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Molecular evidence of a unique lipoamide dehydrogenase in plastids: analysis of plastidic lipoamide dehydrogenase from *Arabidopsis thaliana*[1]

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Abstract Lipoamide dehydrogenase is a subunit of the α-ketoacid dehydrogenases and the glycine decarboxylase complex in mitochondria, and the pyruvate dehydrogenase complex in plastids. We report here the unexpected finding of two plastidic isoforms of lipoamide dehydrogenase from *Arabidopsis thaliana* that are different from the mitochondrial form of the enzyme. The cDNA clones were confirmed by sequence alignment analysis and their location verified by chloroplast import assay. They are single copy genes that appear to be expressed in parallel in different tissues with highest level in developing siliques. Phylogenetic analysis gives further exemplary evidence for the plastidic evolution derived from cyanobacteria. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Lipoamide dehydrogenase; Pyruvate dehydrogenase complex; Glycine decarboxylase complex; Acetyl-CoA; *Arabidopsis thaliana*

1. Introduction

Lipoamide dehydrogenase (LPD) belongs to the flavoprotein disulfide oxidoreductase family [1–3] and is part of the α-ketoacid multienzyme dehydrogenase complexes, pyruvate (PDC), α-ketoglutarate (KDC), and branched-chain α-ketoacid (BCKDC) dehydrogenase complexes [4] as well as part of the glycine decarboxylase complex (GDC) [5]. Those multienzyme complexes are all found in the mitochondria with the exception that PDC also occurs in plastids of plants. PDC, the most studied of the α-ketoacid complexes, consists of four subunits: E1α and E1β forming the pyruvate dehydrogenase subunit, E2, the lipolyltransacetylase subunit, and E3, the LPD subunit. GDC consists of four subunits: P-protein, H-protein (which contains the lipoamide cofactor), T-protein, and L-protein (the LPD). Within these multienzyme complexes, LPD reoxidizes the lipoamide cofactor of E2 for the α-ketoacid dehydrogenases, and of H-protein for the GDC, and passes that electron through FAD to reduce NAD^+ to NADH.

LPD from pea mitochondria has been cloned as a single copy gene by two groups simultaneously [6,7]. It had been suggested by Walker et al. [8], based on monoclonal antibody inhibition profiles, that in pea leaf mitochondria a single isozyme of LPD is functional in both the GDC and the PDC. This has been further substantiated by Bourguignon et al. [9] who used mass spectrometry to show that there is indeed only a single LPD shared between the two complexes.

Less work has been done on LPD of plastidic (pt) PDC. Earlier work [10,11] showed that E3 of pea ptPDC was the same size on SDS–PAGE as the E3 of mtPDC and both reacted with antiserum raised against broccoli mitochondrial (mt) PDC. Oliver and Behal (unpublished) showed that the pt- and mtE3 of peas co-purified during ion exchange and hydroxylapatite chromatography and were both inhibited in parallel upon incubation with a monoclonal antibody against the mtE3.

All of this evidence suggests that in pea the same isoform of LPD cannot only be shared between the different mitochondrial multienzyme complexes, but may also be shared between the mitochondria and the plastids.

There has been one report of the purification of a distinct LPD from pea chloroplasts to near homogenity that was not immunologically related to the mitochondrial protein and had an N-terminal sequence that was unrelated to the proteins whose genes we cloned [12,13]. No further work was reported.

As there is clearly a need for E1 and E2 subunits (alternatively H-, P-, and T- for GDC) to be altered in the different multienzyme complexes to accommodate various substrates, there is no such need for multiple LPDs. It has just recently been shown with the components of the GDC, namely the H- and the L-protein (LPD), that there is no apparent molecular recognition or interaction required between the H-protein and its cognate L-protein and therefore no stable complex between the H- and L-protein exists [14]. LPD only recognizes the lipoyl moiety irrespective of the protein domain bound to it.

