

## Cloning and characterization of a maize cDNA encoding phytoene desaturase, an enzyme of the carotenoid biosynthetic pathway

Zhou-Hui Li<sup>1</sup>, Paul D. Matthews<sup>1</sup>, Benjamin Burr<sup>2</sup> and Eleanore T. Wurtzel<sup>1,\*</sup>

<sup>1</sup>Department of Biological Sciences, Lehman College, The City University of New York, 250 Bedford Park Boulevard, West Bronx, NY 10468, USA (\*author for correspondence); <sup>2</sup>Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA

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### Abstract

To study regulation of the plastid-localized maize carotenoid biosynthetic pathway, a cDNA encoding phytoene desaturase (PDS) was isolated and characterized. The DNA sequence of the maize *Pds* cDNA was determined and compared with available dicot *Pds* genes. The deduced PDS protein, estimated at 64.1 kDa (unprocessed), had a dinucleotide binding domain and conserved regions characteristic of other carotene desaturases. Alignment of available PDS sequences from distantly related organisms suggests that *Pds* has potential as a phylogenetic tool. By use of heterologous complementation in *Escherichia coli*, maize PDS was shown to catalyze two desaturation steps converting phytoene to  $\zeta$ -carotene. RFLP (restriction fragment length polymorphism) mapping was used to place *Pds* on chromosome 1S near *viviparous5* (*vp5*), and RT-PCR (reverse-transcriptase polymerase chain reaction) analysis indicated reduced *Pds* transcript in *vp5* mutant relative to normal endosperm. Other phytoene-accumulating mutant endosperms, *vp2* and *white3* (*w3*), showed no difference in *Pds* transcript accumulation as compared with normal endosperm counterparts. RT-PCR analysis of *Pds* transcript accumulation in developing endosperm showed *Pds* was constitutively expressed. Therefore, endosperm carotenogenesis is not regulated by increasing the level of *Pds* transcripts.

### Introduction

Carotenoids serve multiple functions in plants; as accessory pigments in photosynthesis; as photoprotectors; and as precursors to the hormone, abscisic acid (ABA). In animals, carotenoids are essential precursors to Vitamin A and related

compounds (reviewed in [2]). Carotenoids are synthesized and accumulated in plastids; these plastids include chloroplasts and nonphotosynthetic plastids, such as chromoplasts of fruits, flowers, some endosperms and roots (for reviews, see [5, 12, 29]).

Because of the varied roles and location of

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Database under the accession number U37285.

carotenoids within plastids of different membrane architectures, we expect tissue-specific regulation of the pathway. Maize is an excellent model system to explore regulation of carotenoid biosynthesis because of the many mapped and biochemically characterized mutations blocking the pathway. These include recessive, dominant, and suppressor/modifier alleles [27].

The synthesis of  $C_{40}$  carotenoids begins with condensation of two molecules of geranylgeranyl pyrophosphate to produce phytoene, a step catalyzed by phytoene synthase (PSY). Phytoene synthesis occurs in plastid stroma, whereas subsequent steps leading to synthesis of colored carotenoids occur on plastid membranes [4, 17, 21, 23]. This latter phase includes four sequential desaturations of phytoene. In dicots and cyanobacteria, these steps are catalyzed by two enzymes, PDS (phytoene desaturase) and ZDS ( $\zeta$ -carotene desaturase), each mediating two steps (reviewed in [3]). In other carotenogenic organisms, including fungi, nonphotosynthetic bacteria, and photosynthetic bacteria, one phytoene desaturase enzyme may catalyze up to four desaturation steps (reviewed in [2]). As shown in Fig. 1, recessive alleles of four maize loci, *vp2*, *vp5*, *w3*, and *vp9*, block desaturation both in endosperm and plant [25, 32]. It is presently unclear what functions are represented by these

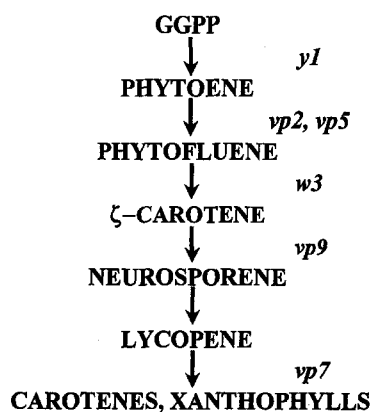


Fig. 1. Lethal recessive mutations blocking the carotenoid biosynthetic pathway of maize. *w3* results in accumulation of both phytoene and phytofluene. Many, but not all, *y1* mutations affect only endosperm and are not lethal to the plant.

genes and whether these genes reflect an alternative array of overlapping or non-overlapping desaturation functions, each mediating a particular number of steps, or encode regulatory or ancillary functions such as pigment-binding proteins or oxidoreductases [22].

With the exception of maize *Y1*, which has been shown by heterologous complementation to encode PSY [6; Yoganathan and Wurtzel, unpublished], there has been little characterization of genes encoding the biosynthetic enzymes in monocots. Genes encoding PDS have primarily been isolated from dicots (reviewed in [3]) and the temporal regulation of their expression examined in chromoplasts [10, 11, 15, 26]. Here PDS transcript abundance appears to correlate with carotenoid accumulation. Therefore, we decided to isolate the maize gene encoding PDS to characterize the temporal regulation of PDS expression in endosperm plastids (amyloplasts). Using RFLP mapping and analysis of steady-state levels of accumulated transcripts, we have associated a known maize carotenoid locus with a specific gene product, PDS. To demonstrate the function and determine the number of desaturation steps catalyzed by maize PDS, we employed a heterologous complementation system. Finally, we showed that the carotenoid biosynthetic pathway in maize endosperm was not regulated by modulation of *Pds* transcript levels.

