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A single polypeptide catalyzing the conversion of phytoene to ζ-carotene is transcriptionally regulated during tomato fruit ripening

(carotenoid biosynthesis/phytoene desaturase/plastids)

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ABSTRACT **The cDNA of the gene *pds* from tomato, encoding the carotenoid biosynthesis enzyme phytoene desaturase, was cloned, and its nucleotide sequence was determined. Cells of *Escherichia coli* that expressed the tomato *pds* gene could convert phytoene to ζ-carotene. This result suggests that one polypeptide, the product of the *pds* gene, can carry out phytoene desaturation in the carotenoid biosynthetic pathway. Transcripts of the *pds* gene accumulate in orange tomato fruit, indicating transcriptional control of *pds* expression during fruit ripening. The deduced amino acid sequence of phytoene desaturase indicates that this enzyme in tomato contains 583 amino acids that are highly conserved with respect to the homologous enzymes in cyanobacteria and algae. The deduced amino acid sequences of the phytoene desaturases from other microorganisms (purple bacteria and fungi) appear to be evolutionarily unrelated to those from green photosynthetic organisms.**

Carotenoid pigments are widely distributed in nature. They are essential components of the photosynthetic apparatus, where they serve in protecting against photooxidative damage and also contribute to light harvesting for photosynthesis. Carotenoids also serve as antioxidants and colorants in plants and animals (1, 2). They are the major source of vitamin A (retinol) in animals (3), are precursors for the synthesis of abscisic acid in plants (4), and may also function as anticancer agents (5) and as immune system enhancers (6).

Carotenoids are synthesized by all photosynthetic organisms as well as by several nonphotosynthetic bacteria and fungi. The first committed step of carotenoid biosynthesis, common to all organisms, is the condensation of two molecules of geranylgeranyl pyrophosphate, yielding *cis*-phytoene, a C_{40} colorless hydrocarbon. Stepwise desaturation (dehydrogenation) reactions convert phytoene to lycopene via phytofluene, ζ-carotene, and neurosporene. Two cyclization reactions convert lycopene to β-carotene. In plants and algae the desaturation and cyclization reactions occur within plastids and are catalyzed by integral membrane enzymes (7). The number of different enzymes involved in each of these steps is unknown.

Clusters of genes devoted to carotenoid biosynthesis have been cloned from the nonsulfur purple photosynthetic bacterium *Rhodobacter capsulatus* (8, 9) and from the nonphotosynthetic bacteria *Erwinia herbicola* (10) and *Erwinia uredovora* (11). Among the genes identified in these prokaryotes is *crtI*, which codes for phytoene desaturase (PDS). A homologous gene, *al-1*, has been cloned from the fungus *Neurospora crassa* (12). However, attempts to clone homologous genes from higher plants by using the bacterial genes as heterologous probes have been unsuccessful.

Recently we cloned the gene *pds*, which encodes PDS, from the cyanobacterium *Synechococcus* PCC7942 (13, 14). We report here the cloning and nucleotide sequence determination of the *pds* homologue from tomato (*Lycopersicon esculentum*).§ Expression of this gene in *Escherichia coli* indicates that it codes for PDS that functions in the conversion of phytoene to ζ-carotene. Comparison of the deduced amino acid sequences of *pds* from a plant, an alga, and a cyanobacterium with the published sequences of *crtI* gene products from other prokaryotes and the *al-1* gene product from *Neurospora* indicates that two distinct, evolutionarily unrelated types of PDS enzymes exist in nature.

MATERIALS AND METHODS

Cloning and Nucleotide Sequence Determination of *pds*. A cDNA library from the green alga *Dunaliella bardawil* in λgt11 was provided by Ada Zamir and Amnon Lers from the Weizmann Institute. This library was probed with a ^{32}P-labeled 0.7-kilobase (kb) *Acc* I–*Hin*dIII fragment of the *pds* gene from *Synechococcus* PCC7942 (14). A positive clone was isolated, phage DNA was extracted, and the cDNA insert was subcloned into the plasmid vector pUC118. Phage infection and DNA extraction were according to established procedures (15).

A cDNA library prepared from ripening tomato fruit (UC82-B) in λ ZAPII, given to us by Lee McIntosh from Michigan State University, was screened with a 0.8-kb *Sac* I internal fragment from the *D. bardawil pds* gene. Infection of *E. coli* XLI-Blue and screening were according to Stratagene protocols. Hybridization was done at low stringency: 35% (vol/vol) formamide/5× standard saline citrate, 5× Denhardt's solution/0.1% SDS/50 mM sodium phosphate for 20 hr at 37°C. Filters were washed twice with 2× standard saline citrate at 37°C.