In this paper, we report the identification and molecular characterization of the plastidic isoforms of LPD, clarifying that despite the biochemical analysis in peas, the ptPDC has its own distinct LPD isoforms different from the one in the mitochondria and giving another example supporting the endosymbiotic theory of plastidic evolution from ancestral cyanobacteria.

2. Materials and methods

2.1. Sequence analysis

Full length clone *ptlpd1* and a partial length clone *ptlpd2* were found as described in Section 3, obtained from the ABRC (*Arabidopsis* Biological Resource Center), and sequenced by the DNA Sequence and Synthesis Facility at Iowa State University. To obtain the missing 5′ end of *ptlpd2*, the SMART® cDNA Library Construction kit by Clontech was used according to the manufacturer's instructions. Using BLAST, ESSA I FCA contig fragment No. 5 (accession number Z97340) from chromosome 4 was found to encode the genomic DNA of *ptlpd2*. Specific forward (5′-TCTCGTACACAATGCAATCG-GTTC-3′) and reverse (5′-CATAACCAGCAGCTGAAACCTGC-

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[1] GenBank/EBI data bank accession numbers are as follows: AF228637 for *ptlpd1* and AF228638 for *ptlpd2*.

PII: S0014-5793(00)02116-5

3′) PCR primers were synthesized. A ∼500 bp fragment was obtained by PCR using these specific primers, isolated and sequenced. DNA alignments and analysis were performed using the Molecular Biology Software 'Vector Nti Suite' by InforMax.

2.2. Southern analysis

Genomic DNA was isolated from 0.1 g *Arabidopsis thaliana* (ecotype Columbia) using PhytoPure Plant DNA Extraction kit by Nucleon® Biosciences according to their protocol. DNA was digested for 4–6 h with the restriction endonucleases indicated and run on a 1% TBE gel, followed by depurination, denaturation and neutralization as described by Maniatis [15]. Further treatments were performed as described below (Northern analysis).

2.3. Northern analysis

Total RNA was isolated from mature *A. thaliana* (ecotype Columbia) grown in greenhouse conditions under continuous light according to the methods described by Kirk [16] with the following changes: 2 mM aurin tricarboxylic acid was added to the lysis buffer [17] and, after precipitation and centrifugation, the supernatant was drained off and the pellet dissolved in 0.1 mM ATA followed by a phenol extraction. Tissue samples were run on a 1% formaldehyde-agarose gel and blotted onto a Zeta Probe nylon membrane (Bio-Rad) by capillary transfer as described by Maniatis [15]. RNA was then auto-cross-linked to the membrane with the UV Stratalinker by Stratagene. Prehybridization and hybridization solutions were modified after Church et al. [18] as described by Xiang et al. [19]. Probes were first PCR-amplified from the clones with 3′ untranslated region (UTR) specific forward and reverse primers, purified, and then labeled with ^{32}P using PCR (10–15 cycles) with only the reverse primer. Procedures were further followed as described by Xiang et al. [19].

2.4. Chloroplast import assay

The chloroplast import assay was performed according to the procedures of Perry et al. [20]. 10 days old pea tissues were used. The in vitro transcription/translation was performed in a single tube using the uncut full length EST clone named *ptlpd1* and the T$_N$T® T7 Coupled Reticulocyte Lysate Systems from Promega. Amersham International Redivue L-[^{35}S]methionine was used as radiolabel. Analysis of the import reactions was done with a 12% SDS polyacrylamide gel, followed by infusion of organic scintillant PPO/DMSO [20] to allow fluorographic detection by X-ray film.

2.5. Phylogenetic analysis

Phylogenetic analysis was performed using the ClustalW WWW service at the European Bioinformatics Institute (EBI) at http://www2.ebi.ac.uk/clustalw. The matrix chosen for the multiple sequence alignment was BLOSUM by Henikoff. The method used for the phylogenetic tree is based on the neighbor joining analysis of Saitou and Nei. The output guide tree was viewed with TreeView downloaded from the Taxonomy and Systematics site at the University of Glasgow at http://taxonomy.zoology.gla.ac.uk, and printed in the neighbor joining format. The amino acid identity matrix was created using the pairwise scores in percentage from the ClustalW multiple sequence alignment results from EBI.

