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Structure and Expression of Chloroplast-Localized Porphobilinogen Deaminase from Pea (*Pisum sativum* L.) Isolated by Redundant Polymerase Chain Reaction[1]

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Porphobilinogen (PBG) deaminase catalyzes the polymerization of four PBG monopyrrole units into the linear tetrapyrrole hydroxymethylbilane necessary for the formation of chlorophyll and heme in plant cells. Degenerate oligonucleotide primers were designed based on amino acid sequence data (generated by mass spectrometry) for purified PBG deaminase from pea (*Pisum sativum* L.) chloroplasts. These primers were used in *Taq*I polymerase-catalyzed polymerase chain reaction (PCR) amplification to produce partial cDNA and nuclear genomic fragments encoding the enzyme. Subsequently, a 1.6-kb cDNA was isolated by screening a cDNA library constructed in λgt11 from leaf poly(A)$^+$ RNA with the PCR products. The cDNA encodes an approximately 40-kD polypeptide containing a 46-amino acid NH_2-terminal transit peptide and a mature protein of 323 amino acids. The deduced amino acid sequence of the mature pea enzyme is similar to PBG deaminases from other species and contains the conserved arginine and cysteine residues previously implicated in catalysis. Northern blot analysis indicates that the pea gene encoding PBG deaminase is expressed to varying levels in chlorophyll-containing tissues and is subject to light induction.

Tetrapyrroles and their derivatives play an important role in plant cell growth and differentiation (Battersby, 1988). Heme is present in all cell types, where it functions as the prosthetic group of integral membrane components (e.g. Cyts of plastidic and mitochondrial electron transfer chains) and as a cofactor to numerous soluble proteins (e.g. catalase, peroxidase). In contrast, the Chls are restricted to the chloroplast thylakoid membranes in photosynthetic cells, where they serve as chromophores in the PSI and PSII reaction centers and light-harvesting apparatus. The first committed step in tetrapyrrole formation is the synthesis of ALA, catalyzed in higher plants, algae, and some bacteria by the cooperative activity of three enzymes and requiring a novel tRNA intermediate (Beale, 1990). Two molecules of ALA are then converted into one molecule of PBG by the activity of ALA dehydratase, and subsequently four monopyrrole PBG units are condensed into a linear tetrapyrrole, 1-hydroxymethylbilane, by the enzyme PBG deaminase (also known as hydroxymethylbilane synthase; EC 4.3.1.8). Hydroxymethylbilane is cyclized and isomerized to yield uroporphyrinogen III, the direct precursor of all biologically active hemes and Chls and the last common intermediate for all cellular tetrapyrroles (for reviews, see Battersby, 1988; Beale and Weinstein, 1990).

The biochemical and kinetic properties of PBG deaminases have been reported for a variety of prokaryotic (Hart et al., 1986) and eukaryotic (Higuchi and Bogorad, 1975; Anderson and Desnick, 1980; Williams et al., 1981; Spano and Timko, 1991) organisms. The exact sequence of steps involved in the assembly of the linear hydroxymethylbilane on the PBG deaminase backbone has been solved (Hart et al., 1987; Warren and Jordan, 1988) and the synthesis of hydroxymethylbilane has been shown to involve a unique enzyme-bound dipyrromethane cofactor (Hart et al., 1987, 1988; Jordan and Warren, 1987). All PBG deaminases characterized thus far are acidic proteins with monomeric molecular masses between 34 and 44 kD and have optimal pH between 8.0 and 8.5. In animal cells and yeast PBG deaminase is a soluble cytosolic protein (Elder, 1976; Labbe-Bois and Labbe, 1990), whereas in higher plants and algae it is found in the chloroplast stroma (Smith, 1988; Shashidhara and Smith, 1991; Spano and Timko, 1991). Cloned cDNAs and nuclear genes encoding PBG deaminase have now been analyzed from several mammalian cells (e.g. Raich et al., 1986; Beaumont et al., 1987; Chretien et al., 1988; Stubnicer et al., 1988), yeast (Gellefors et al., 1986; Keng et al., 1992), bacteria (Thomas and Jordan, 1986; Hansson et al., 1991), and one photosynthetic organism, *Euglena gracilis* (Sharif et al., 1989).

Comparison of the deduced amino acid sequences encoded by these genes revealed considerable similarity in primary protein structure among the enzymes from highly diverged organisms. A number of conserved residues have been shown

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Abbreviations: ALA, 5-aminolevulinic acid; GUS, β-glucuronidase; PBG, porphobilinogen deaminase; PCR, polymerase chain reaction; Uro, uroporphyrinogen.

by chemical modification studies or site-directed mutagenesis of the *Escherichia coli* enzyme to be important for catalytic activity. Among these conserved residues is Cys^{242}, which binds the dipyrromethane cofactor (Jordan et al., 1988; Miller et al., 1988), several Arg's involved in substrate binding (Jordan and Woodcock, 1991; Lander et al., 1991), and putative active site Lys's (Hadener et al., 1990). The recent determination of the three-dimensional structure of the *E. coli* enzyme at 1.9 Å resolution (Louie et al., 1992) will greatly facilitate the study of structure-function relationships in this enzyme.

As a step toward understanding the molecular genetic control of tetrapyrrole formation in plant cells, we report here the molecular cloning and characterization of cDNAs and genomic sequences encoding a chloroplast-localized PBG deaminase from pea (*Pisum sativum* L.). We show that the pea enzyme is formed as a higher molecular mass precursor with a chloroplast-targeting signal at the N terminus. In addition, we describe the tissue-specific and light-regulated expression of the PBG deaminase gene and the relationship between gene expression and enzyme activity.