DNA was sequenced by the dideoxynucleotide chain-termination reaction (16) using T7 DNA polymerase (Promega). *Sac* I fragments of the *D. bardawil pds* gene were subcloned into pUC118 and sequenced by using the universal forward and reverse primers. Appropriate oligonucleotides were synthesized to use as additional internal sequencing primers. Overlapping deletions of the tomato *pds* gene were generated using the Erase-a-Base kit (Promega). Subclones of restriction fragments, obtained by digestion with *Eco*RI, *Hin*dIII, *Pst* I, and *Sph* I, were sequenced with the universal primers. All regions were sequenced in both directions.

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Abbreviation: PDS, phytoene desaturase.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. X59948).

Northern (RNA) Analysis. Total RNA was isolated from 50 g of pericarp from mature green and orange tomato fruit and from 5 g of leaves, according to the guanidine thiocyanate/cesium chloride protocol described in ref. 15 with the following modifications: the tissue was first pulverized in liquid N_2; the homogenization buffer contained 5 M guanidine thiocyanate and 8% (vol/vol) 2-mercaptoethanol; sodium lauryl sulfate was not added to the homogenate. Poly(A)$^+$ mRNA was isolated by binding to oligo(dT)-cellulose beads (17) (Boehringer Mannheim) according to the manufacturer's protocol. Twenty-five micrograms of poly(A)$^+$-enriched RNA of each sample was separated by electrophoresis in a formaldehyde-containing agarose gel and transferred to a GeneScreen membrane (DuPont). The entire cDNA clone of *pds* was labeled with ^{32}P and used as probe. Hybridization was done in 35% (vol/vol) formamide/3× standard saline citrate/1% SDS/2.5× Denhardt's solution/salmon sperm DNA at 100 μg/ml at 37°C. After hybridization, the filter was washed in 1× SSC/0.1% SDS at 37°C.

Amino Acid Sequence Comparisons. Amino acid sequence comparisons were done using the GAP program of the Genetics Computer Group sequence analysis software package (version 6.2, June 1990) and HIBIO PROSIS (Hitachi Software Engineering, Tokyo).

Analysis of Carotenoid Biosynthetic Intermediates. Cells of *E. coli* strain JM109, carrying carotenoid biosynthetic genes on the plasmids pACCRT-BE and pTPDEX (see text and Fig. 2 for details), were grown to stationary phase. Extraction of carotenoids and HPLC analysis were according to the procedures described for *Synechococcus* (18). Pigments were identified by their typical retention time in the HPLC and by analysis of their absorption spectra.

RESULTS

Cloning the *pds* Gene from Tomato. The *pds* gene from the cyanobacterium *Synechococcus* PCC7942 was identified and cloned by genetic complementation of norflurazon resistance (13, 14). The cyanobacterial *pds* gene hybridized very weakly to a tomato genomic DNA blot but hybridized strongly with DNA from the green alga *D. bardawil*. We therefore decided to first clone the algal gene and then to use it as a probe to isolate the plant *pds* gene. A portion of the cyanobacterial gene was therefore used to screen a cDNA library from *D. bardawil*. A positive clone was isolated, and the cDNA insert was subcloned into the plasmid vector pUC118. Sequence analysis revealed an open reading frame coding for a polypeptide, which had an amino acid sequence highly similar to that of the cyanobacterial PDS (data not shown). The cloned *D. bardawil pds* cDNA served as a molecular probe for screening the tomato cDNA library. Four recombinant Bluescript SK$^-$ plasmids that contained the same DNA sequence were identified. The nucleotide sequence of the longest cDNA clone, in a plasmid designated pTPD, was determined. An open reading frame of 583 codons was found, which codes for a polypeptide with a calculated molecular mass of 64.9 kDa (Fig. 1). This coding region is flanked at its 5′ end by an untranslated sequence of >440 nucleotides.

Expression of the Tomato *pds* Gene in *E. coli*. Normally, *E. coli* cells do not synthesize carotenoids. However, cells of *E. coli* JM109, which carry the plasmid pACCRT-BE, produce high quantities of phytoene (19). This plasmid contains the genes *crtE* and *crtB* from *E. uredovora* (11), which code for geranylgeranyl pyrophosphate synthase and phytoene synthase, respectively, on the vector pACYC184 (Fig. 2). An expression vector carrying the *pds* gene from tomato, pTPDEX, was constructed by subcloning a truncated *pds* gene into the plasmid vector Bluescript SK$^-$. This procedure generated an in-frame fusion of *pds* with the amino terminus of *lacZ* (Fig. 2). *E. coli* cells that contained the genes *crtB* and *crtE* from *E. uredovora* and the tomato *pds* cDNA accumulated a light yellow pigment that was clearly seen in colonies. Carotenoids produced in *E. coli* cells carrying the plasmid pACCRT-BE, alone or together with pTPDEX, were extracted and analyzed by HLPC. Fig. 3 shows that the

