing was performed either manually or using an automated Biosystem Applied sequencer. DNA sequence analysis was performed using programs of the University of Wisconsin Genetics Computer Group. Database searches used the National Center for Biotechnology Information server (NCBI; BLAST programs).

2.3. Expression in E. coli

Transformed *E. coli* JM101 cells were grown at 28°C in the presence of the appropriate antibiotics for 48 h when carotene formation was anticipated. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added (1mM) at the end of the logarithmic growth phase. Plasmids derived from pACYC184 were pACCRT-EB which harbors the *Erwinia uredovora crtE* and *crtB* genes and mediates the formation of phytoene, pACCAR-EBP carrying in addition the *Synechoccocus pds* gene and mediating the formation of ζ-carotene, or pACCAR-EBI with the *crtI* gene from *Rhodobacter capsulatus* [8]. The latter plasmid is responsible for the synthesis in *E. coli* of neurosporene. A JM101 strain containing

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one of these plasmids (chloramphenicol) was used as the host cells for cDNAs inserted in pBluescriptKS$^-$ (ampicillin) in the sense orientation with respect to the *lacZ* promoter. Plasmid pCapZDS contains the full-length ζ-carotene desaturase cDNA from *C. annuum*. The presence of an *Nco*I site after the transit peptide coding region (methionine-55) allowed deletion of this region and fusion of the remaining part of the cDNA to the unique *Kpn*I site of the polylinker (after these sites were rendered blunt in the presence of T4 pol). In this construction (pCapZDSΔTP), the cDNA is translationally fused to the *lacZ* initiation codon.

2.4. HPLC analysis of pigments

The harvested *E. coli* cells were freeze-dried. Pigments were extracted from the cell powder with 6% KOH in methanol by heating at 60°C for 20 min. Carotenoids were partitioned into 10% ether in petrol and separated and quantified by HPLC on a Sherisorb ODS-1 column with acetonitrile/methanol/2-propanol 85:10:5 [9]. Identification and quantification was carried out with authentic standards. The spectra of each individual carotene was recorded using a diode array detector on-line from the elution peaks.

3. Results

3.1. cDNA cloning

Since the dehydrogenation of ζ-carotene to produce neurosporene and lycopene is a reaction which by its mechanism closely resembles the dehydrogenation of phytoene to produce phytofluene and ζ-carotene, we hypothesized that the amino acid sequences of the enzymes catalyzing these reactions are likely to resemble each other. Therefore, we screened the databases for sequences resembling known plant PDS polypeptides but which are not identical to them. An *A. thaliana* expressed sequence tag (EST) deposited under the accession number T46272 [10] was found by this search. This partial sequence shows limited amino acid sequence similarity to position 370 to 428 of the query sequence, namely *C. annuum* PDS [11]. Total RNA from *A. thaliana* was used in a RT-PCR amplification in the presence of 2 oligonucleotides (see section 2) in order to produce the corresponding cDNA fragment. This fragment was subcloned and its sequence verified.

This fragment was then used to screen ca. 10^5 λ clones from a *C. annuum* cDNA library. Thirteen positive clones were obtained. The largest cDNA insert was identified after PCR amplification of the inserts and this clone was then plaque-purified.

3.2. Sequence comparison

After subcloning of the cDNA insert, its sequence was determined. This cDNA contains an open reading frame of 588 codons encoding a precursor polypeptide of MW 64.7 kDa. The deduced amino acid sequence is shown in Fig. 1 (where it is tentatively called CapZDS).

The NH_2-terminal portion of the protein resembles a typical transit peptide for plastid targeting, with a possible cleavage site at the position indicated in Fig. 1. This region does not show any significant primary sequence identity with known sequences (apart from the occurence of numerous hydroxylated and positively charged residues) which is a normal feature of transit peptides. The predicted MW of the mature polypeptide would therefore be ca 59.4 kDa and its pI 7.4.

Significant amino acid sequence similarity was found with plant and cyanobacterial PDS, starting from position 80 (which corresponds to the initiation methionine of the cyanobacterial PDS) up to the COOH-end. The overall sequence identity (starting at position 80 and after introduction of gaps) is 35.5% (59% similarity) with *C. annuum* PDS [11] and 33.5% identity (58% similarity) with *Synechococcus* PDS [12]. The identity/similarity percentages are not significantly different when compared with other known PDS sequences from plants or cyanobacteria (for a review see [1]). Sequence comparison are also shown in the homology plots presented in Fig. 2.

Like in PDS, a typical dinucleotide binding site is found near the NH_2-end of the mature polypeptide. When compared to *Anabaena* ZDS [6], only a limited number of short regions of similarity were found (see Figs. 1 and 2), which includes the potential dinucleotide binding site.