MATERIALS AND METHODS

Plant Materials and Growth

For the purification of the enzyme PBG deaminase, pea seeds (*Pisum sativum* L. var Progress No. 9) (obtained from W.A. Burpee and Co., Warminster, PA) were allowed to imbibe distilled water overnight at room temperature and planted in moistened vermiculite. They were grown for 10 to 14 d at 28°C under white light illumination (4000 lux) prior to harvesting of leaves (Spano and Timko, 1991).

Alternatively, pea seedlings (*Pisum sativum* L. var Feltham First; obtained from Sanders Seeds Ltd, Cambridge, UK) were grown in Fison's Levington compost for 7 to 8 d at 18 to 22°C, under a 16-h day:8-h night regime (for the isolation of genomic DNA for PCR amplification), or for 3 to 6 weeks to prepare different organ samples for expression studies. For the study of the effect of light, the plants were grown in complete darkness for 7 d, then illuminated at 4000 lux for various times.

Purification of PBG Deaminase and Protein Microsequencing

PBG deaminase was isolated from pea leaf chloroplasts as described previously (Spano and Timko, 1991). Enzymically active chromatographic fractions were pooled and 100 pmol of the protein in 50 μL of buffer was digested with 0.5 μg of trypsin (Boehringer Mannheim) at 37°C for 26 h. The reaction was stopped by the addition of 5 μL of acetic acid and the tryptic peptides were separated by reverse-phase HPLC on a narrow-bore RP300 Aquapore column (2.1 × 30 mm). Mass spectra were recorded on a triple-quadrapole mass spectrometer (TSQ-70, Finnigan Mat, San Jose, CA) (Hunt et al., 1986; Griffin et al., 1989) and a Fourier transform mass spectrometer (Hunt et al., 1987). Peptide methyl esters were prepared as described (Hunt et al., 1986). Automated Edman degradation analysis was performed by standard methods on an Applied Biosystems model 473 protein microsequencer (Matsudaira, 1987).

Oligonucleotide Synthesis, PCR Amplification, and Isolation of cDNAs

Using the peptide sequence data for pea PBG deaminase obtained by MS, degenerate sense and antisense oligonucleotide primers were designed. These were prepared by the Protein and Nucleic Acid Chemistry Facility (PNACF), Department of Biochemistry, University of Cambridge, UK. They were used either individually or in pair-wise combinations as indicated in the text, in *Taq*I polymerase-catalyzed PCRs. Template DNA was either genomic DNA extracted from pea leaves by the method of Thompson et al. (1983), or phage DNA prepared by the plate lysate method of Sambrook et al. (1989) from a cDNA library of pea leaf poly(A)$^+$ RNA constructed in λgt11 (Gantt and Key, 1986). Each reaction contained 100 ng of template and appropriate sense or antisense primers at a final concentration of 0.15 μM. To optimize product formation in our initial amplification reactions, the concentration of $MgCl_2$ was varied. The reaction mixture was heated to 96°C for 6 min and then subjected to 30 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 20 s, and polymerization at 72°C for 1 min.

Amplification products were analyzed by agarose gel electrophoresis and fragments of interest were excised from the gel and cloned into pGEM-3Zf using the methods of Sambrook et al. (1989). The PCR products were used as probes (after labeling with ^{32}P by the random oligonucleotide method of Feinberg and Volgelstein, 1983) to screen the pea leaf cDNA library (Gantt and Key, 1986) to isolate additional cDNA clones using the method of Huynh et al. (1985). Approximately 2×10^5 independent recombinant phage were screened, and three positives were identified. After two additional rounds of screening, the plaques were homogenous. The λDNA was then purified and digested with *Eco*RI, and the inserts from each were subcloned into pGEM-3Zf for further analysis.

Nucleotide Sequencing and Analyses

The DNA sequence of both strands of DNA in the individual cDNAs and genomic DNA fragments was determined by the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase kit (United States Biochemical). Recombinant plasmids used as sequencing templates were generated by restriction digestion of the original cDNAs or genomic fragments and ligated into pGEM vectors for subsequent sequencing. Analysis of sequence data was carried out using the software of the Genetics Computer Group, Madison, WI (Devereux et al., 1984).

RNA Extraction and Northern Blot Analysis

Total RNA was prepared from etiolated and greening pea leaf tissues and from light-grown leaves, stems, and roots as described by Thompson et al. (1983). The quantity of the total RNA recovered and used in northern blot analysis was determined spectrophotometrically. Aliquots of total RNA

were denatured with glyoxal, fractionated on agarose gels, and subsequently transferred to GeneScreen (NEN Research Products) for northern analysis essentially as described by Sambrook et al. (1989). The filter was prehybridized in 10% (w/v) dextran sulfate, 1 M NaCl, 1% SDS at 65°C for 3 h, and then hybridized overnight at 65°C in the same buffer containing radiolabeled probe, generated by the random oligonucleotide method of Feinberg and Vogelstein (1983). The filters were washed twice in 2× SSC for 5 min at room temperature, then twice in 2× SSC, 1% SDS for 30 min at 42°C, before exposure to x-ray film. The extent of hybridization on the individual filters was determined by densitometry and compared with the intensity of signal obtained with a probe against polyubiquitin from pea (Watts and Moore, 1991) hybridized to the same blot under identical conditions.