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   1 CATCTTCATCATAAATTAGTTTGTTTATTTATACAGAATTATACGCTTTTACTAGTTATAGCATTCGGTATCTTTTTCTGGGTAACTGCCAAACCACCACAAATTTCAAG
 111 TTTCCATTTAACTCTTCAACTTCAACCCAACCAAATTTATTTGCTTAATTGTGCAGAACCACTCCCTATATCTTCTAGGTGCTTTCATTCGTTCCGAGGTAAGAAAAGAT
 221 TTTTGTTTCTTTGAATGCTTTATGCCACTCGTTTAACTTCTGAGGTTGTGGATCTTTTAGGCGACTTTTTTTTTTTTTTGTATGTAAAATTTGTTTCATAAATGCTTCTC
 331 AACATAAATCTTGACAAAGAGAAGGAATTTTACCAAGTATTTAGGTTCAGAAATGGATAATTTTCTTACTGTGAAATATCCTTATGGCAGGTTTTACTGTTATTTTTCAG
 441 TAAAATGCCTCAAATTGGACTTGTTTCTGCTGTTAACTTGAGAGTCCAAGGTAGTTCAGCTTATCTTTGGAGCTCGAGGTCGTCTTCTTTGGGAACTGAAAGTCGAGATG
 147    M  P  Q  I  G  L  V  S  A  V  N  L  R  V  Q  G  S  S  A  Y  L  W  S  S  R  S  S  S  L  G  T  E  S  R  D  G
 551 GTTGCTTGCAAAGGAATTCGTTATGTTTTGCTGGTAGCGAATCAATGGGTCATAAGTTAAAGATTCGTACTCCCCATGCCACGACCAGAAGATTGGTTAAGGACTTGGGG
 184   C  L  Q  R  N  S  L  C  F  A  G  S  E  S  M  G  H  K  L  K  I  R  T  P  H  A  T  T  R  R  L  V  K  D  L  G
 661 CCTTTAAAGGTCGTATGCATTGATTATCCAAGACCAGAGCTGGACAATACAGTTAACTATTTGGAGGCTGCATTTTTATCATCAACGTTCCGTGCTTCTCCGCGCCCAAC
 221 P  L  K  V  V  C  I  D  Y  P  R  P  E  L  D  N  T  V  N  Y  L  E  A  A  F  L  S  S  T  F  R  A  S  P  R  P  T
 771 TAAACCATTGGAGATTGTTATTGCTGGTGCAGGTTTGGGTGGTTTGTCTACAGCAAAATATTTGGCAGATGCTGGTCACAAACCGATACTGCTGGAGGCAAGGGATGTTC
 257   K  P  L  E  I  V  I  A  G  A  G  L  G  G  L  S  T  A  K  Y  L  A  D  A  G  H  K  P  I  L  L  E  A  R  D  V  L
 881 TAGGTGGAAAGGTAGCTGCATGGAAAGATGATGATGGAGATTGGTACGAGACTGGTTTGCATATATTCTTTGGGGCTTACCCAAATATTCAGAACCTGTTTGGAGAATTA
 294   G  G  K  V  A  A  W  K  D  D  D  G  D  W  Y  E  T  G  L  H  I  F  F  G  A  Y  P  N  I  Q  N  L  F  G  E  L
 991 GGGATTAACGATCGATTGCAATGGAAGGAACATTCAATGATATTTGCAATGCCAAGCAAGCCAGGAGAATTCAGCCGCTTTGATTTCTCCGAAGCTTTACCCGCTCCTTT
 331 G  I  N  D  R  L  Q  W  K  E  H  S  M  I  F  A  M  P  S  K  P  G  E  F  S  R  F  D  F  S  E  A  L  P  A  P  L
1101 AAATGGAATTTTAGCCATCTTAAAGAATAACGAAATGCTTACATGGCCAGAGAAAGTCAAATTTGCAATTGGACTCTTGCCAGCAATGCTTGGAGGGCAATCTTATGTTG
 367   N  G  I  L  A  I  L  K  N  N  E  M  L  T  W  P  E  K  V  K  F  A  I  G  L  L  P  A  M  L  G  G  Q  S  Y  V  E
1211 AAGCTCAAGATGGGATAAGTGTTAAGGACTGGATGAGAAAGCAAGGTGTGCCGGACAGGGTGACAGATGAGGTGTTCATTGCTATGTCAAAGGCACTCAACTTTATAAAC
 404   A  Q  D  G  I  S  V  K  D  W  M  R  K  Q  G  V  P  D  R  V  T  D  E  V  F  I  A  M  S  K  A  L  N  F  I  N