3. Results and discussion

3.1. Identification of ptlpd genes from A. thaliana

With the publication of the entire plastid genome of the red alga *Porphyra purpurea* plastidic homologous to the mtE1α and mtE1β subunits of PDC were found by Randall's group

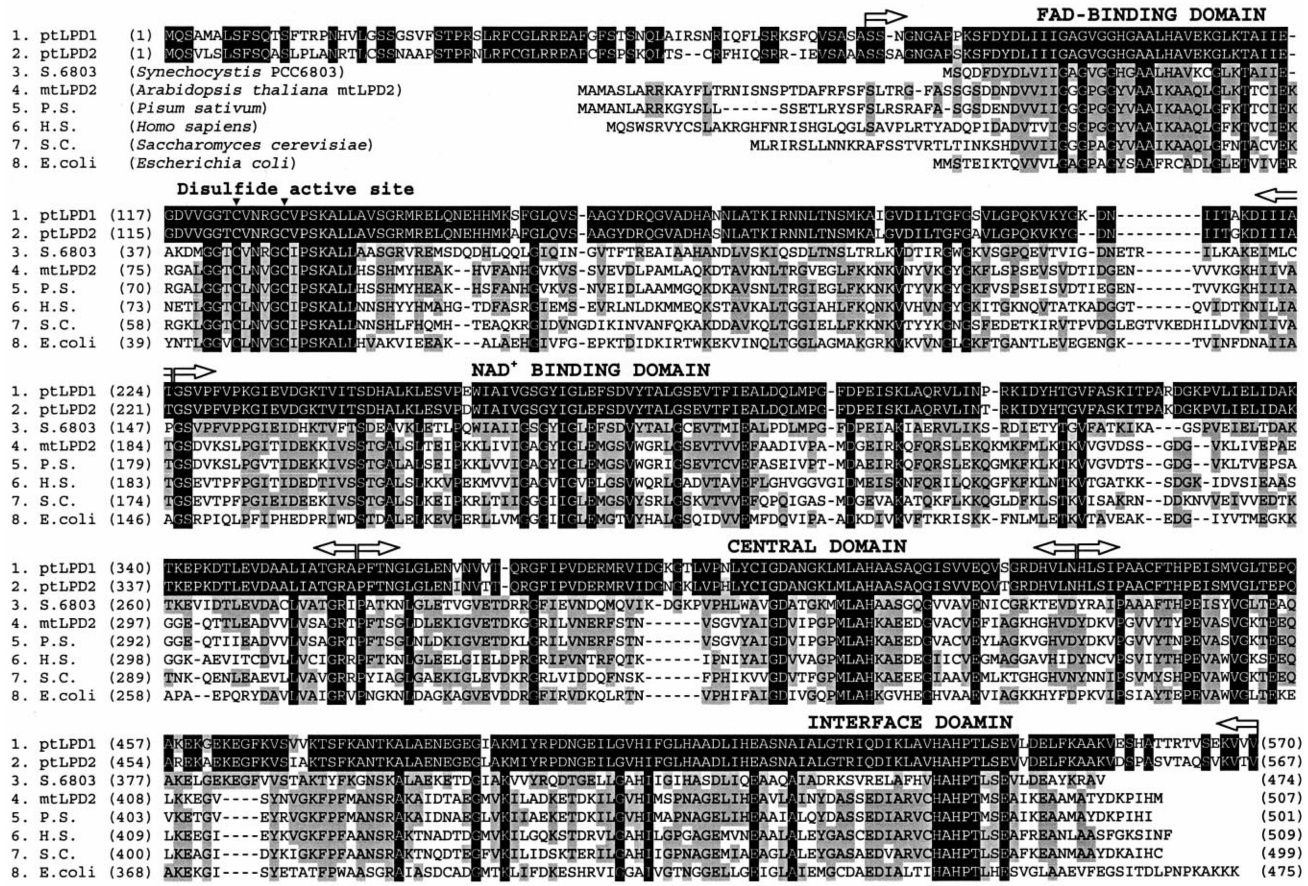


Fig. 1. Sequence comparison of LPDs from different organisms. Alignment of the amino acid sequences deduced from the two *ptlpd* genes from *Arabidopsis ptlpd1* and *ptlpd2* is compared with LPD from *Synechocystis* PCC 6803 (S. 6803), *E. coli*, and mitochondrial proteins from *Arabidopsis*, pea, human, and yeast. The specific domains of LPDs are marked with arrows with ptLPDs as indicator. Black boxes represent identities between ptLPDs as well as identities between all organisms selected. Gray boxes represent conserved amino acids as well as amino acids that are identical to the ptLPDs.

[21] as well as by our group [22]. Those *Arabidopsis* plastidic subunits were distinct from those found in the mitochondria to the point that they were more closely related to those of the red alga plastids than to their *Arabidopsis* mitochondrial counterparts. Recently, the ptE2 subunit has also been reported [23]. This led us to search for the gene encoding LPD with the homologous one from the cyanobacterium *Synechocystis* PCC 6803 [24] using the *Arabidopsis* Database via BLAST at TAIR (The *Arabidopsis* Information Resource). Three EST clones from *Arabidopsis* encoding putative ptLPD were found. DNA sequencing revealed a full length clone (AIMS stock # 36A9T7) termed *ptlpd1* and a partial length clone (AIMS stock # 120P10T7) termed *ptlpd2*. The third EST clone (AIMS stock # 169G7T7) was found to be a partial length clone identical to *ptlpd1*.

Ptlpd1 has a length of 2205 bp encoding 570 amino acids with a calculated molecular mass of 60 753 Da. The full length gene for *ptlpd2* was obtained by a reverse transcription PCR-based approach. The reconstructed full length *ptlpd2* has a length of 1840 bp, encoding 567 amino acids with a calculated molecular mass of 60 142 Da.

The three amino acids difference in the two ptLPDs is due to ptLPD2 being three amino acids shorter in its presequence. Alignments of ptLPDs also show a one amino acid gap in both ptLPD2 within a non-conserved region of the FAD-binding domain.

3.2. *LPD sequence alignments analysis clearly shows ptLPD identities*