3.3. E. coli *expression*

Functional expression of the putative ζ-carotene desaturase cDNA in *E. coli* was determined by analysis of the carotenes formed after cotransformation with other plasmids carrying different combinations of carotenoid genes. The latter mediate the synthesis of either phytoene, ζ-carotene or neurosporene at approximately 250 fg/mg dry weight. In the presence of the additional plasmid expressing the full-length form of the newly cloned cDNA (pCapZDS), phytoene was not converted to any desaturation product whereas ζ-carotene and also neurosporene, the intermediate of ζ-carotene desaturation, were both converted to lycopene (Table 1). In the presence of a plasmid expressing a truncated form of the cDNA (pCapZDSΔTP) from which the first 54 codons (which encode the proposed transit peptide) have been deleted, a much more efficient conversion of ζ-carotene to neurosporene and lycopene (about 18%) was observed. The new carotene products were identified by HPLC including cochromatography with authentic standards and their absorbance maxima recorded on-line from the elution peaks, which were 415, 440, 471 nm for neurosporene and 445, 472, and 505 nm for lycopene.

The most likely interpretation of these data is that we have cloned a cDNA encoding a new plant carotene desaturase which is able to catalyze the conversion of ζ-carotene to lycopene via neurosporene.

Table 1
Carotenoid formation in *E. coli* cells carrying different plasmids for carotene synthesis together with pCapZDS or pCapZDSΔTP

E. coli background	Plasmid	Phytoene	ζ-Carotene	Neurosporene	Lycopene
Phytoene	pCapZDS	278.2	0	0	0
ζ-Carotene	pCapZDS	–	252.0	3.8	1.0
Neurosporene	pCapZDS	–	–	105.8	26.1
ζ-Carotene	pCapZDSΔTP	–	221.2	28.2	19.8

Concentrations in fg/g. Product carotenes were identified by cochromatography with authentic standards and by their absorbance maxima recorded on-line from the elution peaks. Neurosporene: 415, 440 and 471 nm; lycopene: 445, 472 and 505 nm.