Analytical Methods

PBG deaminase activity was assayed according to the method of Williams et al. (1981). Protein content was determined by either the procedure of Bradford (1986), the bicinchoninic acid method (Smith et al., 1985), or spectrophotometrically by reading A_{280}.

RESULTS

Protein Microsequence Analysis

We previously reported the purification of the mature pea chloroplast PBG deaminase and determination of the amino acid sequence of its NH_2 terminus (Spano and Timko, 1991). Purified protein of similar quality was digested with trypsin and the amino acid sequences of various tryptic peptide fragments were determined by tandem MS as shown in Table I. Of the nine peptide sequences obtained, six (peptides a, c, d, e, g, and h) could be aligned with some confidence with the sequences of PBG deaminases from other organisms, whereas the location of the remaining three peptides (b, f, and j) were ambiguous and were presumed to be from more variable regions, such as the carboxy terminus of the protein (Sharif et al., 1989). Subsequent molecular cloning and analysis (discussed below) resolved the location of all peptide sequences and confirmed both the identity of the isolated cDNA and nuclear fragments as encoding the pea PBG deaminase and the deduced amino acid sequences of these clones.

Table I. *Amino acid sequence analysis of peptides from purified pea PBG deaminase*

Shown are the amino acid sequences of the various peptide fragments derived from purified pea PBG deaminase determined by tandem MS (peptides b–j) along with the peptide mass given as *m/z*. The NH_2-terminal sequence of the protein (peptide a) was determined by limited Edman degradation (Spano and Timko, 1991).

	Peptide	*m/z*
a.	SLAVEQQT(Q/C)Q(D/Q)XTAG	
b.	TALIR	573.7
c.	ILSQPLADIGGK	1212.4
d.	EIDEALINGDIDIAVHSMK	2084.4
e.	GLVASPDGTR	973.1
f.	IGSYTYEDMMK	1338.6
g.	DAFISLS	752.8
h.	KLSEGVVK	860.1
j.	MAEYLASLNHEETR	1664.8

Use of PCR Amplification to Isolate Nuclear and cDNA Sequences Encoding Pea PBG Deaminase

Based upon our peptide sequence information, together with comparisons of PBG deaminase sequences from other organisms, three degenerate oligonucleotides, two in the sense orientation (primers 1 and 2) and one in the antisense orientation (primer 3) were designed (Table II). Primer 1 included a codon for Lys at the 5′ end because this is conserved in all known PBG deaminases from other organisms. Primers 2 and 3 included additional nucleotides at their 5′ ends to provide either an *Xba*I or *Bam*HI site, respectively, to facilitate subsequent cloning steps. These primers were used in *Taq*I polymerase-catalyzed PCRs to amplify sequences encoding PBG deaminase either directly from pea genomic DNA or from DNA prepared from a λgt11 cDNA library generated from leaf poly(A)$^+$ RNA.

In preliminary amplification reactions, the sense and antisense primers were used in combination and individually to define the specificity of the reactions. When primers 1 and 3 were used, several amplification products were obtained from total genomic DNA (Fig. 1, lane 4). However, most of these products were also obtained when primer 1 was used alone (Fig. 1, lane 1). Amplifications using primers 2 and 3 yielded products of 1.1, 0.5, and 0.3 kb (lane 5); neither primer 2 nor 3 alone gave any discernible product (lanes 2 and 3). To test whether any of the primer 1/primer 3 reaction products were specific for PBG deaminase, the reaction products were fractionated by electrophoresis on 2% agarose gels and DNA from the various size fractions was recovered from the gel. A portion of these fractions was then used as a template for a nested PCR amplification with primers 2 and 3. Only reactions containing template DNA from the largest size class (approximately 1.0–1.2 kb) showed a product, a single band of 1.1 kb, the same size as the largest fragment produced independently with primers 2 and 3. This product (Pg2) was purified by agarose gel electrophoresis, digested with *Xba*I and *Bam*HI, and ligated into pGEM-3Zf. When template DNA in the amplification reactions was prepared from a λgt11 cDNA library prepared from pea leaf poly(A)$^+$ RNA (Gantt and Key, 1986), amplification reactions containing primers 2 and 3 gave a single 650-bp product (Fig. 1, lane 6). This product is smaller than the corresponding genomic fragment but within the size range compatible with the predicted size of the pea PBG deaminase mRNA. This too was excised from an agarose gel, digested with *Xba*I and *Bam*HI, and ligated into pGEM-3Zf to give plasmid pPc1. Analysis of the PCR products Pg2 and Pc1 revealed that they had contiguous restriction maps and that the fragments cross-hybridized by Southern blot analysis. Neither fragment hybridized with the smaller PCR products obtained from pea genomic DNA using primers 2 and 3, or with probes prepared from sequences encoding PBG deaminase from *E. coli*, *Euglena*, or human (data not shown).

Nucleotide Sequence Analysis and Isolation of a Full-Length cDNA

The nucleotide sequences of both the cDNA and genomic PCR products were determined on both strands. Both were found to encode a protein containing four of the peptide sequences (peptides e, g, h, and j) determined by tandem MS for the pea PBG deaminase, as well as to resemble other known PBG deaminases (Fig. 2). The difference in size between Pc1, the cDNA product (650 bp), and Pg2, the PCR product from genomic DNA (1.1 kb), can be accounted for by the presence of three introns, of 86, 214, and 89 bp, in the genomic DNA. The sequences of the genomic fragment and cDNA differ at three nucleotides. Whether these reflect true differences or are the result of amplification artifacts resulting from the inherent inaccuracy of *Taq*I polymerase, which has an error rate of about 1 in 10^3 (Tindall and Kunkel, 1988), is uncertain (but see below).