Alignments of the two *ptlpd*s show 81% identity on the DNA level and 88% identity on the protein level. Alignments with the *Synechocystis* LPD show 61% identity on the protein level. LPD belongs to the family of the flavoprotein disulfide oxidoreductases, as does glutathione reductase. Glutathione reductase is the best studied enzyme of this family and, as the tertiary structures are very similar between the two, the characteristic domains are assigned to LPD based on those established for the human glutathione reductase [2]. Very recently, the crystal structure of the L-protein of pea has been published [14,25]. All four characteristic domains established for glutathione reductase can be found in the two ptLPD proteins. Starting from the N-terminus, these are the FAD-binding domain (including the redox active site), the NAD$^+$-binding domain, the central domain and the interface domain (Fig. 1). The FAD-binding domain with the functional motif (GxGxxG/AxxxG/A) for dinucleotide-binding of the so called Rossmann fold [26] and the redox active site (CL/VNxGC) are present in ptLPDs (residues 71–224 for ptLPD1). It is to be mentioned that typically in the redox active site, the first cysteine is followed by a leucine in LPDs, and by a valine in glutathione reductases (as in both ptLPD sequences). Glutathione reductase, in contrast to LPD, has its two subunits covalently bound by a disulfide bridge. This cysteine residue is missing in LPDs as well as in the ptLPDs described here. The NAD$^+$-binding domain can be found from residues 225 to 359 with the motif (GxGxIGxExxxVxxxxG) followed by the central domain from residues 360 to 433. The interface domain (residues 434–570) contains the active base histidine and the stabilizing hydrogen bond partner glutamic acid in the signature motif (HAHPTxxE). LPD in its active form is a homodimer with two redox centers. While it could theoretically be converted to a four electron reduced form, it is only reduced by two electrons in a charge-transfer complex [1,3]. As only one proton can be shared between the two thiols, the second proton is taken up by the active base histidine. This histidine is contributed by one subunit and the redox active site by the other subunit. The very C-terminal is important for stability. Fig. 1 shows protein alignments of LPDs from different representative species. The two ptLPDs show all the characteristic domains of a LPD.