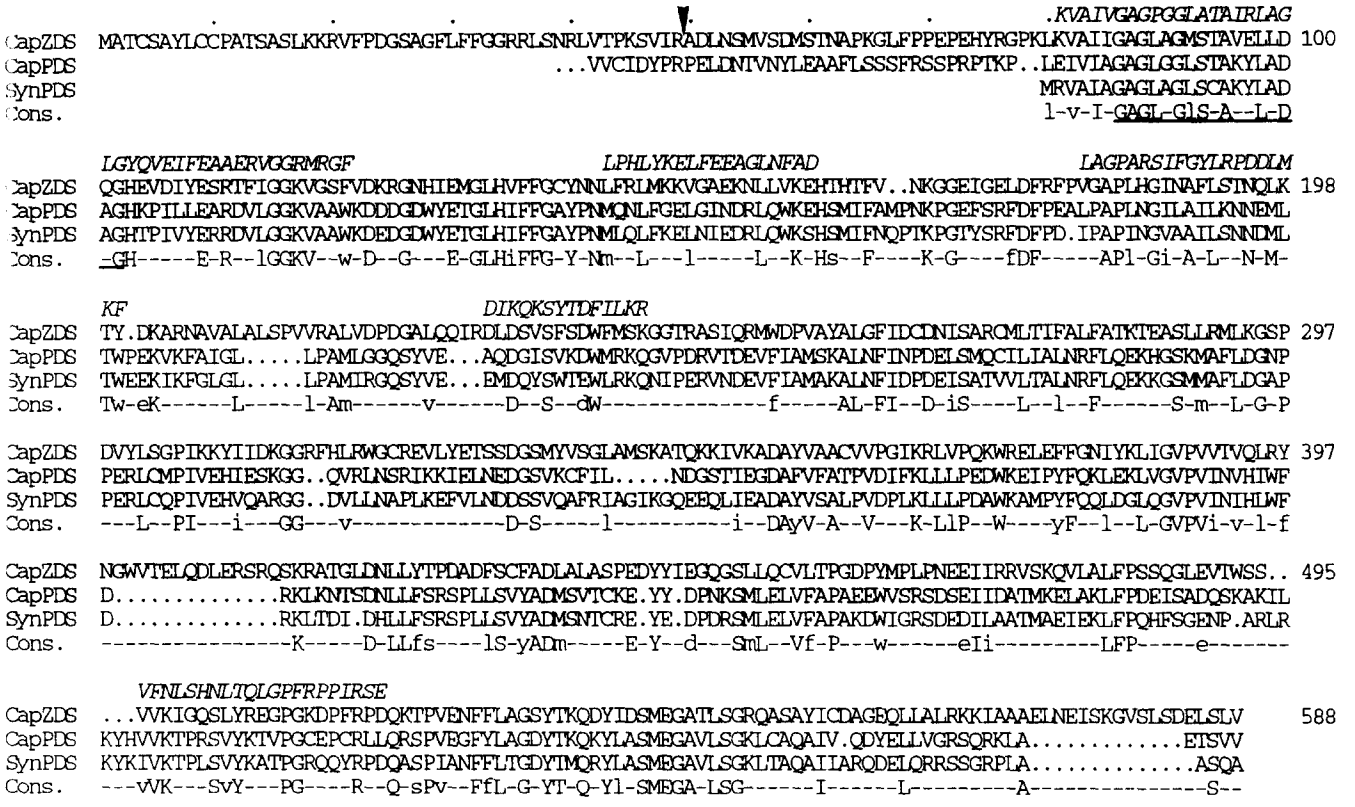

Fig. 1. Amino acid sequence alignment of *Capsicum annuum* ζ-carotene desaturase (CapZDS) and phytoene desaturase (CapPDS) and *Synechococcus* phytoene desaturase (SynPDS). The consensus line (cons.) shows residues conserved in all 3 sequences (upper case letters) or residues which are identical in 2 sequences and replaced by an equivalent amino acid in the third sequence (lower case). Numbering refers to the CapZDS sequence. The NH_2-end of CapPDS [11] is not shown. The SynPDS sequence [12] starts at the initiation methionine. The arrowhead indicates a potential transit peptide cleavage site. The core sequence of a dinucleotide binding site is underlined. Sequence portions given above this alignment (in italics) represent motifs from *Anabaena* ZDS [6].

4. Discussion

During the past few years, a number of carotene desaturases have been cloned and characterized. First, CRT-I type of phytoene desaturases have been cloned from the bacteria *Rhodobacter capsulatus* [13], *Erwinia uredovora* and *E. herbicola* (*crtI* genes) [14,15] and from the fungus *Neurospora crassa* (*al-1* gene) [16] and sequenced. Then, the genes encoding phytoene desaturases from cyanobacteria [12,17], green algae and plants [11,18,19] have been isolated (*pds* genes). Sequence data indicate that the CRT-I type of desaturases and the PDS-type of desaturases belong to 2 separate groups of enzymes. This is also reflected by the fact that CRT-I desaturases catalyze 3–4 desaturation steps, whilst PDS catalyzes only the first two reactions. The search for desaturases able to catalyze the remaining desaturation steps, namely the conversion of ζ-carotene into lycopene has led to the cloning of a gene from the cyanobacterium *Anabaena* which codes for an enzyme (called ZDS) which is more closely related to the CRT-I enzymes [6].

We have now cloned a cDNA from the plant *C. annuum*, which encodes a novel enzyme related to carotene desaturation. After expression in *E. coli* cells accumulating ζ-carotene or neurosporene, the newly formed protein mediated specifically the in situ conversion of ζ-carotene as well as neurosporene into lycopene, the end product of these desaturation reactions (Table 1). These are essential features expected for a ζ-carotene/neurosporene desaturase. Therefore, we tentatively propose the name ZDS for this enzyme. Our cDNA has only limited sequence similarity to *Anabaena* ZDS but higher similarity to the PDS-type of desaturases. This suggests that the plant ZDS and PDS genes originated from a common ancestral gene. It can be speculated that this ancestral gene was encoding an enzyme catalyzing 4 desaturation steps and that this gene became duplicated, each copy giving rise to a specialized desaturase gene (*pds* and plant-type *zds*). The reason for such a duplication and specialization of desaturase genes is unclear but could have resulted in more efficient conversion of phytoene to lycopene in plant cells. Since *C. annuum* ZDS is almost as closely related to cyanobacterial PDS as it is to plant PDS, it seems likely that this duplication event occurred early in evolution. Characterization of other cyanobacterial ZDS genes should reveal whether this gene duplication event has already occured in cyanobacteria, or whether all cyanobacteria contain a ZDS gene resembling the one from *Anabaena*. The fact that the latter gene does not cross-hybridize with other cyanobacterial DNAs would suggest that the occurence of such a gene in *Anabaena* is a special feature of this cyanobacterium, and that other cyanobacteria could contain a ZDS gene related to *pds*. As the *crtI*-type *zds* has only been cloned to date from a heterocyst-forming cyanobacterial species, one could speculate that the function of the related enzyme is restricted to this organelle with its specialized metabolism involved in N_2 fixation. A more systematic search for the occurrence of both types of ζ-carotene desaturase genes especially in cyanobacteria will

