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Cloning and molecular analyses of the *Arabidopsis thaliana* plastid pyruvate dehydrogenase subunits [1]

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Abstract

Herein we report the first molecular description of the pyruvate dehydrogenase component of the higher plant plastid pyruvate dehydrogenase complex. The full-length cDNAs for the E1α (1530 bp) and E1β (1441 bp) subunits of the *Arabidopsis thaliana* plastid pyruvate dehydrogenase contain open reading frames that encode polypeptides of 428 and 406 amino acids, respectively, with calculated molecular weight values of 47 120 and 44 208. The deduced amino acid sequences for *Arabidopsis* plastid E1α and E1β have 61% and 68% identity to the *odpA* and *odpB* genes of the red alga *Porphyra purpurea*, respectively, but only 31% and 32% identity to the plant mitochondrial counterparts. Results of Southern analyses suggest that each subunit is encoded by a single gene. Northern blot analyses indicate expression of mRNAs of the appropriate size in *Arabidopsis* leaves. © 1997 Elsevier Science B.V.

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The pyruvate dehydrogenase complex (PDC) is a large multi-enzyme structure composed of three primary component enzymes, pyruvate dehydrogenase (PDH) (E1, EC 1.2.41); dihydrolipoamide acetyltransferase (E2, EC 2.3.1.12); and dihydrolipoamide dehydrogenase (E3, EC 1.8.1.4)[1]. In the well-characterized mammal complex, 60 subunits of E2 comprise the central core and the E1 and E3 components decorate the outer surface of this core [2]. E1 is a heterotetramer composed of two α and two β subunits. The E3 component, a homodimer, associates with the complex via an E3 binding protein. [3].

The PDC catalyzes the irreversible oxidative decarboxylation of pyruvate according to the equation:

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{Acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

In mitochondria, this reaction represents the irreversible commitment of carbon to the citric acid cycle, and therefore is a logical point for regulation. Previous experiments have shown that plant mitochondrial PDC activity is, in fact, regulated by product inhibition, metabolites, and reversible phosphorylation [4–7] as is the mammalian complex [2].

In prokaryotes, PDC is localized in the cytoplasm, while in eukaryotes it is within the mitochondrial

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matrix. Plants, however, are unique in that a second form of the complex exists in the plastids [8–10]. Based upon enzymology [11–13] and immunochemical analyses [14,15], it is clear that plastid PDC is distinct from its mitochondrial counterpart. In plants de novo fatty acid biosynthesis occurs exclusively in the plastids [16–20]. The plastid form of PDC can provide the fatty acid precursor, acetyl-CoA [16,17,21].

The cDNAs that encode the E1α and E1β subunits of plant mitochondrial PDH have been cloned [22–24]. Recently, Reith and Munholland [25] reported the sequence of the entire plastid genome of the red alga *P. purpurea*. Encoded in this genome are open reading frames homologous to PDH α and β subunits. We obtained expressed sequence tag (EST) clones [25] from the Arabidopsis Biological Resource Center (ABRC), at Ohio State University, that have significant homology to the algal plastid sequences. These partial cDNAs were used to isolate full-length cDNAs for both the plastid α and β subunits from an *A. thaliana* cDNA library.

Selection of *A. thaliana* EST clones was accomplished searching the *Arabidopsis* EST database using the BLASTP PROGRAM of the National Center for Biotechnology Information. We reasoned that EST clones encoding the plastid PDH subunits would have relatively high homology to the *P. purpurea odpA* and *odpB* genes [25], and at the same time relatively low homology to mitochondrial PDH E1α and E1β [22–24] sequences. Two clones (GenBank accessions T75600 and N65566) were thus identified as potentially encoding the plastid E1α and E1β subunits.