## Material and methods

### Plant materials

For developmental studies, maize plants were grown under standard field conditions at the Black Rock Forest (Cornwall, NY). For other studies, plants were grown either in the field or in greenhouses at Lehman College, City University of New York. Inbred lines were obtained from Dr S. Briggs (Pioneer Hy-Bred) and mutant lines were provided by Dr D. Robertson (Iowa State University) and from the Maize Coop (University of Illinois, Urbana, IL). The *vp5* mutant used in this study was generated in a *Mutator* background, by Dr Robertson, and is therefore designated *vp5-*

*Mum*. By introduction of this mutation into another genetic background in which the *Mutator* element was inactive, a stable phenotype was obtained and revertant sectors no longer observed. *vp2* and *w3* are stable mutations. Developing endosperms were collected and frozen at  $-80^{\circ}\text{C}$  prior to use.

#### *Amplification of the maize Pds gene*

The CLUSTAL program [14] from PC/Gene software (Intelligenetics, Mountain View, CA) was used to align *Pds* sequences from tomato (GenBank accession numbers X59948, S36691), soybean (M64704), pepper (X68058) and *Arabidopsis* (L16237). A region of high homology, corresponding to nucleotides 1905–2099 of tomato *Pds*, was shared by all available dicot *Pds* genes. Degenerate oligonucleotide primers, 5'-CCTG-ATGAAATC(T)TCG(A,T)GCG(A,T)GAC(T)CA-3' and 5'-ACAGCA(G)CCTTCCATG(T)GAAGCC(T)AA-3', were used to amplify the corresponding region of maize *Pds* from maize B73 genomic DNA as follows: DNA, 0.1  $\mu\text{g}$ , in 20  $\mu\text{l}$  PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1 mM  $\text{MgCl}_2$ , 160  $\mu\text{M}$  each dNTP, 0.4  $\mu\text{M}$  each primer; 1  $\mu\text{g}/\mu\text{l}$  bovine serum albumin (BSA), 0.1 unit/ $\mu\text{l}$  *Taq* polymerase (Gibco-BRL, Gaithersburg, MD)) was incubated for 1 cycle at  $94^{\circ}\text{C}$  (3 min), followed by 40 cycles at  $94^{\circ}\text{C}$  (30 s),  $48^{\circ}\text{C}$  (30 s),  $72^{\circ}\text{C}$  (30 s) and one cycle of  $72^{\circ}\text{C}$  (10 min).

#### *Isolation of maize Pds cDNA clones*

One to two million clones of a  $\lambda\text{gt}11$  cDNA library [9], prepared from RNA extracted from maize endosperm dissected at 14 days after pollination (DAP), were screened with the PCR-amplified maize *Pds* fragment [28]. Seven positive clones were isolated, phage DNA extracted according to Sambrook *et al.* [28], and inserts amplified using primers, 5'-AGGCACATGGCTGAATATCG-3' and 5'-CGGCAGTACAATGGATTCC-3'. Lambda DNA, 0.1  $\mu\text{g}$ , in

20  $\mu\text{l}$  PCR buffer (20 mM Tris pH 8.2, 10 mM KCl, 2.5 mM  $\text{MgCl}_2$ , 1 mM  $(\text{NH}_4)_2\text{SO}_4$ , 200  $\mu\text{M}$  each dNTP; 1  $\mu\text{M}$  each primer, 1  $\mu\text{g}/\mu\text{l}$  BSA, 0.1% Triton X-100, 0.025 unit/ $\mu\text{l}$  *Pfu* DNA polymerase (Stratagene)) was incubated for one cycle at  $94^{\circ}\text{C}$  (2.5 min), then 40 cycles at  $94^{\circ}\text{C}$  (30 s),  $55^{\circ}\text{C}$  (30 s),  $72^{\circ}\text{C}$  (2 min) and one cycle of  $72^{\circ}\text{C}$  (10 min).

#### *DNA sequence analysis*

The *fmol*<sup>TM</sup> DNA Sequencing System (Promega, Madison, WI) was used for initial sequencing of phage DNA inserts using primers described for PCR. The plasmid deletion series prepared for the complementation analysis was used for making single-stranded templates for sequence analysis of the entire gene. Complete sequencing of maize *Pds* was carried out using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH). Sequence analysis and homology comparisons were carried out using PCGene software (Intelligenetics). Alignments were carried out using the program CLUSTAL. *Pds* sequences used for comparisons shown in Figs. 3 and 4 are as follows: maize, this paper (GenBank U37285); for *Arabidopsis*, pepper, soybean, tomato, see GenBank numbers listed above; *Synechocystis*, X62574; *Synechococcus*, X55289; sequence encoding CRTI from *Erwinia herbicola*, GenBank M87280; sequence encoding CRTI and CRTD from *Rhodobacter sphaeroides*, X82458; sequence encoding ZDS from *Anabaena*, S43324. Maize and pepper *rbcL* sequences for DNA and protein comparisons described in the discussion were deposited under accession numbers Z11973 and U08610, respectively.