Because the cDNA and genomic fragments encoded only part of the PBG deaminase protein, the insert from Pc1 was used as a hybridization probe to screen the pea leaf λgt11 cDNA library. Of 200,000 recombinant phage screened, 3 independent phages remained positive through two rounds of rescreening. The inserts from these three phages (designated PD1, PD2, and PD3) were removed from the λgt11 vector sequences by digestion with *Eco*RI and found to be approximately 1.6, 1.5, and 0.8 kb in length, respectively. The inserts from all three phages hybridized with Pc1 in Southern blot analysis (data not shown). The longest of the cDNAs, PD1, was cloned into *Eco*RI-cut pGEM3-Zf, and its nucleotide sequence was determined. The nucleotide and predicted amino acid sequence is shown in Figure 2, together with that for the PCR products Pc1 and Pg2. These are identical to that of the cDNA except at the three nucleotide positions, which were different between Pc1 and Pg2. This strongly supports the conclusion that the differences are the result of errors by *Taq*I polymerase.

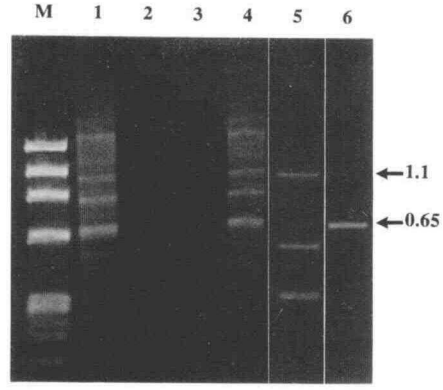

Figure 1. Agarose gel electrophoretic analysis of PCR amplification products. PCR amplification was carried out as described in "Materials and Methods" with templates of either pea genomic DNA (lanes 1–5) or DNA from a pea leaf cDNA library (lane 6) and various combinations of primer. M, ΦX174 DNA digested with *Hae*III; lane 1, primer 1; lane 2, primer 2; lane 3, primer 3; lane 4, primers 1 and 3; lanes 5 and 6, primers 2 and 3.

The first ATG codon in PD1 is at nucleotide position 88 and is the start of an open reading frame of 369 amino acids, which includes the open reading frame of Pc1. This ATG lies within the sequence CCAAATG, similar to the dicot consensus of aAaA/CATG calculated by Cavener and Ray (1991), although it does not have a purine at −3. At the immediate 5′ end of the cDNA is an unusual sequence of 52 Ts; this is, coincidentally, the same as the number of As at the 3′ end of the cDNA and could be the result of a cloning artifact during construction of the cDNA library. It is not known if

Table II. *Structure of synthetic oligonucleotide primers based upon peptide sequence data from purified pea PBG deaminase*

The nucleotide sequences of the sense and antisense primers synthesized for these studies are shown. Given above each is the corresponding amino acid sequence determined by tandem MS. The inclusion of a codon for Lys at the beginning of primer 1 was because this residue is completely conserved in the known PBG deaminases from other organisms. Degenerate base positions are listed below each oligonucleotide. I, Inosine. Nucleotide sequences for primers 1 and 2 are given as 5′–3′; primer 3 is given as 3′–5′ (antisense orientation to the determined amino acid sequence). Additional nucleotides encoding the *Xba*I and *Bam*HI sites in primers 2 and 3, respectively, are underlined. The degree of degeneracy is given for each.

Primer	Structure	Comments
Primer 1	K E I D E A 5′-AAA GAA ATT GAT GAA GC-3′ G G C C G A	48-fold degenerate
Primer 2	D I A V H S M K 5′-GCTCTA GAT ATT GCI GTI CAT TCI ATG AA-3′ *Xba*I C C C	12-fold degenerate 3 inosine substitutions
Primer 3	Y T Y E D M M 3′-ATA TGA ATA CTT CTA TAC TAC CTAGGGC-5′ G G G C G *Bam*HI C T	64-fold degenerate