Oligonucleotides were designed based on sequences common to *P. purpurea odpA* and *odpB* and the two *Arabidopsis* EST sequences and synthesized: E1α 5′ primer, (CGGTAC▼CAAGTCTGACTCTGTCGTT), 3′ primer, (CCTTCGA◆AGGTTCCATCTCCGAAAAA); E1β 5′ primer, (CGGTAC▼CTTCGAGGCTCTTCAGGAA), 3′ primer, (CCTTCGA◆ACGGGCCTTAGACCAGT). The symbols denote restriction sites (▼ *Kpn*I and ◆ *Hin*dIII) added for subcloning. Thermal cycling was used to amplify cDNA fragments from *A. thaliana* using first strand cDNA. Thermal cycling reactions (50 μl total volume) contained 10 mM Tris-HCl, pH 7.9, 1.25 mM $MgCl_2$, 25 μM dNTPs, 5 units *Taq* polymerase (Promega, Madison, WI), 2 μg *A. thaliana* first strand cDNA, and 10 ng of each primer. Thermal cycling was performed with a Perkin-Elmer model 480, with rapid ramp times set at 1°C/s. Cycling conditions were 94°C for 20 s, 50°C for 30 s, 72°C for 2 min with 6 s extensions each cycle and 30 rounds of cycling. Under these conditions, products of the expected size, 288 base pairs (E1α) and 215 base pairs (E1β) were obtained. The products were subcloned into pGEMT (Promega, Madison, WI) and sequenced to confirm their identity. Thermal cycling was also used to generate probes radiolabelled with (α^{32}P)-dCTP, using reaction mixtures identical to those previously described except for a 1000-fold reduction in the concentration of non-radioactive dCTP. Before use, the probes were desalted using Sephadex G-50 columns to remove unincorporated nucleotides. An *Arabidopsis* cDNA library (λ-PRL2, obtained from the ABRC) was plated at a density of 2.25×10^4 plaques per plate for a total of 2.25×10^5 plaques. BioTrace NT nylon filters (Gelman, Ann Arbor, MI) were used for plaque-lifts and were processed according to the manufacturer′s specification. Hybridizations were done according to Current Protocols in Molecular Biology [26]. After three rounds of screening, 7 potential E1α and 12 potential E1β cDNA clones were isolated, ranging in size from 1100 to 1550 base pairs. Plaque-purified λ phage were treated according to the manufacturer′s instructions (Gibco BRL, Gaithersburg, MD) in order to excise the pZL-1 recombinant clones.

DNA sequencing was performed using an ABI prism Model 377 sequencer, and analyzed using IntelliGenetics GeneWorks DNA analysis program version 2.5 on a Macintosh computer. Dye-deoxy terminating cycle sequencing reactions were carried out on both strands of full-length cDNA inserts and deletion fragments derived therefrom.

DNA isolation, Northern and Southern blotting, and Southern hybridizations were carried out according to Current Protocols in Molecular Biology (2.9.1, 4.3.1 and 4.9.1) [26]. RNA isolation was accomplished with the RNAgents, total RNA isolation kit (Promega, Madison, WI). Northern blot prehybridization (3 h), hybridization (12 h), and 4 washes were done with 2.5 × SSPE (1 × = 0.15 mM NaCl, 0.02 mM Na_2PO_4, 2 μM EDTA, pH 7.4), 1% SDS, 1% non-fat dry milk, and 250 μg/ml salmon sperm DNA at 68°C. Blots were exposed on Kodak X-

OMAT/AR film (Rochester, New York) at −70°C with an intensifying screen.

Two EST clones (accessions T75600 and N65566) which encode proteins more highly related to the *P. purpurea odpA* and *odpB* sequences than to the *Arabidopsis* mitochondrial sequences were used to isolate two cDNAs as potential E1α and E1β clones. E1α cDNA (1530 bp) has a 106 bp 5′ untranslated region, a 1284 bp open reading frame encoding a polypeptide of 428 amino acids (Fig. 1A), and a 140 bp 3′ untranslated region. The E1β cDNA (1441 bp) has a 6 bp 5′ untranslated region, a 1218 bp open reading frame encoding a polypeptide of 406 amino acids (Fig. 1B), and a 217 bp 3′ untranslated region. The calculated molecular weight and isoelectric point values for the E1α and E1β polypeptides encoded by the open reading frames are 47 120 with a p*I* of 7.25, and 44 208 with a p*I* of 5.89, respectively. The deduced amino acid sequence for E1α has 61% and E1β 68% identity with *P. purpurea odpA* and *odpB*, respectively.