#### *Subcloning, expression and functional complementation of maize Pds*

Amplified  $\lambda\text{gt}11$  inserts were purified by adsorption to Glass-Milk (GeneClean II kit, BIO 101, Vista, CA) following the manufacturer's direc-

tions, treated at 37 °C for 1 h with Klenow fragment and 10 mM dNTP to create blunt ends, and then ligated to *Sma* I-linearized vector, pBlue-script II SK(-) (Stratagene). One clone, found to be in the sense orientation with respect to *lacZ*, on the basis of sequencing and restriction mapping, was designated pMPDS3. This plasmid was purified by CsCl equilibrium density centrifugation according to Sambrook *et al.* [28]. To create an in-frame fusion with *lacZ*, the plasmid was linearized with *Not* I and *Bst* XI, and subjected to 5'-end deletions using the Exonuclease III and Mung Bean Nuclease Deletion Kit (Stratagene, La Jolla, CA). Religated plasmids were transformed into *Escherichia coli* JM101 containing plasmid pACCRT-EB, encoding GGPPS and PSY from *Erwinia uredovora* [19]. Transformants containing both the deletion derivative of pMPDS3 and pACCRT-EB were selected by resistance to ampicillin and chloramphenicol and then grown in liquid culture (LB medium) with appropriate antibiotics. Expression of the LacZ-PDS fusion proteins was induced by addition of isopropylthio- $\beta$ -D-galactoside (IPTG) (1 mM final concentration) during log phase. After growth to stationary phase, pigments were extracted and analyzed by HPLC.

#### *Pigment extraction and HPLC analysis*

Fifteen ml stationary phase cultures of *E. coli* cells were pelleted and resuspended in 20 ml methanol and carotenoids extracted according to Sandmann [30], except that 15 ml petroleum ether was used in place of diethyl ether. Pigments were separated by reverse phase HPLC, using a 25 cm  $\times$  4.6 mm Spherisorb ODS-1 5u C18 column (Phenomenex, Torrance, CA), and a solvent of acetonitrile/methanol/isopropanol (85:10:5) with flow rate of 1 ml/min using a Series 410 BIO LC Pump (Perkin Elmer, Norwalk, CT). Peaks were detected using an LC-480 Auto Scan photodiode array detector (Perkin Elmer). Alternatively, a Waters HPLC system with 600 controller and pump, a 996 photodiode array detector, and WISP 717 autosampler were used. Peaks were

identified on the basis of co-migration and shared spectrophotometric profiles with known standards.

#### *RNA extraction and RT-PCR*

Total RNA of maize was extracted from endosperms collected at various DAP and from leaves of young plants (2–3 leaf stage) [20]. RNA pellets were resuspended in DEPC (diethyl pyrocarbonate)-treated water, centrifuged 5 min at 14 000 rpm in an Eppendorf centrifuge, and the supernatants collected. RNA concentration was estimated spectrophotometrically and total RNA (1  $\mu$ g) used as template for cDNA synthesized with the SuperScript Preamplification System (Gibco-BRL) for first-strand cDNA synthesis. One fourth (5  $\mu$ l) of product, ca. 1–4 ng cDNA, was used for PCR in a final volume of 25  $\mu$ l. The amount of total RNA used was first tested to ensure linearity of response in the RT-PCR reaction (data not shown). Primers used for amplification were: *Pds*, 5'-GGAAGTGTGAAACACTTCGC-3' and 5'-GAAACCTTCGATAGGTGACC-3'; *Sh1*, 5'-ATCCCTGAGAAAGGCAGAGG-3' and 5'-AGTGACTCCCAACTTGTGCG-3' (GenBank accession number X02382). The conditions for PCR were: 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 100  $\mu$ g/ $\mu$ l BSA; 2 mM DTT (dithiothreitol), 0.1 mM each dNTP, 0.4  $\mu$ M each primer, 0.1  $\mu$ l/ $\mu$ l *Taq* DNA polymerase (BRL). The protocol for PCR was: one cycle of 94 °C (2 min), followed by 40 cycles of 94 °C (30 s), 52 °C (30 s), 72 °C (30 s) and one cycle of 72 °C (10 min). 10  $\mu$ l of each PCR reaction was analyzed by electrophoresis on 1.8% agarose gels in 0.5  $\times$  TBE. The sizes of the PCR products for *Sh* and for *Pds* were 673 and 528 bp, respectively.

## **Results**

#### *Isolation of a maize Pds cDNA*

Since we observed that dicot *Pds* genes hybridized poorly to monocot sequences, we chose to amplify maize *Pds* from genomic DNA using de-