1321 CCTGACGAACTTTCAATGCAGTGCATTTTGATCGCATTGAACAGGTTTCTTCAGGAGAAACATGGTTCAAAAATGGCCTTTTTAGATGGTAATCCTCCTGAGAGACTTTG
 441 P  D  E  L  S  M  Q  C  I  L  I  A  L  N  R  F  L  Q  E  K  H  G  S  K  M  A  F  L  D  G  N  P  P  E  R  L  C
1431 CATGCCGATTGTTGAACACATTGAGTCAAAAGGTGGCCAAGTCAGACTGAACTCACGAATAAAAAAGATTGAGCTGAATGAGGATGGAAGTGTCAAGAGTTTTATACTGA
 477   M  P  I  V  E  H  I  E  S  K  G  G  Q  V  R  L  N  S  R  I  K  K  I  E  L  N  E  D  G  S  V  K  S  F  I  L  S
1541 GTGACGGTAGTGCAATCGAGGGAGATGCTTTTGTGTTTGCCGCTCCAGTGGATATTTTCAAGCTTCTATTGCCTGAAGACTGGAAAGAGATTCCATATTTCCAAAAGTTG
 514   D  G  S  A  I  E  G  D  A  F  V  F  A  A  P  V  D  I  F  K  L  L  L  P  E  D  W  K  E  I  P  Y  F  Q  K  L
1651 GAGAAGTTAGTCGGAGTACCTGTGATAAATGTACATATATGGTTTGACAGAAAACTGAAGAACACATATGATCATTTGCTCTTCAGCAGAAGCTCACTGCTCAGTGTGTA
 551 E  K  L  V  G  V  P  V  I  N  V  H  I  W  F  D  R  K  L  K  N  T  Y  D  H  L  L  F  S  R  S  S  L  L  S  V  Y
1761 TGCTGACATGTCTGTTACATGTAAGGAATATTACAACCCCAATCAGTCTATGTTGGAATTGGTTTTTGCACCTGCAGAAGAGTGGATATCTCGCAGCGACTCAGAAATTA
 587   A  D  M  S  V  T  C  K  E  Y  Y  N  P  N  Q  S  M  L  E  L  V  F  A  P  A  E  E  W  I  S  R  S  D  S  E  I  I
1871 TTGATGCAACGATGAAGGAACTAGCAACGCTTTTTCCTGATGAAATTTCAGCAGATCAAAGCAAAGCAAAAATATTGAAGTACCATGTTGTCAAAACTCCGAGGTCTGTT
 624   D  A  T  M  K  E  L  A  T  L  F  P  D  E  I  S  A  D  Q  S  K  A  K  I  L  K  Y  H  V  V  K  T  P  R  S  V
1981 TATAAAACTGTGCCAGGTTGTGAACCCTGTCGGCCTTTACAAAGATCCCCAATAGAGGGGTTTTATTTAGCCGGTGACTACACGAAACAGAAATACTTGGCTTCAATGGA
 661 Y  K  T  V  P  G  C  E  P  C  R  P  L  Q  R  S  P  I  E  G  F  Y  L  A  G  D  Y  T  K  Q  K  Y  L  A  S  M  E
2091 AGGCGCTGTCTTATCAGGAAAGCTTTGTGCTCAAGCTATTGTACAGGATTATGAGTTACTTGTTGGACGTAGCCAAAAGAAGTTGTCGGAAGCAAGCGTAGTTTAGCTTT
 697   G  A  V  L  S  G  K  L  C  A  Q  A  I  V  Q  D  Y  E  L  L  V  G  R  S  Q  K  K  L  S  E  A  S  V  V  *
2201 GTGGTTATTATTTAGCTTCTGTACACTAAATTTATGATGCAAGAAGCGTTGTACACAACATATAGAAGAAGAGTGCGAGGTGAAGCAAGTAGGAGAAATGTTAGGAAAGC
2311 TCCTATACAAAAGGATGGCATGTTGAAGATTAGCATCTTTTTAATCCCAAGTTTAAATATAAAGCATATTTTATGGAATTC
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FIG. 1. Nucleotide sequence of the cDNA of *pds* and the deduced amino acid sequence of PDS from *L. esculentum*. The 22 amino acids that may comprise the carotenoid-binding domain are underlined.