The first 68 residues of E1α and 73 residues of E1β exhibit characteristics of chloroplast transit peptides but not those of mitochondrial targeting sequences [27,28]. To determine structural motifs of the transit peptides, GeneWorks (IntelliGenetics, Mountain View, CA) protein algorithm was used to identify possible α-helix and β-strands. Both plastid E1α and E1β have the potential to form amphiphilic β-strands consistent with plastid targeting sequences, but did not fit the amphiphilic α-helix which is characteristic of mitochondrial targeting sequences (data not shown).

Overall, there is 28% sequence identity between *Arabidopsis* plastid PDH E1α and its mammalian counterparts. However, in specific regions the degree of sequence conservation is much higher. The PDH component of PDC requires thiamin pyrophosphate (TPP) as a cofactor for decarboxylation of pyruvate [2]. It has been reported that TPP binds to the E1α subunit of mammalian PDH at a site containing a structural motif common to pyrophosphate-binding enzymes [1]. A similar motif (50% identity with the bovine E1α TPP-binding domain) is found in the *A. thaliana* plastid E1α sequence at residues 160–213 (Fig. 2A).

A highly conserved Cys residue (Cys 62 of mature human E1α, Fig. 2A) has been identified in eukaryotic PDH E1α sequences, and it has been proposed that this Cys is an essential component of the enzyme's active site [29]. The *A. thaliana* plastid E1α sequence contains a similar motif, i.e. the same immediate flanking residues at 112–116, but the otherwise conserved Cys is replaced with a Val (Fig. 2A). Additional experiments will be necessary to determine if a distal Cys fulfills the role of the residue absent in the plastid sequence.

Mitochondrial PDCs are regulated in part by reversible phosphorylation of three conserved Ser residues in the E1α sequence by a specific, complex-associated PDH-kinase [1]. The Ser residues phosphorylated in mammalian mitochondrial PDH are also conserved in the plant mitochondrial [23], yeast [30], and nematode [31] amino acid sequences. However, while the plant mitochondria PDC is reversibly phosphorylated [5,6], all evidence to date indicates that plastid PDC activity is not regulated by phosphorylation [15]. Despite this difference, the regulatory Ser residues and their flanking sequences are present in plastid E1α sequence (Fig. 2A). Korotchkina and Patel [32] have reported the results from

A

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MATAFAPTKL TATVPLHGSH ENRLLLPIRL APPSSFLGST RSLSLRRLNH SNAIRRSPVV   60
SVQEVVKEKQ STNNTSLLIT KEEGLELYED MILGRSFEDM CAQMYYRGKM FGFVHLYNGQ  120
EAVSTGFIKL LTKSDSVVST YRDHVHALSK GVSARAVMSE LFGKVTGCCR GQGGSMHMFS  180
KEHNMLGGFA FIGEGIPVAT GAAFSSKYRR EVLKQDCDDV TVAFFGDGTC NNGQFFECLN  240
MAALYKLPII FVVENNLWAI GMSHLRATSD PEIWKKGPAF GMPGVHVDGM DVLKVREVAK  300
EAVTRARRGE GPTLVECETY RFRGHSLADP DELRDAAEKA KYAARDPIAA LKKYLIENKL  360
AKEAELKSIE KKIDELVEEA VEFADASPQP GRSQLLENVF ADPKGFGIGP DGRYRCEDPK  420
FTEGTAQV                                                           428
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B