1 ATGGACACTGGCTGCCTCATCTATGAATATTACTGGAGCTAGCCAG  
 1 M D T G C L S S M N I T G A S Q  
 49 ACAAGATCTTTTGGGGGCAACTTCTCCTCAGAGATGTTTTGGGAGT  
 17 T R S F A G Q L P P Q R C F A S  
 97 AGTCACTATAACAAGCTTTGCCGTGAAAAACTTGTCTCAAGGAATAAA  
 33 S H Y T S F A V K K L V S R N K  
 145 GGAAGGAGATCACACCGTAGACATCCTGCCTTGCAGGTTGTCTGCAAG  
 49 G R R S H R R H P A L Q V V C K  
 193 GATTTCCAAGACCTCCACTAGAAGCACATAAACTATTTGGAAGT  
 65 D F P R P P L E S T I N Y L E A  
 241 GGACAGCTCTTTCATTTTTTAGAAACAGCGAACGCCCCAGTAAGCCG  
 81 G Q L S S F F R N S E R P S K P  
 289 TTGCAGGTCGTGGTTGCTGGTGCAGGATTGGCTGGTCTATCAACAGCG  
 97 L Q V V V A G A G L A G L S T A  
 337 AAGTATCTGGCAGATGCTGCCATAAACCCATATTGCTTGAGCAAGA  
 113 K Y L A D A G H K P I L L E A R  
 385 GATGTTTTGGGTGAAAGTAGCTGCTTGGAAAGATGAAGATGGAGAT  
 129 D V L G G K V A A W K D E D G D  
 433 TGGTACGAGACTGGCTTCATATATTTTTTGGAGCTTATCCCAACATA  
 145 W Y E T G L H I F F G A Y P N I  
 481 CAGAATCTGTTGGCGAGCTTAGGATTGAGGATCGTTTGCAGTGGAAA  
 161 Q N L F G E L R I E D R L Q W K  
 529 GAACACTCTATGATATTGCGCATGCCAAACAAGCCAGGAGATTTCAGC  
 177 E H S M I F A M P N K P G E F S  
 577 CGGTTGATTTCCAGAACTTTGCCAGCACCTATAAATGGGATATGG  
 193 R F D F P E T L P A P I N G I W  
 625 GCCATATTGAGAAACAATGAATGCTTACTTGGCCGGAGAGGTTGAAG  
 209 A I L R N N E M L T W P E K V K  
 673 TTTGCAATCCGACTTCTGCCAGCAATGGTGGTGGTCAACCTTATGTT  
 225 F A I G L L P A M V G G Q P Y V  
 721 GAAGCTCAAGATGGCTTAACCGTTTTCAGAATGGATGAAAAGCAGGGT  
 241 E A Q D G L T V S E W M K K Q G  
 769 GTTCTGATCGGTTGAACGATGAGGTTTTTATGCAATGTCACAGGCA  
 257 V P D R V N D E V F I A M S K A  
 817 CTCARATTCATAAATCCTGATGAGCTATCTATGCAGTGCATTTTGATT  
 273 L N F I N P D E L S M Q C I L I  
 865 GCTTTGAACCGATTCTTTCAGGAGAAGCATGGTCTAAAATGGCATTTC  
 289 A L N R F L Q E K H G S K M A F  
 913 TTGGATGGTATCCGCCCTGAAAGGCTATGCATGCCTATTGTTGATCAC  
 305 L D G N P P E R L C M P I V D H  
 961 ATTCGGTCTAGGGTGGAGAGGTCGCCCTGAATTCCTGATTTAAAAG  
 321 I R S R G E V R L N S R I K K  
 1009 ATAGAGTGAATCCTGATGGAACTGTAAACACTTCCGCACTTAGTGAT  
 337 I E L N D G T V K H F A L S D  
 1057 GGAACCTCAAATAACTGGAGATGCTTATGTTGTGCAACACAGCTGCAT  
 353 G T Q I T G D A Y V C A T P V D  
 1105 ATCTTCAAGCTTCTTGTACCTCAAGAGTGGAGTGAATTAATTAATTC  
 369 I F K L L V P Q E W S E I T Y F  
 1153 AAGAACTGGAGAAGTGGTGGGAGTTCCTGTTATCAATGTTTCATATA  
 385 K K L E K L V G V P V I N V H I  
 1201 TGGTTTGCAGAAAACCTGAACAACACATATGACCACCTTCTTTTCAGC  
 401 W F D R K L N N T Y D H L L F S  
 1249 AGGAGTTCACCTTTAAGTGTCTATGCAGACATGTCAGTAACTGCAAG  
 417 R S S L L S V Y A D M S V T C K  
 1297 GAATACTATGACCCAAACCGTTCAATGCTGGAGTGGTCTTTGCTCCT  
 433 E Y Y D P N R S M L E L V F A P  
 1345 GCAGACGAATGGATTGGTGAAGTGCAGTGAATCATCGATGCAACT  
 449 A D E W I G R S D T E I I D A T  
 1393 ATGGAAGAGCTAGCCAAAGTATTCTCTGATGAAATGGCTGCTGATCAG  
 465 M E E L A K L F P D E I A A D Q  
 1441 AGTAAAGCAAAGATCTTAAAGTATCATATTGTGAAGACACCGAGATCG  
 481 S K A K I L K Y H I V K T P R S  
 1489 GTTTACAAAAGTGTCCAAACTGTGAGCCTTGCAGGCTCTCAAAGG  
 497 V Y K T V P N C E P C R P L Q R  
 1537 TCACCTATCGAAGGTTTCTATCTAGCTGGTATTACACAAAGCAGAAA  
 513 S P I E G F Y L A G D Y T K Q K  
 1585 TACCTGGCTTCTATGGAAGGTGCAGTCCCTATCCGGGAAGCTTTGTGCC  
 529 Y L A S M E G A V L S G K L C A  
 1633 CAGTCCATAGTGCAGGATTATAGCAGGCTCGCACTCAGGAGCCAGAAA  
 545 Q S I V Q D Y S R L A L R S Q K  
 1681 AGCCTACAATCAGGAGAAGTTCGCCCTCCATCTTAGTTGATGTTGGCT  
 561 S L Q S G E V P V P S \*  
 1729 TTAGCTATCGTCATCCCACTGGGTGCTATCTTATCTCCTATTTCAAT  
 1777 GGAACCCACCAATGGTGCATGTTGGAGACAACACCTGTTATGGTCTCT  
 1825 TTGACCTCTCGGGTACTGTAGTTGATGTCATATTCCGATATATAT  
 1873 GTAAAAGGACCTGCATAGCAATGTTAGACCTTGGAAAAAAA