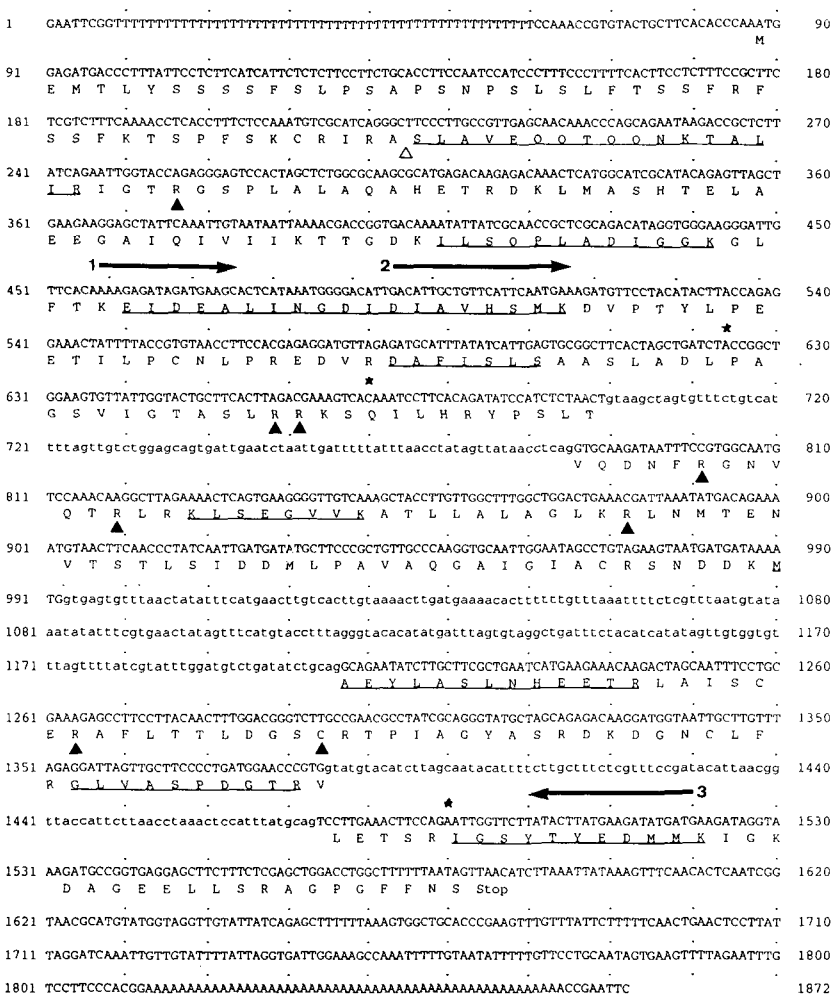

Figure 2. Nucleotide and deduced amino acid sequences of cDNA and nuclear genomic fragments encoding the pea PBG deaminase. The figure shows a composite nucleotide sequence of the full-length cDNA encoded by PD1 and the genomic PCR product Pg2, with the deduced amino acid sequence below. The positions of the primers are shown as numbered arrows above the sequence. Primers 2 and 3 delineate the 5′ and 3′ ends of the PCR products. The position of the introns (shown in lowercase letters) were determined by comparison of Pg2 to the cDNA sequences (Pc1 and PD1) over this region. Nucleotide differences between the cDNA and PCR products are noted by asterisks: at position 624, Pg2 contains a G rather than an A; at position 670, Pc1 contains a T rather than a C; at position 1490, Pg2 contains a T rather than an A. The amino acid sequence at the NH_2 terminus of the mature protein and those determined for the tryptic peptides by tandem MS are underlined. The open triangle indicates the position of cleavage of the transit peptide from the mature protein, and the solid triangles indicate conserved residues referred to in the text.

there are introns in the pea PBG deaminase gene other than those in Pg2. However, the *Arabidopsis* PBG deaminase gene contains all three of the introns in Pg2 and one other as well in the 5′ region of the gene (S.H. Lim and A.G. Smith, unpublished data). All of the identified splice sites at the exon/intron borders in the pea gene conform to the eukaryotic consensus.

The polypeptide encoded by PD1 has a calculated molecular mass of 39,918 D and contains all nine peptide sequences derived from the purified pea PBG deaminase. Based upon our previously reported amino acid sequence of the NH_2 terminus of the mature pea PBG deaminase protein (Spano and Timko, 1991), the cDNA encodes an additional 46 amino acids at the NH_2 terminus. This region has a high percentage (39%) of hydroxylated residues characteristic of chloroplast transit peptides. At the junction of the NH_2-terminal extension and the mature protein is the sequence IRA S (open triangle in Fig. 2), which conforms with the consensus cleavage site recognized by the stromal processing peptidase (Gavel and von Heijne, 1990) and is similar to the conserved tetrapeptide motif (V/IRA S/Q/E) recognized in precursors of other Chl biosynthetic enzymes, for example ALA dehydratase (Boese et al., 1991) and Pchlide reductase (Spano et al., 1992a, 1992b).

Similarity to Other Known PBG Deaminases

Figure 3 shows the alignment of the amino acid sequence of pea PBG deaminase with those from other organisms, where completely conserved residues are indicated with an asterisk (*) and conservative substitutions with an apostrophe (′). It can be seen that there is considerable conservation throughout the sequence, although the least similarity is observed at the COOH terminus, where the human enzyme appears to have an additional sequence of some 15 to 20 residues. The similarity between the sequences was calculated using the GAP program of the Genetics Computer Group suite of sequence analysis programs (Devereux et al., 1984) after pairwise comparisons for each sequence with each of the others. The pea enzyme has identical residues in about 40% of the equivalent positions in PBG deaminases from other species. This value increases to 60% if conservatively substituted residues are included. It is interesting that, although the enzymes in both *Euglena* (Shashidhara and Smith, 1991; Shashidhara et al., 1992) and pea (Smith, 1988; Spano and Timko, 1991) are plastid localized, their primary sequences are no more similar to one another than to that of *E. coli* PBG deaminase. This suggests that relatively strong selective pressure must exist on the three-dimensional struc-