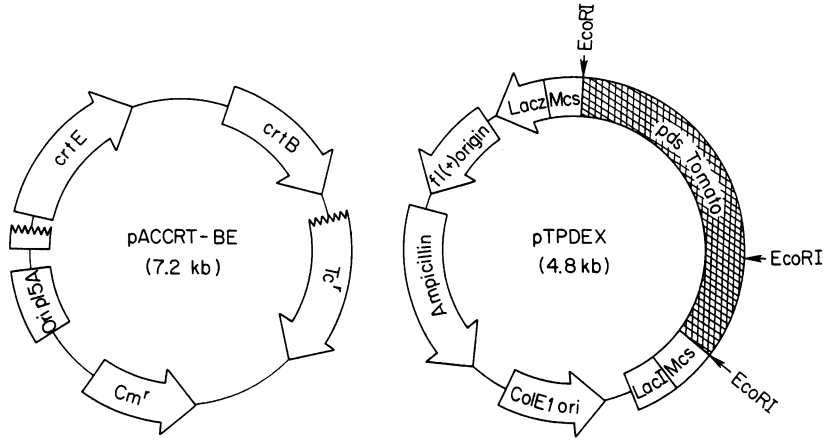

FIG. 2. Structure of plasmids that express carotenoid biosynthetic genes in *E. coli*. Plasmid pACCRT-BE contains *crtE* and *crtB* genes from *E. uredovora*, which code for geranylgeranyl pyrophosphate synthase and phytoene synthase, respectively. The plasmid was derived from pCAR16 (11) by deleting the 2.3-kb *Bst*EII–*Sna*BI and the 0.75-kb *Bam*HI–*Sac* I fragments from the carotenoid gene cluster and subcloning the insert in the *Eco*RV site of pACYC184. Plasmid pTPDEX was constructed by subcloning a partial *Eco*RI cleavage product of pTPD, which contains the cDNA clone of *pds* from tomato, into the plasmid vector pBluescript SK. This procedure generates a chimeric gene that codes for a fusion protein of 580 amino acid residues: 37 residues at the amino terminus are from the *lacZ* gene of *E. coli*, and 543 residues are from the PDS cDNA, beginning at Asn-41 and reading to the end of the open reading frame. The 40 amino acids from the amino terminus of PDS that were removed in this construct are part of the putative transit peptide (see text). Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Mcs, multiple cloning site.

expression of the *pds* cDNA results in the conversion of phytoene to ζ-carotene in *E. coli*. This activity was blocked by the herbicide flurtamone [5-methylamino-2-phenyl-4-(3-trifluoromethylphenyl)-3(2H)furasone], which is a specific inhibitor of PDS (20).

Expression of the *pds* Gene During Fruit Ripening. Carotenoids are produced in plastids of all green tissues. However, increased carotenogenesis and accumulation of specific carotenoids occur in chromoplasts found mainly in fruit and flowers. In tomato there is a dramatic increase in carotenogenesis during fruit ripening. Accumulation of PDS mRNA in leaves and fruit was analyzed by Northern hybridization by using the entire coding region of the PDS cDNA as probe (Fig. 4). Densitometry of the autoradiogram shown in Fig. 4 indicated that orange fruit contains 10-fold more PDS-specific RNA than mature green fruit. Our Northern analysis has not detected PDS RNA in leaves.

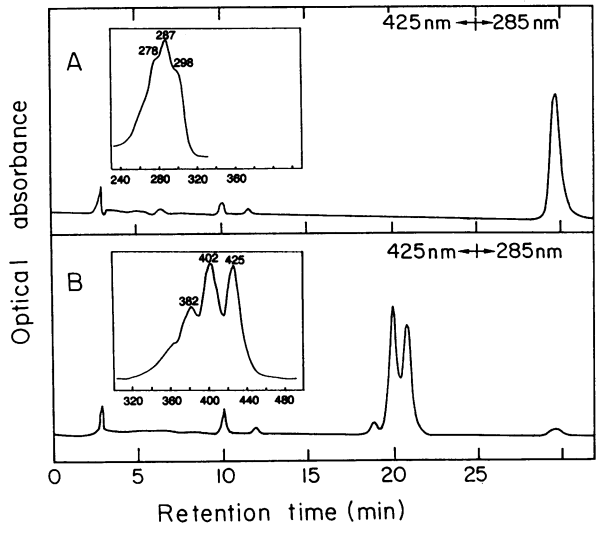

FIG. 3. HPLC separation of carotenoids accumulating in *E. coli* cells carrying plasmid pACCRT-BE (*A*) or plasmids pACCRT-BE and pTPDEX (*B*). Carotenoids were extracted from cells at stationary phase in the presence of isopropyl β-D-thiogalactoside. Absorbance was recorded at 425 nm for the first 25 min and then at 285 nm. The carotenoids were identified by the retention time of peaks, which matched those of standards, and by their absorption spectra. The peak at 29.8 min showed a typical phytoene absorption spectrum, and the peaks at 18.8, 20.1, and 20.9 min showed ζ-carotene absorption spectra (displayed in *Insets*).