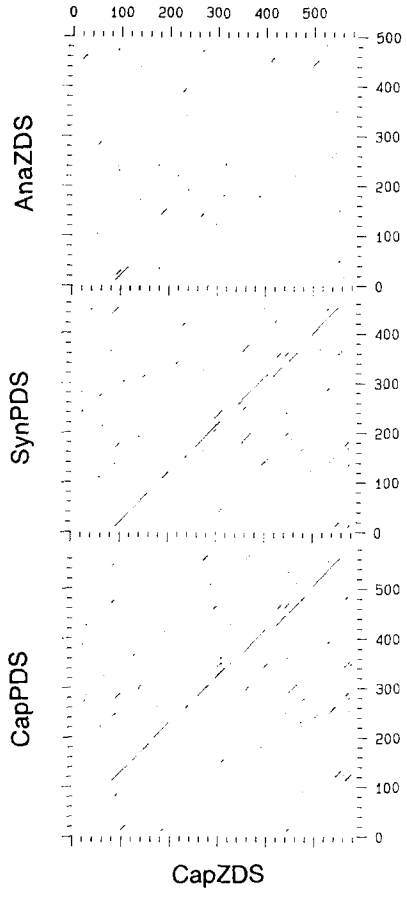

Fig. 2. Protein homology plots comparing *C. annuum* ζ-carotene desaturase (CapZDS) to *Anabaena* ζ-carotene desaturase (AnaZDS), *Synechococcus* (SynPDS) and *C. annuum* phytoene desaturase (CapPDS). Amino acids were compared using a window of 21. Twelve identical or similar residues were scored as a dot.

contribute to our understanding of the evolution of these different types of desaturase genes.

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References

[1] Sandmann, G. (1994) Eur. J. Biochem. 223, 7–24.
[2] Goodwin, T.W. (1980) The Biochemistry of Carotenoids, vol. 1, Chapman-Hall, New York.
[3] Camara, B., Hugueney, P., Bouvier, F., Kuntz, M. and Monéger, R. (1995) Int. Rev. Cytol. (in press).
[4] Mathews-Roth, M.M., Lausen, N., Drouin, G., Richre, A. and Krinski, N.I. (1991) Oncology 48, 177–179.
[5] Linden, H., Vioque, A., and Sandmann, G. (1993) FEMS Microbiol. Lett. 106, 99–104.
[6] Linden, H. Misawa, N., Saito, T. and Sandmann, G. (1994) Plant Mol. Biol. 24, 369–379.
[7] Kuntz, M., Römer, S., Suire, C., Hugueney, P., Weil, J.H., Schantz, R. and Camara, B. (1992) Plant J. 2, 25–34.
[8] Linden, H. Misawa, N., Chamovitz, D., Pecker, I., Hirschberg, J. and Sandmann, G. (1991) Z. Naturforsch. 46c, 1045–1051.
[9] Sandmann, G. (1991) Arch. Microbiol. 155, 229–233.
[10] Becker, K.G. and Drew, P.D. (1995) Nucleotide sequence databases, accession number T46272.
[11] Hugueney, P., Römer, S., Kuntz, M. and Camara, B. (1992) Eur. J. Biochem. 209, 399–407.
[12] Chamovitz, D., Pecker, I. and Hirschberg, J. (1991) Plant Mol. Biol. 16, 967–974.
[13] Armstrong, G.A., Alberti, M., Leach, F. and Hearst, J.E. (1989) Genetics 216, 254–268.
[14] Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K. and Harashima, K. (1990) J. Bacteriol. 172, 6704–6712.
[15] Armstrong, G.A., Alberti, M. and Hearst, J.E. (1990) Proc. Natl. Acad. Sci USA 87, 9975–9979.
[16] Schmidhauser, T.J., Lauter, F.R., Russo, V.E.A. and Yanofsky, C. (1990) Mol. Cell Biol. 10, 5064–5070.
[17] Martinez-Férez, I.M. and Vioque, A. (1992) Plant Mol. Biol. 18, 981–983.
[18] Bartley, G.E., Viitanen, P.V., Pecker, I., Chamovitz, D., Hirschberg, J. and Scolnik, P.A. (1991) Proc. Natl. Acad. Sci. USA 88, 6532–6536.
[19] Pecker, I., Chamovitz, D., Linden, H., Sandmann, G. and Hirschberg, J. (1992) Proc. Natl. Acad. Sci. USA 89, 4962–4966.