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Pea SLAVEQQTQQNKTALIRIGTRGSPLALAQAHETRDKLMASHTELAEEGAIQIVIIKTTGDKILSQPLADIGGKGLFTKEIDEALIN----GDIDIAVHSM  96
Eug    STTGSNIGAGKTVRVATRKSPLAMWQAEFIQSELERLWPGITVE----LQPMSTRGDKILDSPLAKVGGKGLFVKELETALLE----NRSDIAVHST  89
Hum              MRVIRVGTRKSQLARIQTDSVVATLKASYPGLQFE----IIAMSTTGDKILDTALSKIGEKSLFTKELEHALEK----NEVDLVVHSL  80
Ye            MGPETLHIGGRKSKLAVIQSNHVLKLIEEKYPDYDC----KVFTLQTLGDQIQFKPLYSFGGKALWTKELEDHLYHDDPSKKLDLIVHSL  86
Ec            MLDNVLRIATRQSPLALWQAHYVKDKLMASHPGLVVE----LVPMVTRGDVILDTPLAKVGGKGLFVKELEVALLE----NRADIAVHSM  82
Bs             MMRTIKVGSRRSKLAMTQTKWVIQKLKEINPSFAFE----IKEIVTKGDRIVDVTLSKVGGKGLFVKEIEQALLN----EEIDMAVHSM  81

Pea KDVPTYLPEETILPCNLPREDVRDAFI---SLSAASLADLPAGSVIGTASLRRKSQILHRYPSLTVQDNFRGNVQTRLRKLSEG--VVKATLLALAGLKR 191
Eug KDVPMELPEGLVLGVICKRHDPCDAIVFPKGSNLKSLEDLPHGARVGTSSLRRQCQLLLKRPDLKFLE-LRGNVNTRLAKLDSG--DYDAIILAAAGLKR 186
Hum KDLPTVLPPGFTIGAICKRENPHDAVVFHPKFVGKTLETLPEKSVVGTSSLRRAAQLQRKFPHLEFRS-IRGNLNTRLRKLDEQQ-EFSAIILATAGLQR 178
Ye  KDMPTLLPEGFELGGITKRVDPTDCLVMPFYSAYKSLDDLPDGGIVGTSSVRRSAQLKRKYPHLKFES-VRGNIQTRLQKLDDPKSPYQCIILASAGLMR 185
Ec  KDVPVEFPQGLGLVTICEREDPRDAFV---SNNYDSLDALPAGSIVGTSSLRRQCQLAERRPDLIIRS-LRGNVGTRLSKLDNG--EYDAIILAVAGLKR 176
Bs  KDMPAVLPEGLVIGCIPEREDPRDALI---SKNRVKLSEMKKGAVIGTSSLRRSAQLLIERPDLTIKW-IRGNIDTRLQKLETE--DYDAIILAAAGLSR 175

Pea LNMTENV----TSTLSIDDMLPAVAQGAIGIACRSNDDKMAEYLASLNHEETRLAISCERAFLTTLDGSCRTPIAGYASRDKDGNCLFRGLVASPDGTRV 287
Eug LGFSDRVLPGETNIIDPNVMCPAAGQGALSIELRTNDPEIAALLEPLHHIPDAVTVACERAMNRRLNGGCQVPISGFAQLKDGQLRME-ARVGSVTGKGP 285
Hum MGWHNRV----GQILHPEECMYAVGQGALGVEVRAKDQDILDLVGVLHDPETLLRCIAERAFLRHLEGGCSVPVAVHTAMKDGQLYLT-GGVWSLDGSDS 273
Ye  MGLENRI----TQRFHSDTMYHAVGQGALGIEIRKGDTKMMKILDEICDLNATICCLSERALMRTLEGGCSVPIGVESKYNEETKKLL-LKAIVVDVEGT 280
Ec  LGLESRI----RAALPPEISLPAVGQGAVGIECRLDDSRTRELLAALNHHETALRVTAERAMNTRLEGACQVPIGSYAELIDGEIWLR-GLVGAPDGSQI 271
Bs  MGWKQDVV---TEFLEPERCLPAVGQGALAIECRESDEELLALFSQFTDEYTKRTVLAERAFLNAMEGGCQVPIAGYSVLNGQDEIEMTGLVASPDGKII 272

Pea LETSRIGSYTY----------------------------------EDMMKIGKDAGEE-LLSRAGPGFFNS      323
Eug LI-IQSKTFRLP----------------WSGRTWPQLQKESEALGVEVADMLLADGAQAYLDEAYASRTLGWA    341
Hum IQETMQATIHVPAQHEDGPEDDPQLVGITARNIPRGPQLAAQNLGISLANLLLSKGAKTILDVARQLNDAH      344
Ye  EAVEDEIEMLIENVKED----------------------SMACGKILAERMIADGAKKILDEINLDRIK       327
Ec  IRGE---------------------------RRGAPQDAEQMGISLAEELLNNGAREILAEVYNGDAPA       313
Bs  FKETVTGNDP-------------------------------EEVGKRCAALMADKGAKDLIDRVKRELDEDGK   314
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Figure 3. Alignment of PBG deaminase amino acid sequences. The sequences are from pea (this work), *E. gracilis* (Eug; Sharif et al., 1989), human erythrocytes (Hum; Raich et al., 1986), yeast (Ye; Keng et al., 1992), *E. coli* (Ec; Thomas and Jordan, 1986), and *Bacillus subtilis* (Bs; Jansson et al., 1991). *, Match across all sequences; ', conservative substitution.

ture of this protein. Supporting this notion is the observation that residues essential for catalysis identified by site-directed mutagenesis of *E. coli* PBG deaminase (Hadener et al., 1990; Jordan and Woodcock, 1991; Lander et al., 1991) are conserved in the pea enzyme. These include pea residues that correspond to *E. coli* Arg's at positions 11, 131, 132, 149, 155, 176, and 232, which are involved in substrate binding, and Cys^{242}, to which the dipyrromethane cofactor is bound (solid triangles in Fig. 2).