Comparison of Amino Acid Sequences of PDS from Different Species. Comparison of the deduced amino acid sequence of PDS from tomato with that of *D. bardawil* revealed a high degree of conservation: 69% identity and 81% similarity. The PDS of *Synechococcus* PCC7942 is 64% identical and 78% similar to tomato PDS. This conservation is clearly illustrated by homology plots (Fig. 5). The homology between the eukaryotic and cyanobacterial PDS begins only at residues 112 and 65 of the tomato and *D. bardawil* proteins, respectively, corresponding to the first residue in the cyanobacterial protein. In contrast, very little homology was found between tomato PDS and the *crtI* gene products of *E. herbicola* or *N. crassa*. The latter two show strong conservation in their amino acid sequences to the PDS from *R. capsulatus*.

The tomato PDS contains a βαβ dinucleotide-binding motif found in many dehydrogenases that use NAD or NADP as cofactors (21). This region is also found in the amino terminus of the cyanobacterial PDS, as well as in all of the *crtI* genes (Fig. 6).

DISCUSSION

We have presented the nucleotide sequence of a carotenoid biosynthetic gene from a higher plant. Expression of this gene

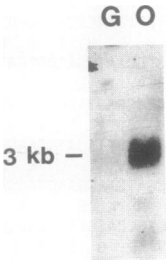

FIG. 4. Expression of *pds* in ripening fruit of tomato. RNA extracted from pericarp tissues of mature-green (G) and orange (O) fruit was analyzed by Northern hybridization by using the ^{32}P-labeled cDNA of *pds* as probe. Equal amounts (25 μg) of poly(A)$^+$ RNA of each type were separated by gel electrophoresis and blotted.

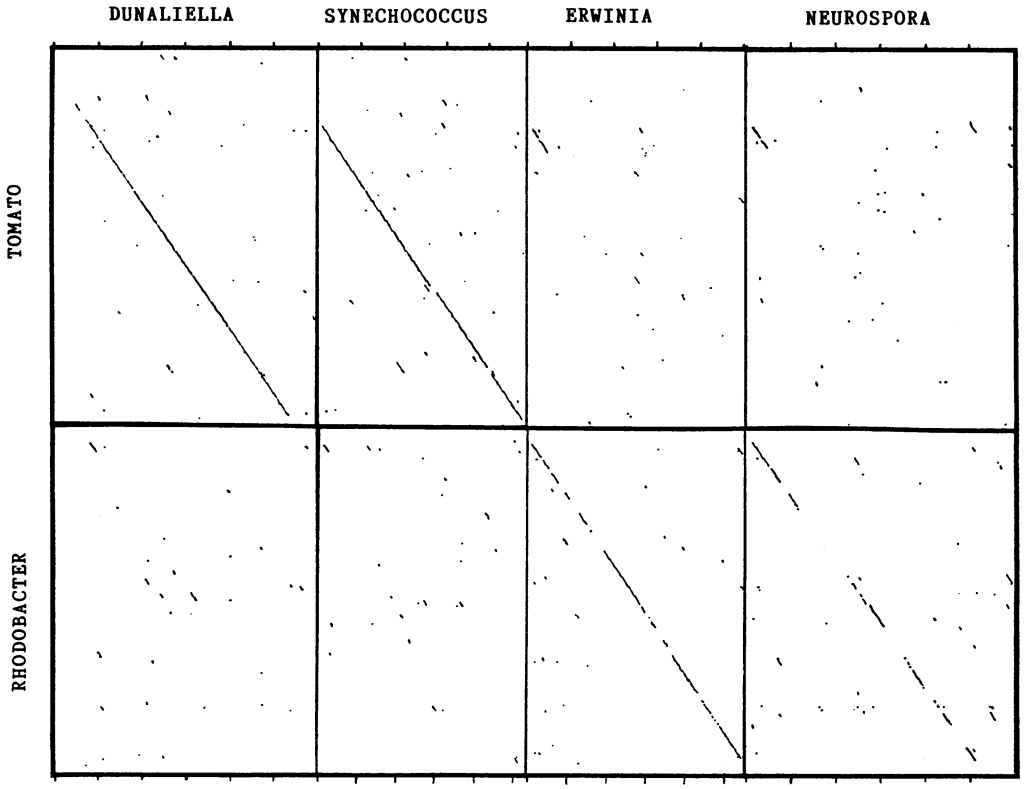

FIG. 5. Protein homology plots of PDSs from various organisms. The amino acid sequence of PDS from *D. bardawil* was deduced from a cDNA sequence cloned as described in text (I.P. and J.H., unpublished work). Amino acid sequences of PDSs from *Synechococcus* PCC7942 (14), *Rhodobacter* (8, 9), *Erwinia* (10, 11), and *Neurospora* (12) have been published. Amino acid sequences were compared through the entire length of the polypeptides for identities or similarities in a window of 15 residues. Seven identical or similar amino acids were scored as a dot.

in *E. coli* establishes its identity as *pds*, which encodes the enzyme PDS. This result suggests that one polypeptide is responsible for PDS activity in tomato and cyanobacteria. Whether as a single polypeptide or as a homodimeric (or oligomeric) protein, this enzyme catalyzes the introduction of two symmetric double bonds into the carbon chain of phytoene. This result implies that ζ-carotene is a *bona fide* product of a single enzyme in the carotenoid biosynthetic pathway in which phytofluene is predicted to be an intermediate between phytoene and ζ-carotene.