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MSSIIHGAGA ATTTLSTFNS VDSKKLFVAP SRTNLSVRSQ RYIVAGSDAS KKSFGSGLRV   60
RHSQKLIPNA VATKEADTSA STGHELLLFE ALQEGLEEEM DRDPHVCVMG EDVGHYGGSY  120
KVTKGLADKF GDLRVLDTPI CENAFTGMGI GAAMTGLRPV IEGMNMGFLL LAFNQISNNC  180
GMLHYTSGGQ FTIPVVIRGP GGVGRQLGAE HSQRLESYFQ SIPGIQMVAC STPYNAKGLM  240
KAAIRSENPV ILFEHVLLYN LKEKIPDEDY ICNLEEAEMV RPGEHITILT YSRMRYHVMQ  300
AAKTLVNKGY DPEVIDIRSL KPFDLHTIGN SVKKTHRVLI VEECMRTGGI GASLITAAIN  360
NFHDYLDAPV MCLSSQDVPT PYAGTLEEWT VVQPAQIVTA VEQLCQ                 406
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Fig. 1. A and B. The deduced amino acid sequences of the *A. thaliana* plastid E1α and E1β cDNA clones receptively. Presumptive targeting sequences are underlined.

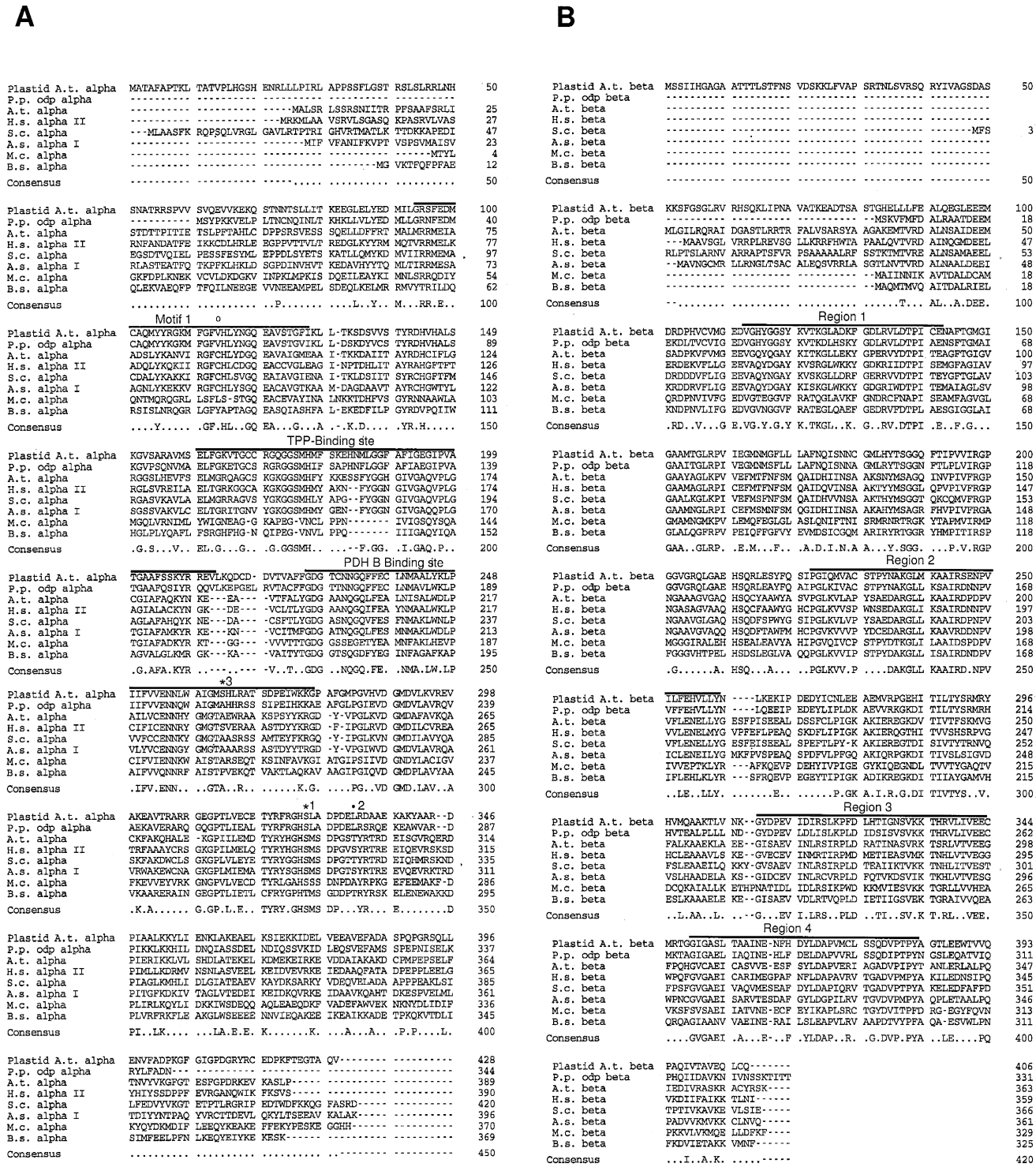

Fig. 2. A and B. Alignment of the deduced amino acid sequences of PDH E1α and E1β. Abbreviations are the same as in Fig. 5. *, conserved; ●, non-conserved phosphorylation sites; ○, the conserved Cys 62 of the mature *H.s.* E1α sequence.

mutagenesis of these phosphorylation sites, and concluded that site one is closer to the active site or lies on the pathway to the main catalytic conformational change. This might explain why this region is so highly conserved. The amino acid-motif corresponding to phosphorylation site one in mitochondrial PDH sequences is present in the plastid polypeptide (Tyr 320-Pro 330 or Tyr 287-Pro 297 in the *H.s.