Fig. 2. Nucleotide and amino acid sequence of the maize *Pds* cDNA. Deduced amino acid sequence is shown as single letters below the nucleotide sequence. Bold letters indicate the putative dinucleotide binding domain in the protein sequence

generate oligonucleotide primers designed by alignment of all available dicot *Pds* sequences. We sequenced the maize PCR product to verify homology to other *Pds* genes, and this PCR fragment was then used to screen a maize cDNA library of 1–2 million clones. We obtained seven clones. Based on preliminary sequence analysis and alignment with the dicot *Pds* sequences, we chose a 2.0 kb clone for further characterization.

#### Sequence analysis of maize *Pds* and comparison with other dicot *Pds* genes

We sequenced the maize *Pds* cDNA as shown in Fig. 2. Based on the deduced amino acid sequence, maize PDS, including its putative transit peptide, was found to be 571 amino acid residues with a mass of 64.1 kDa. The sequence determined here, is almost identical to a sequence of a *Pds* cDNA from another maize line, Funk F, except at 14 nucleotide positions, only four of which resulted in a change of amino acid sequence (residues 61–63, 68, and 555) [13]. However, at amino acid position 61–63, the maize *Pds* protein sequence reported here is identical to the dicot sequences shown in Fig. 3A, whereas the Funk F *Pds* protein sequence is different due to a shift in reading frame.

Figure 3A shows the comparison of the N-terminal sequence of available dicot and cyanobacterial PDS proteins. Based on this comparison, we estimate that the maize PDS transit peptide is approximately 96 residues or 10.6 kDa and therefore the plastid-localized PDS should be about 53.5 kDa. The highest homology found between the dicot and monocot putative transit sequences corresponds to residues 59–96 of maize PDS. The comparison of the proposed dinucleotide binding domain, shared by carotene desaturases (PDS, CRTI, CRTD, ZDS) found in phylogenetically distant carotenogenic organisms, is shown in Fig. 3B. Figure 3C shows the region

and the oligonucleotide primers used for PCR in the nucleotide sequence. The sequence as shown represents the entire sequence available from the cloned cDNA.

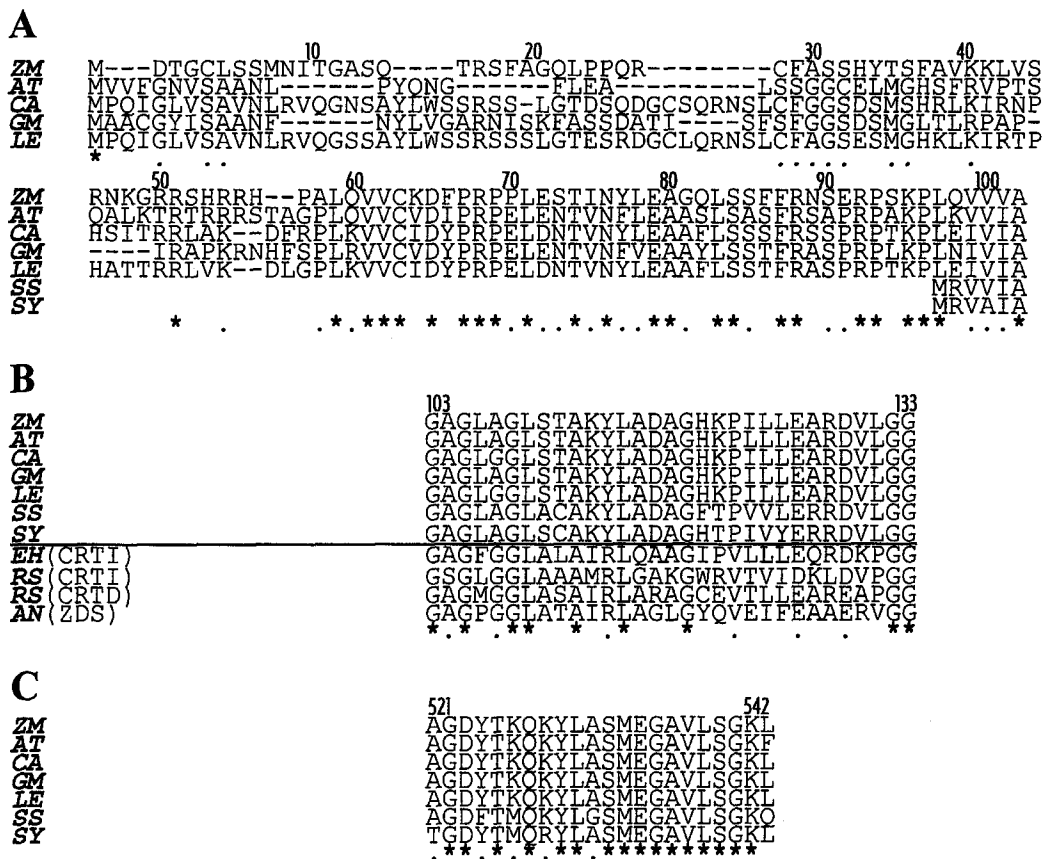


Fig. 3. PDS amino acid sequence alignments. A, N-terminus and transit sequence; B, putative dinucleotide-binding domain; C, highly conserved region used for design of degenerate oligonucleotide primers used for amplification of the maize gene. Asterisks indicate identical residues and dots indicate similar residues. ZM, maize; AT, *Arabidopsis*; CA, pepper; GM, soybean; LE, tomato; SS, *Synechocystis*; SY, *Synechococcus*; EH, *Erwinia herbicola*; RS, *Rhodobacter sphaeroides*; AN, *Anabaena*.