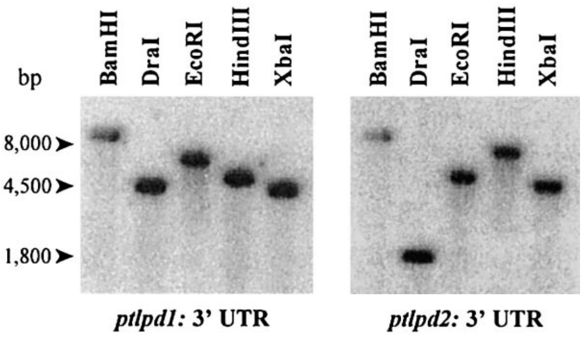


Fig. 2. Southern blot of *ptlpd1* and *ptlpd2*. 5 µg total *A. thaliana* DNA per lane was separated on an agarose gel, transferred to a nylon membrane and probed with *ptlpd1/ptlpd2*. Hybridization was performed using probe made from the 3′ UTR of each gene.

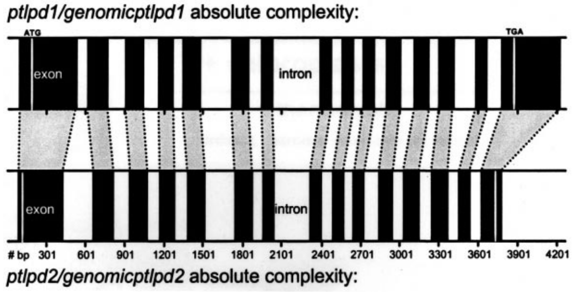


Fig. 3. Comparison of intron patterns of *Arabidopsis ptlpd1* and *ptlpd2*. Alignments of the genomic sequences with the cDNAs show 15 exons and 14 introns with similar spacing for each gene.

3.3. *ptLPDs are encoded by single nuclear genes*

A. thaliana genomic DNA was digested with: *Bam*HI, *Dra*I,

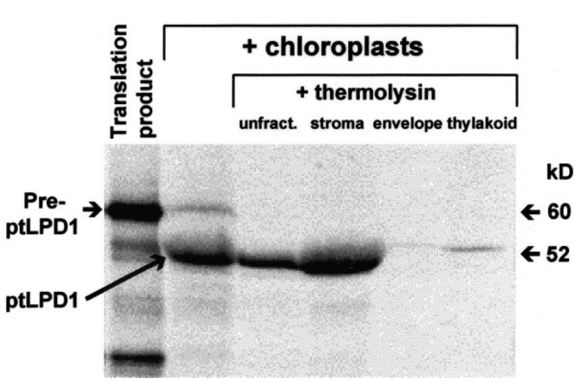


Fig. 4. Uptake of ptLPD1 protein by pea chloroplasts. A chloroplast import assay was performed using isolated pea chloroplasts and ptLPD1. Labeled ptLPD1 (60 kDa) was made by in vitro transcription and translation. A mature protein of about 52 kDa can be found in the stromal fraction of the chloroplasts. Thermolysin treatment confirms that ptLPD1 is indeed transported into the chloroplasts.