* sequence, Fig. 2A). Two of the four substitutions are by residues with conserved properties. The sequence of the plastid E1α corresponding to phosphorylation site two lacks a Ser and the region is dominated by five acidic and two basic residues (Asp 329-Asp 339). The *Arabidopsis* plastid E1α sequence contains a Ser at site 3 (Ala 259-Ala 267), but the flanking residues are dissimilar to the mammalian site 3 (Fig. 2A). While two of the three Ser are in the appropriate positions, it is most likely then that plastid PDC is not regulated by phosphorylation due to the lack of plastid PDH-kinase [15].

Wexler et al. [33] compared alignments of three PDH and three branched-chain α-keto acid dehydrogenase sequences. Among E1β sequences, four regions of sequence conservation were observed. Region one, the proposed E2 interaction site, is present in the *Arabidopsis* plastid PDH E1β sequence (Fig. 2B). Conserved regions two and three share high homology with other decarboxylating enzymes, suggesting a role in decarboxylation of pyruvate [33]. A functional role has not yet been attributed to region four (Fig. 2B). Eswaran et al. [34] have described Arg 239 as being an essential residue near or at the active site of the bovine E1β. This residue is conserved throughout the eukaryotic PDH sequences (e.g. Arg 269 of *H.s.* sequence in Fig. 2B) and is present in the *A. thaliana* plastid E1β sequence at position 318.

The genomic organization of *Arabidopsis* E1α and E1β was determined by Southern blot analysis. An E1α cDNA probe hybridized to a single restriction fragment in each lane, suggesting one gene (Fig. 3A). An E1β cDNA probe, on the other hand, hybridized to multiple fragments in a pattern consistent with the restriction digest of E1β cDNA (data not shown). The *Xba* I lane contained multiple hybridizing bands which could be due to a second gene or an intron containing an *Xba* I restriction site (Fig. 3B).