of high homology at the C-terminus, which was used for design of the degenerate oligonucleotide primers used initially to amplify the maize *Pds* gene. Overall nucleotide homology between the maize and other dicot *Pds* genes ranges from 70.5–72%, whereas the amino acid homology based on identical or similar residues is about 77% and 82.8–84.2%, respectively. An alignment of all available PDS protein sequences was carried out and the results are shown in Fig. 4.

*Functional analysis of maize PDS by heterologous complementation in E. coli*

To test the function of the maize *Pds* gene product, we subcloned the 2.0 kb insert into pBluescript II

SK– and designated the clone pMPDS3. By creating progressive 5'-end deletions, we obtained

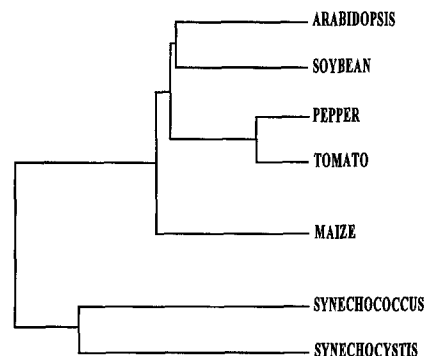


Fig. 4. Phylogenetic tree based on alignment of PDS amino acid sequences in monocots, dicots, and cyanobacteria.

several plasmids in which the insert was cloned in-frame with the *lacZ* gene. One of these, pMPDSd3-33, was introduced into *E. coli* cells carrying the plasmid pACCRT-EB encoding GGPPS (geranylgeranyl pyrophosphate synthase) and PSY from the nonphotosynthetic bacterium, *Erwinia uredovora* [19]. The enzymes GGPPS and PSY will together catalyze the synthesis of phytoene in *E. coli*. Therefore, if maize PDS is a two-step desaturase, as is the case for the dicot PDS enzyme, transformants expressing both the *Erwinia* genes and maize *Pds* will accumulate  $\zeta$ -carotene (refer to Fig. 1). Phytoene and  $\zeta$ -carotene are distinguished on the basis of retention times on reversed-phase HPLC (high-performance liquid chromatography) as well as by their unique spectrophotometric profiles. As shown in Fig. 5A, *E. coli* cells containing only the genes encoding GGPPS and PSY, accumulated

phytoene, detected at 285 nm at about 19 min. Whereas, in cells carrying genes for GGPPS, PSY and maize PDS, phytoene was converted to a mixture of  $\zeta$ -carotene isomers, which were detected at 400 nm at about 14–15 min, as shown in Fig. 5B. These results indicate that the cloned maize *Pds* encodes a two-step desaturase which catalyzes the desaturation of phytoene to  $\zeta$ -carotene.

#### RFLP mapping of maize *Pds* to chromosome

As shown in Fig. 1, mutant alleles of at least three genetic loci cause phytoene accumulation in both endosperms and green tissues, and therefore one of these loci might encode PDS. To identify the correct maize locus, we used RFLP analysis of a recombinant inbred family produced from a cross

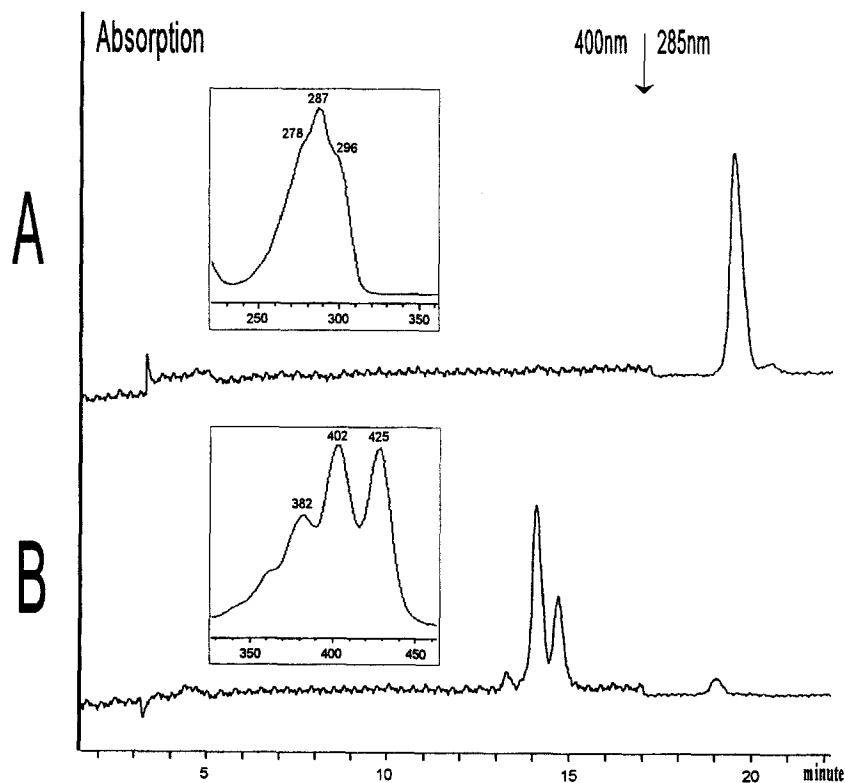


Fig. 5. HPLC analysis of products in *E. coli* heterologous complementation system. Pigments were extracted from *E. coli* cells transformed with either (A) pACCRT-EB only or (B) pACCRT-EB and pMPDSd3-33. The insets show spectrophotometric profiles of the major peaks. Phytoene has a retention time of 19 minutes and  $\zeta$ -carotene, 14–15 min.