One noteworthy difference occurs within a region of the protein that has been shown by crystallographic analysis of the *E. coli* enzyme (Louie et al., 1992) to be part of the active site cleft (*E. coli* residues 126–132; pea residues 186–192). This region, GTSSLRR, is completely conserved in PBG deaminases from other bacteria and animals, but is GTASLRR in pea and GTSSVRR in yeast. The importance of this variation is not known, but molecular modeling suggests that substitution of Ser with Ala in the pea enzyme does not disrupt this region (M. Witty and A.G. Smith, unpublished observations).

The complete nucleotide sequences for the two shorter cDNAs (PD2 and PD3) identified during screening of the λgt11 library with Pc1 as a probe were not determined, but over the regions analyzed, both were found to start internal to PD1 at their 5′ ends (nucleotides 116 and 823 of PD1, respectively) and are presumably truncated versions of this cDNA. The 3′ untranslated regions of PD2 and PD3 were identical to that of PD1, but were 231 and 314 bp long compared with 226 bp in PD1, suggesting that the polyadenylation site is not precise. The lack of difference in nucleotide sequences over their coding and untranslated regions suggests that all three were derived from the same gene.

Developmental and Light-Regulated Expression of PBG Deaminase

To determine the extent to which light and cell type affect the expression of PBG deaminase, enzyme activity and steady-state mRNA levels were measured in various tissues and at different stages of light-induced leaf development. PBG deaminase activity was found in all tissue types analyzed (Table III). As might be expected, the highest level of activity was seen in green leaves, but it was readily detectable even in roots where no Chl synthesis takes place. Enzyme activity in etiolated leaves was greater than in roots or green stems, but about 3-fold less than in green leaves. However, on exposure to light the levels increased, so that after 48 h of illumination, enzyme activity was comparable with that in fully green leaves.

Northern analysis of total RNA isolated from either etiolated leaves or leaves of greenhouse-grown plants detected a single size class of message approximately 1.6 kb in length when hybridized with ^{32}P-labeled insert from PD1 (Fig. 4A), which is the same size as the full-length cDNA. The steady-state level of message clearly increases during greening, and when the intensity of the band was quantified using densitometry (as described in "Materials and Methods"), it was found to increase some 10- to 15-fold during the first 48 h following illumination (Fig. 4B). Although this is a more

Table III. *PBG deaminase activity in pea seedlings*

PBG deaminase activity was determined by the method of Williams et al. (1981) in soluble protein extracts from different tissues from 7-d-old pea seedlings (var Feltham First) grown under 16-h day:8-h night, and from etiolated leaves of seedlings grown in complete darkness and greened for differing lengths of time. Values are means of triplicate samples.

Tissue	Enzyme Activity
	nmol Urol h^{-1} g^{-1} fresh weight
Green leaves	210.00
Upper stems	17.03
Mid stems	2.02
Rhizoderms	8.82
Root tips	13.03
Etiolated leaves	61.98
1 h illuminated	59.12
4 h illuminated	88.87
7 h illuminated	77.95
24 h illuminated	133.30
48 h illuminated	170.93

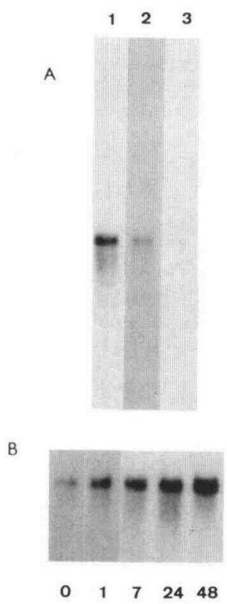

Figure 4. Northern blot analysis of pea PBG deaminase. Total RNA was extracted from leaf and stem tissue from 7-d-old peas, glyoxylated, and electrophoresed on agarose gels before transfer to nylon membrane. The membrane was hybridized with ^{32}P-labeled PD1, washed as described in "Materials and Methods," and then autoradiographed with an intensifying screen. Each blot was then reprobed with a ^{32}P-labeled polyubiquitin clone (Watts and Moore, 1991) to normalize the amounts of RNA. A, lane 1, 1 μg of green leaf RNA; lane 2, 15 μg of etiolated leaf RNA; lane 3, 15 μg of green stem RNA. The film was exposed for 18 d. B, Fifteen micrograms of leaf RNA from seedlings illuminated for 0, 1, 7, 24, and 48 h, respectively. The film was exposed for 9 d.

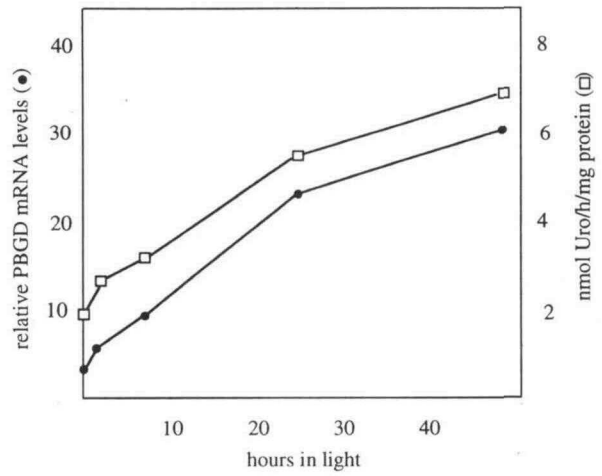

Figure 5. Comparison of PBG deaminase mRNA and enzyme activity. The graph shows the changes in PBG deaminase activity expressed in nmol Uro h^{-1} mg^{-1} protein and in relative mRNA levels (determined densitometrically with reference to the extent of hybridization of a polyubiquitin probe [Watts and Moore, 1991] to the same blot) in leaves of pea seedlings grown for 7 d in complete darkness and then illuminated for up to 48 h.

marked effect than for enzyme activity, both follow a similar time profile during the greening process (Fig. 5).