In order to evaluate expression of the *A. thaliana*

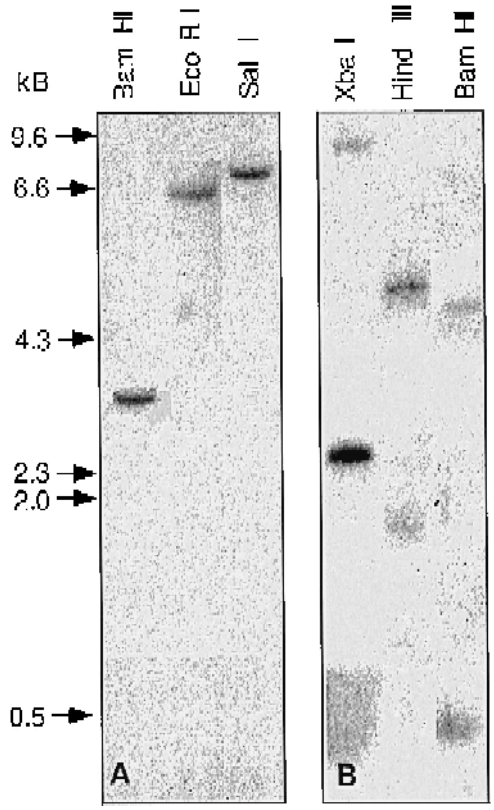

Fig. 3. Southern analyses of genomic DNA isolated from mature *A. thaliana* leaves. Each lane was loaded with 10 μg of DNA digested with *Bam*HI, *Hin*dIII, *Sal*I, *Eco*RI or *Xba*I as indicated. A and B. Genomic Southern blots hybridized with random primed probes generated from gel-excised E1α and E1β cDNAs, respectively. (α^{32}P)-dCTP was incorporated using an oligolabelling kit (Pharmacia, Uppsala, Sweden). The positions of λ DNA markers digested with *Hin*dIII are indicated to the left of the figure.

plastid PDH genes, 10 μg total RNA obtained from young leaves were resolved by formaldehyde gel electrophoresis. Northern blot analyses confirmed the expression of a single mRNA species of 1.65 kb for E1α and 1.5 kb for E1β (Fig. 4A and B).

The two cDNAs reported here have been tentatively identified as encoding plastid rather than mitochondrial proteins based on their high homology with the *P. purpurea* chloroplast genes, the presence of N-terminal sequences characteristic of plastid transit peptides, and their relatively low homology with plant mitochondrial E1 subunits [22–24]. Predictions of the mature N-terminal sequences were based on homology with the mature *odp* and mitochondrial E1 sequences. The mature *A. thaliana* plastid E1α and

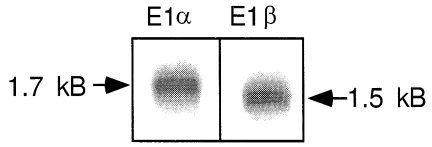

Fig. 4. Northern blot analyses of *A. thaliana* RNA. Total RNA was isolated from young leaves of *A. thaliana* plants. 10 μg of total RNA was run on formaldehyde gels then transferred to nylon membranes. Probes were prepared as described in the legend for Fig. 3. RNA markers were used to determine the sizes of the hybridizing bands.

E1β amino acid sequence (Fig. 2A and B), have the highest homology (68%) with the *P. purpurea* chloroplast *odpA* and *odpB* sequences, respectively, but only 31 and 32% identity with the respective *A. thaliana* mitochondrial E1 sequences. The homology with other eukaryotic mitochondrial E1 sequences is lower yet. Additionally, a monoclonal antibody prepared against mitochondrial E1α does not recognize chloroplastic E1α [35] nor does the monoclonal antibody recognize the recombinant plastid E1α on immunoblots (data not shown).

Dendrogram analyses show that *A. thaliana* plastid E1, *P. purpurea* chloroplast *odp* and *Synechocystis sp.* (a cyanobacterium) *pdh* sequences segregate as a family distinct from mitochondrial and bacterial sequences (Fig. 5A and B). A similar separation has also been shown for plastid and mitochondrial ribosomal RNA sequences [36]. The *A. thaliana* plastid cDNAs and *P. purpurea odp* genes are the only sequences reported thus far for plastid forms of PDH.