between T232 and CM37 [7]. On the basis of strong hybridization to one fragment, we mapped maize *Pds* to chromosome 1S, near *vp5* (data not shown). Additional weak hybridization signals mapped to three other loci; 4L (1 map unit from *o2*); 2L (1 map unit from *bnl 17.25*); 1L (1 map unit from *dup103*).

#### RT-PCR analysis of *Pds* in phytoene-accumulating mutant endosperms

RT-PCR was used to examine *Pds* transcript levels in mutant endosperms accumulating phytoene. The low transcript abundance required the use of this sensitive technique over conventional northern analysis. Endosperms of the genotypes *vp5*, *vp2*, and *w3* accumulate phytoene and were tested for *Pds* transcript accumulation in comparison to normal endosperm counterparts. As shown in Fig. 6A, only *vp5* endosperms showed a visible difference in transcript accumulation as compared with transcripts accumulating in the normal endosperms. For normalization, we amplified *Sh1* (*Shrunken1*) sequences from the same cDNA; no differences in the amount of *Sh* amplification product were observed.

#### Determination of *Pds* transcript accumulation in leaves and developing endosperms

RT-PCR analysis was used to assess the temporal pattern of *Pds* transcript accumulation in developing endosperms. Also the level of transcript accumulation in endosperm was compared to that in leaves. In comparison, we also examined the accumulation of the maize *Sh* transcript which has been previously studied in developing endosperm and in leaves using northern analysis [31, 34]. As shown in Fig. 6B, the abundance of *Pds* transcripts in maize leaves and in endosperms were of comparable levels. Figure 6C shows that *Pds* was expressed in the unfertilized ear and transcript levels did not vary substantially (less than a 1.5-fold difference determined densitometrically) over the entire period of endosperm

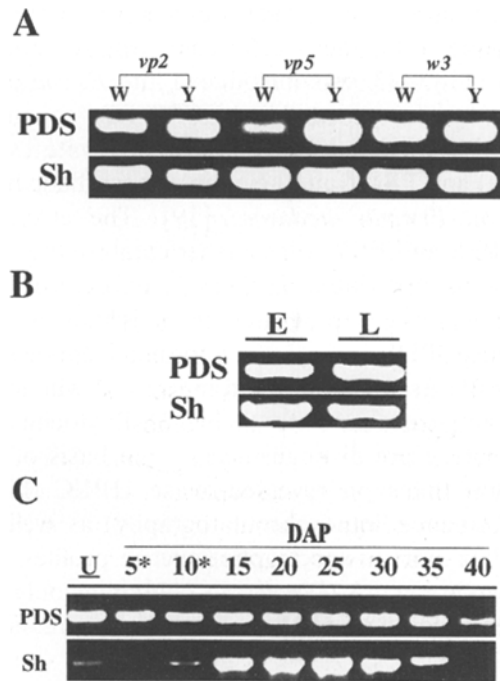


Fig. 6. RT-PCR analysis of *Pds* and *Sh* transcripts. RNA for RT-PCR was extracted from A, white carotenoid mutant (W) and normal yellow endosperms (Y) segregating on maize ears harvested at 20 DAP; B, endosperms harvested at 20 DAP and young leaves; C, developing endosperms harvested at varying DAP (as shown by corresponding numbers) and unfertilized ear (U). \* indicates that some maternal tissue may also be present in the endosperm sample. Identity of amplification products are shown at left of bands in each panel.

development, except at 40 DAP (days after pollination), when an almost three-fold reduction was observed. In contrast, accumulation of the *Sh* transcript appeared to be under temporal control; the *Sh* amplification product was first detected at 10 DAP, increased at 15 DAP and remained at a constant level between 15–30 DAP, at which point the level dropped, and no product was obtained from the 40 DAP endosperm. The temporal pattern of *Sh* transcript accumulation in developing endosperm detected by RT-PCR was consistent with previous results using northern analysis [34].

#### Discussion

A maize *Pds* cDNA clone was isolated, and by using a heterologous complementation system in



*E. coli*, shown to encode a two-step desaturase, an enzyme catalyzing the desaturation of phytoene to  $\zeta$ -carotene in two steps. The presence of a two-step desaturase in maize, a monocot, as well as in several dicots, suggests that all higher plants must encode such a two-step desaturase.