Comparison of the deduced amino acid sequences of PDS from tomato, *D. bardawil*, and *Synechococcus* PCC7942 reveals a striking amino acid identity of >65% with many conservative changes. However, the overlap of homology between the cyanobacterial PDS and that of *D. bardawil* and tomato is found only after a leader region in the two eukaryotic PDSs. Because *pds* is a nuclear gene, we postulate that this region serves as a transit peptide for the transport of PDS into plastids. Results obtained with the PDS from soybean (22), which is 89% identical to the tomato PDS, support this prediction. The soybean polypeptide was shown to be imported into isolated chloroplasts while being processed to a smaller form. Our preliminary results indicate that *in vitro*-translated tomato PDS is a polypeptide with apparent mo-

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                                                                    20
L.e. (pds)                                                MPQIGLVSAVNLRVQGSSAYLWSSRSSSLGTL
G.m. (pds)                                                      MAACGYISAANFNYLVGARNI

                          40                  60                  80                  100
                        *  * ** ***  *      *   *      ** *** ******* ****  *** **
L.e. (pds)          ESRDGCLQRNSLCFAGSESMGHKLKIRTPHATTRRLVKDLGPLKVVCIDYPRPELDNTVNYLEAAFLS
G.m. (pds)          SKFASSDATISFSFGGSDSMGLTLRPAPIRAP.KRNHFS..PLRVVCVDYPRPELENTVNFVEAAYLS
D.b. (pds)                MQTMQGRAHAQTLNRQPVTQVAGRTQRRVGRS..RLQVYARDFPPPQFDGTASYQDAVALS

                                  120                 140                 160
                    ********* **     ###  #   #  #   #         #  ##
L.e. (pds)          STFRASPRPTKPLEIVIAGAGLGGLSTAKYLADAGHKPILLEARDVLGGKVAAWKDDDGDWYETGLHI
G.m. (pds)          STFRASPRPLKPLNIVIAGAGLAGLSTAKYLADAGHKPILLEARDVLGGKVAAWKDKDGDWYETGLHI
D.b. (pds)          TKLQNAPRPAKPQRVVIAGAGLAGLSAAKYLSDAGHIPIVLEARDVLGGKVAAWKDEDGDWYETGLHI
Syn. 7942 (pds)               MRVAIAGAGLAGLSCAKYLADAGHTPIVYERRDVLGGKVAAWKDEDGDWYETGLHI
Syn. 6803 (pds)               MRVAIAGAGLAGLACAKYLADAGFTPVVLERRDVLGGKIAAWKDEDGDWYETGLHI
Rb.c. (crtI)            MSKNTEGMGRAVVIGAGLGGLAAAMRLGAKGYKVTVVDRLDRPGGRGSSITKGGHRFDLGPTIV
E.h. (crtI)                   MKKTVVIGAGFGGLALAIRLQAAGIPTVLLEQRDKPGGRAYVWHDQGFTFDAGPTVI
E.d. (crtI)                   MKPTTVIGAGFGGLALAIRLQAAGIPVLLLEQRDKPGGRAYVYEDQGFTFDAGPTVI
N.c. (al-1)             MAETQRPRSAIIVGAGAGGIAVAARLAKAGVDVTVLEKNDFTGGRCSLIHTKAGYRFDQGPSL
NADP(H) binding motif                 G-G--A---A------G-------R-----R
NAD(H) binding motif                  G-G--G---G------G------E------B
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FIG. 6. Alignment of sequences in the amino termini of PDSs from different species. Amino acid sequences of PDSs from soybean (G.m.) (22), *Synechococcus* PCC7942 (Syn. 7942) (14), *R. capsulatus* (Rb.c.) (8, 9), *E. herbicola* (E.h.) (10), *E. uredovora* (E.u.) (11), and *N. crassa* (N.c.) (12) have been published. The sequences of *pds* from *D. bardawil* (D.b.) (I.P. and J.H., unpublished work), *Synechocystis* PCC6803 (Syn. 6803), and tomato (L.e.) were determined in our laboratory. Consensus motifs for the binding sites of NAD(H) and NADP(H) (21) are given below the sequences. The arginine residues thought to interact with the 2′-phosphate of NADP(H) are underlined. The conserved glutamic acid residue, involved in binding the 2′-OH of the ribose in NAD(H), is doubly underlined. Residues conserved in all PDSs are marked with #. Residues in the putative transit sequences conserved between the tomato and *Dunaliella* PDS polypeptides are overlined and the residues conserved between tomato and soybean are marked with asterisks. B = proline (P), alanine (A), serine (S), or glycine (G). Numbering refers to tomato PDS.

lecular mass of 64 kDa, which is processed to 50 kDa during its import into isolated pea chloroplasts. This is approximately the expected size of the polypeptide that would be obtained were pre-PDS cleaved around residue 113. If that were the case, the mature functional enzyme in tomato would have the same size as its counterpart in cyanobacteria.