As additional cDNAs and genes for plastid and mitochondrial specific isozymes are determined, insight as to the lineage of plastid genes will be gained. Mitochondrial rRNA genes show convincing similarity to purple-photosynthetic bacterial rRNA sequences. In contrast, plastid rRNA has similarity with cyanobacterial rRNA. This relationship between plastids and cyanobacteria also has been noted for genes encoding the transcriptional and translational apparatus [36]. The new sequences reported here should contribute to understanding if the emergence of mitochondria and plastids was the result of single or multiple primary (i.e. eubacteria/eukaryotic) endosymbioses, or if secondary (i.e. eukaryotic/eukaryotic) endosymbioses led to the establishment of these organelles [36].

In conclusion, we have cloned and sequenced *A. thaliana* cDNAs whose properties indicate that they are the plastid forms of PDH E1α and E1β subunits. Having these cDNAs will allow us to perform trans-

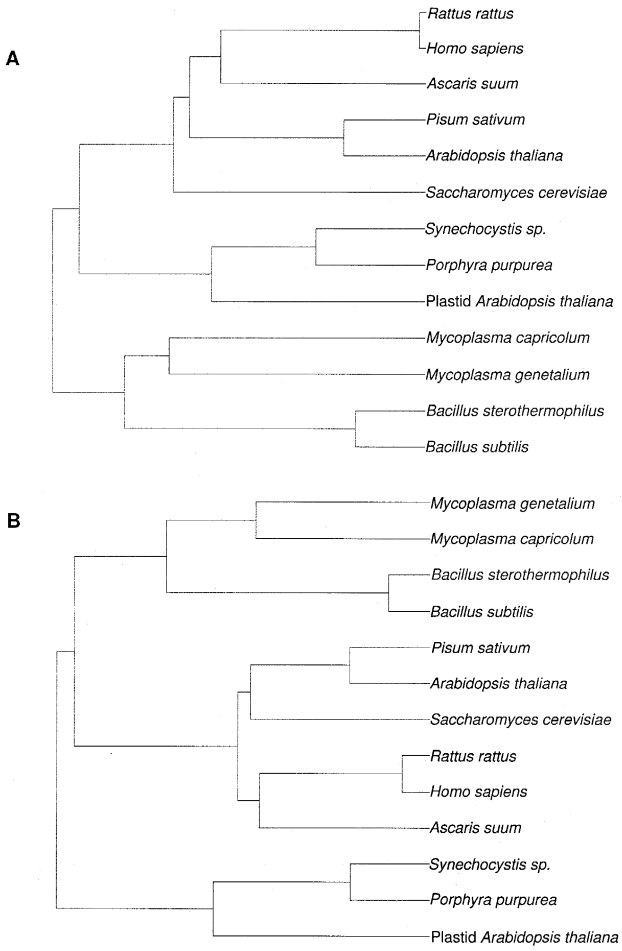

Fig. 5. A and B. Dendrogram analysis of the deduced amino acid sequence of PDH E1α and E1β subunits, respectively. Abbreviations and accession numbers to the sequences are: *P. p., Porphyra purpurea odp* (U38804); *S. sp., Synechocystis sp.* (D90915); *A. t., Arabidopsis thaliana* (U21214, U09137); *P. s., Pisum sativum* (U51918, U56697); *H. s., Homo sapiens* (L13318, D90086); *R. r., Rattus rattus* (Z12158, P49432); *S. c., Saccharomyces cerevisiae* (P16387, M98476); *A. s., Ascaris suum* (M76554, M38017); *M. gen., Mycoplasma genetalium* (U39706); *M. c., Mycoplasma capricolum* (U62057); *B. su., Bacillus subtilis* (M57435); and *B. s., Bacillus stearothermophilus* (X53560). Dendrogram analyses was accomplished with GeneWorks CLUSTAL V method (IntelliGenetics, Mountain View, CA). CLUSTAL V parameters were as follows: cost to open gap = 5, cost to lengthen gap = 25, gap penalty = 3, number of top diagonals = 5, window size = 5, PAM matrix = PAM250, K-tuple = 1, and consensus cutoff = 50%.

genic plant and developmental expression experiments to determine the role of plastid PDC in fatty acid biosynthesis.

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