A transcript of 1.6 kb for PBG deaminase was also detected in total RNA extracted from stems (Fig. 4A), but no hybridization was seen to total RNA from either root tips or older regions of the root (rhizoderm), even after prolonged exposure of the autoradiograph (data not shown). This is despite the fact that PBG deaminase activity was observed in both tissues, albeit at levels some 15 to 20 times lower than in leaves.

DISCUSSION

Initial attempts to isolate cDNAs encoding the pea PBG deaminase from λgt11 expression libraries prepared from leaf poly(A)$^+$ RNA by direct hybridization with heterologous DNA probes encoding the enzyme from *Euglena* (Sharif et al., 1989), *E. coli* (Alefounder et al., 1988), and humans (Raich et al., 1986) were not successful. This is perhaps not surprising given the nucleotide sequence identity between the different cDNAs, which is less than 50%. More unexpected, however, was a similar inability to successfully isolate phage encoding the PBG deaminase by immunoscreening of these λgt11 expression libraries using antiserum prepared against purified PBG deaminase from either *Euglena* or pea chloroplasts (Sharif et al., 1989; Spano and Timko, 1991). As a consequence, we adopted an alternative approach, in which primary protein sequence information was used to design a set of degenerate oligonucleotides that were subsequently used in *Taq*I polymerase-catalyzed PCR amplifications from pea genomic DNA and cDNA.

Cloned cDNAs and nuclear genomic fragments encoding PBG deaminase have now been characterized from pea and have provided the first reported sequence for the enzyme from higher plants. Comparisons of PBG deaminases from

photosynthetic and nonphotosynthetic organisms show that they are well conserved at the amino acid level (approximately 50% identical), in particular maintaining Arg and Cys residues implicated in catalysis and dipyrromethane cofactor binding. More informative comparisons among organisms will be possible when additional sequences become available. In this regard, we have recently used a similar approach based upon redundant PCR amplification with our primers designed against the pea protein sequence to isolate part of a PBG deaminase gene from *Arabidopsis*, but have not been successful with tobacco (M. Witty and A.G. Smith, unpublished observations). The reason is not known, but is likely to be the result of subtle nucleotide differences between the various genomic DNA templates.

Our nucleotide and peptide sequence analyses support the identification of the PD1 gene product as a higher molecular mass precursor to a chloroplast-localized form of PBG deaminase. The presence of PBG deaminase in plastids is consistent with previous studies indicating that the bulk of tetrapyrrole formation in higher plants is associated with the chloroplast fraction of cells (Smith, 1988; Smith et al., 1993). As expected for an enzyme involved predominantly in Chl synthesis, PBG deaminase mRNA and enzyme activity can be detected in light-grown leaves, stems, and green seed pods. The observed increase in steady-state levels of PBG deaminase mRNA upon illumination of etiolated leaves reported in this paper is consistent with the previously reported increases in enzyme activity during this same time frame (Smith, 1986; Spano and Timko, 1991), but contrasts somewhat with our earlier observation of a lack of significant increase in immunodetectable enzyme protein in greening etiolated tissues (Spano and Timko, 1991). This may reflect the operation of one or more posttranscriptional mechanisms in the control of enzyme levels. We have also recently found that the expression of several genes encoding enzymes of the Chl biosynthetic pathway (e.g. ALA dehydratase, Pchlide reductase) is highly regulated in both light and darkness by leaf developmental age and degree of plastid development (Z. He, J. Li, and M.P. Timko, unpublished data). Thus, the discrepancy between enzyme abundance, activity, and transcript accumulation among these studies might also reflect differences in age of the seedlings used (10–14 d old in our previous work compared with 7 d old in this study).

The observation of a single-sized message in all tissues examined suggests that there may be only a single gene product present in green plants. However, we cannot rule out the presence of additional gene products encoding more divergent forms of the enzyme, whose mRNA may not be recognized by PD1. Such might be the case in nonphotosynthetic tissues, such as roots, where even though there are measurable amounts of enzyme activity, no message is detected by northern analysis. Arguing against this possibility are recent data showing that although no hybridization of PD1 is observed to mRNA from petals or tendrils, both of these tissues contain chloroplasts and PBG deaminase activity (A.D.M. Wallace-Cook and A.G. Smith, unpublished observations). Thus, the lack of a hybridization signal in some tissues might simply reflect insufficient probe sensitivity. Additional support for this conclusion comes from transgenic plant studies where it has been observed that chimeric genes consisting of the promoter for an *Arabidopsis* gene encoding chloroplast PBG deaminase fused to GUS exhibit significant GUS expression in roots, although at much lower levels than in leaves or stems (S.H. Lim and A.G. Smith, unpublished observations). The expression of GUS in roots can be greatly enhanced by exposing them to light, indicating that the gene is light inducible.

The results of this study will be useful in designing future experiments to examine the molecular genetic mechanisms controlling general tetrapyrrole metabolism in plant cells. They should allow us to resolve more thoroughly the cellular distribution of tetrapyrrole biosynthetic enzymes and the relative contributions of the plastidic and extraplastidic pathways to cellular heme and Chl formation.

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