Recessive alleles of four unlinked loci, *vp2*, *vp5*, *w3*, and *vp9*, condition a block in the desaturation steps; the first three condition an accumulation of phytoene, the substrate of PDS. Therefore, we proposed that one of these three loci might encode PDS. RFLP mapping results showed that *Pds* mapped near *vp5* on chromosome 1S. Furthermore, transcript analysis, using RT-PCR, showed that only *vp5* endosperms had lower levels of *Pds* transcripts accumulating in comparison to normal endosperms segregating on the same ear. Taken together, these results suggest that maize PDS may be encoded by the *vp5* locus. Consistent with these results is a previous genetic experiment showing *vp5* to encode a cell-autonomous product and not some diffusible regulator [33]. Since *vp2* and *w3* endosperms also accumulate phytoene, but do not affect *Pds* transcript accumulation as detected by RT-PCR (Fig. 6A), it is unlikely that these loci encode transcriptional regulators. These other loci may encode a phylogenetically diverged *Pds*, such as found in the case of *Psy* genes of tomato [3]. However, DNA hybridization results obtained using maize *Pds* as a probe suggest *Pds* is a single-copy gene (not shown). Other weak hybridization signals did not map to loci associated with blocks in the desaturation steps. Therefore, *vp2* and *w3* are not as likely to be structural genes and might encode ancillary functions such as pigment binding proteins or oxidoreductases [22]. The *vp9* gene might encode or regulate expression of ZDS, since recessive alleles confer accumulation of  $\zeta$ -carotene (see Fig. 1).

Like Rubisco (ribulose biphosphate carboxylase), which has been widely used for plant evolutionary studies, PDS shows high homology in comparing amino acid sequences of dicots with that of maize, a monocot. For example, amino acid identity and similarity for maize and pepper PDS proteins is 77.2 and 84.2%, respectively. In

contrast, Rubisco identity and similarity are 90.6 and 95%, respectively. The phylogenetic tree (Fig. 4) produced by alignment of cyanobacterial, monocot, and dicot PDS amino acid sequences is consistent with current hypotheses of plant evolutionary relationships. However, unlike the chloroplast encoded *rbcL*, which is highly conserved at the nucleotide level between monocots and dicots, the nuclear-encoded *Pds* nucleotide sequence is more variable. Nucleotide homology between maize and pepper *Pds* genes is 72%, whereas for *rbcL*, the homology is 85.4%.

The *rbcL* gene has been an important tool for plant evolutionary studies. However, its high degree of conservation limits its utility for evolutionary studies concerning lower ranked taxonomic groupings. In contrast, *Pds* is an essential, nuclear-encoded gene with greater variability, suggesting that it holds great potential for studies at lower taxonomic ranks than shown in Fig. 4.

A major question regarding control of carotenoid biosynthesis is whether the pathway is differentially regulated in various tissues, i.e. in different plastid types. During endosperm development, plastids triple in number [24] and total colored carotenoids increase dramatically (Yu and Wurtzel, unpublished) during the period of 10–20 days after pollination. Using RT-PCR, we studied the expression of *Pds* transcripts in developing maize endosperm during the period of carotenoid accumulation. No marked change in *Pds* transcript level was found between 5–35 DAP, as compared with the temporally regulated expression of *Sh*. At 40 DAP, there was a reduction in *Pds* transcripts. However, this stage is late in endosperm development and well past the greatest period of carotenoid accumulation. This constitutive expression of *Pds* during the period of carotenoid accumulation in developing maize endosperm is in contrast to the temporal control of *Psy* and *Pds* transcript accumulation in developing tomato fruit; during development of tomato chromoplasts from chloroplasts, carotenoid accumulation is accompanied by a 25-fold increase in *Psy* transcripts and a 3–10 fold increase in *Pds* transcripts [11, 26]. This difference is not unexpected, since carotenoid-containing plastids

of endosperm (amyloplasts) and fruit (chromoplasts) are the products of different developmental processes [16].

It is not surprising that maize endosperm carotenoid accumulation is not regulated by specific induction of *Pds* transcript accumulation. The induction of carotenoid accumulation in the endosperm may not necessarily be regulated at the level of transcript accumulation. Alternatively, the endosperm pathway may be regulated by transcriptional control of *Psy*. Furthermore, the endosperm pathway may not be regulated by controlling expression of the enzymes within the pathway, but alternatively by controlling the flow of substrates to the pathway. Such upstream control (above PSY, the first enzyme specific to carotenogenesis) has been previously documented. Albrecht and Sandmann [1] demonstrated by *in vitro* labeling experiments that the phytochrome-mediated accumulation of carotenoids during the course of conversion of an etioplast to a chloroplast is regulated upstream of the pathway, via activation of IPP (isopentenyl pyrophosphate) isomerase. Another example of upstream regulation of carotenogenesis, occurs in developing pepper fruits; the abundance of transcripts encoding GGPP synthase show a concomitant increase, followed by an increase in enzyme activity, that is associated with carotenoid accumulation [18]. Both IPP and GGPP are precursors to a variety of terpenoid pathways [8]. Therefore, evidence is mounting that the pathway is not only regulated with respect to tissue specificity, but is controlled both within the pathway as well as upstream of the pathway.

The genetically identified mutant alleles affecting carotenoid synthesis in maize endosperm, but not leaves, will be useful for determining how the carotenoid biosynthetic pathway is regulated in different plastid/tissue types, where the role and localization of carotenoids varies. Furthermore, an understanding of the molecular regulation of carotenoid biosynthesis in endosperm is of great value for engineering the pathway in endosperms of other cereal crops that are otherwise poor nutritional sources of carotenoids. The stable expression of *Pds* transcripts in developing en-

dosperm may also serve as a useful internal experimental control for future studies of endosperm gene expression, including other genes involved in carotenogenesis.

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