The deduced amino acid sequences of the PDS-encoding *crtI* genes of bacteria and fungi are highly conserved (Fig. 5). This observation led Armstrong *et al.* (10) to propose a common evolutionary origin for these genes. A similar conclusion can be reached for the *pds* gene products of cyanobacteria, algae, and plants. However, the two groups of enzymes have clearly unique primary structures. This finding suggests that the two types of PDS have no common ancestor and that they originated through convergent evolution independently in green photosynthetic organisms (*pds* type) and in nongreen organisms (*crtI* type).

Several lines of biochemical evidence further support this hypothesis. All *pds*-type enzymes are inhibited by certain bleaching herbicides, whereas those of the *crtI* type are unaffected (23). PDS catalyzes the introduction of two double bonds into phytoene to form ζ-carotene, whereas the *crtI*-type gene product introduces either three double bonds to form neurosporene in *Rhodobacter* or four double bonds to form lycopene in *Erwinia* (19). Similar convergent evolution has been suggested for serine proteases from bacilli and mammals, which have different primary structures but are functionally similar in substrate binding and catalysis (24). Convergent evolution of two nucleotide-binding proteins was proposed for *E. coli* thioredoxin reductase and human gluthatione reductase (25). These enzymes share the same catalytic mechanism and have similar tertiary structures but diverge in their primary structure and their active sites.

The only significant similarity found between *crtI* and *pds* gene products was in the nucleotide-binding consensus motifs found in their amino termini (Figs. 5 and 6). It is not possible to determine the specificity for either NAD, NADP, or FAD solely by the amino acid sequence. However, the consensus sequence in enzymes using NADP differs in several residues from that of NAD enzymes (21). From comparison of these consensus sequences with the various PDSs, it appears that all of them contain a composite sequence that is ambiguous in its specificity for either NAD or NADP. Enhancement of the phytoene desaturation in a cyanobacterial cell-free system was seen after adding either NAD or NADP, but no significant preference for either one was found (26).

Despite the lack of homology at the amino acid level between the two classes of PDS, the predicted hydropathy structures of the carboxyl termini of enzymes of the two classes are similar (data not shown). This domain contains the conserved "crt box" that is found in all of the *crtI* gene products and in the *R. capsulatus crtD* gene product, which encodes neurosporene dehydrogenase. Armstrong *et al.* (8) postulated that this is the carotenoid-binding domain. The amino acid sequence of the corresponding domain in the *pds*-type enzymes differs from the *crtI* type. However, this sequence contains a stretch of 22 amino acids (shown in Fig. 1) that is conserved in PDS of cyanobacteria, algae, and plants. Therefore this sequence could be the carotenoid-binding region in PDS.

Accumulation of PDS transcripts in tomato fruit at the "orange" stage, which are actively synthesizing large amounts of carotenoids, is ≈10 times higher than in green fruit. This finding suggests that transcriptional control may regulate the expression of *pds* during fruit ripening. However, as carotenoids are also synthesized in all photosynthetic tissues, it is surprising that no PDS transcripts could be detected in tomato leaves. This phenomenon was described also in soybean, where no PDS transcripts could be detected in any tissue (22). We assume simply that the expression of the *pds* gene in foliar tissues is below the detection level by Northern analysis. Although it may be speculated that there are two types of *pds* genes, one for fruit ripening and one for constitutive synthesis in green tissues, it must be noted that the amino acid sequence of the tomato PDS highly resembles the soybean PDS (89% identity, 95% similarity). The two polypeptides are most probably coded by homologous genes. Because the soybean cDNA of *pds* was derived from cotyledon mRNA and the tomato *pds* is expressed in fruit, the same enzyme is probably involved in carotenoid biosynthesis in chloroplasts and chromoplasts.

Mutations in the *pds* gene from *Synechococcus* PCC7942, which lead to amino acid substitutions in PDS, were found to confer resistance to a number of PDS-inhibiting herbicides (ref. 14; D.C. and J.H., unpublished work). These amino acid residues are conserved in the tomato PDS. We have recently observed that overexpression of *pds* in cyanobacteria increased carotenoid content of the cells (D.C. and J.H., unpublished result). The cloning of *pds* from tomato opens the door for a variety of genetic manipulations of carotenoid synthesis in important crop plants.

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