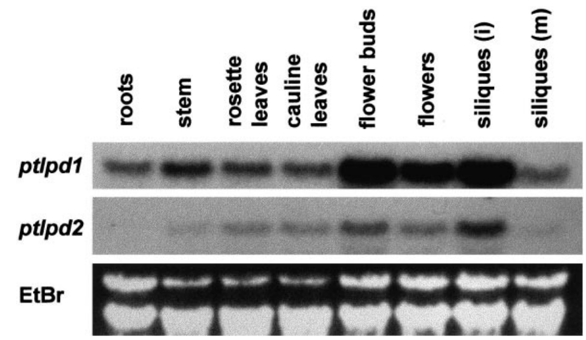


Fig. 5. Northern blot analysis of mRNA for *ptlpd1* and *ptlpd2*. 10 μg total *A. thaliana* RNA per lane from different tissues was separated on a denaturing gel, transferred to a nylon membrane and probed with the 3′ UTR of *ptlpd1*/*ptlpd2*. EtBr staining was used to confirm equal loading.

*Eco*RI, *Hin*dIII and *Xba*I, run on a gel and blotted. The blot was first hybridized with the 3′ UTR of *ptlpd1*, and then stripped and rehybridized with *ptlpd2*. Both autoradiograms showed different single bands in each lane (Fig. 2). Therefore both ptLPDs are encoded by different single copy genes. The database at TAIR was searched for the genomic DNA of these genes. *Ptlpd1* was found to be on chromosome 3 (TAC clone K14A17 accession number AB026636) and *ptlpd2* on chromosome 4 as described in Section 2. Both genes are encoded by 15 exons and therefore contain 14 introns. Interestingly, comparing the two intron patterns reveals similar spacing ranging from 77 to 375 bp for *ptlpd1* and 83 to 276 bp for *ptlpd2* (Fig. 3). Therefore our conclusion is that there are two genes encoding ptLPD due to a fairly recent gene duplication.

3.4. *Chloroplast import assay proves location of ptLPD1 in plastids*

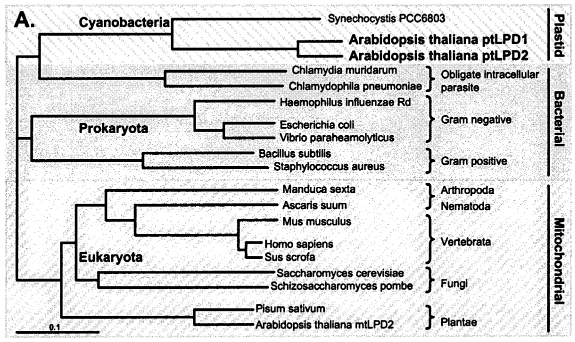
TargetP (http://www.cbs.dtu.dk/services/TaregtP) was first used to predict the organelle to which ptLPD1 and ptLPD2 were targeted. Both presequences where predicted with very high probability (0.977 for ptLPD1 and 0.962 for ptLPD2) to be targeted to the plastids. The probability for any other organelle was below 0.068. ChloroP (http://www.cbs.dtu.dk/services/ChloroP) further confirmed the plastidic transit peptides and predicted the putative cleavage sites for ptLPD1 to be between amino acids 70 and 71 with a score of 9.594. The program did not predict a confident cleavage site for ptLPD2. Due to the low score for ptLPD2 and the high identities between the *ptlpd*s, we believe that the putative cleavage site for ptLPD2 is located between amino acids 67 and 68. Comparing the ptLPDs with the LPD from *Synechocystis* (Fig. 1) also indicates that the mature protein starts at around amino acids 68–82, as the *Synechocystis* LPD does not contain any presequences and this protein is 80 (for ptLPD1) and 78 amino acids (for ptLPD2) shorter at the N-terminus compared to ptLPDs (Fig. 1).

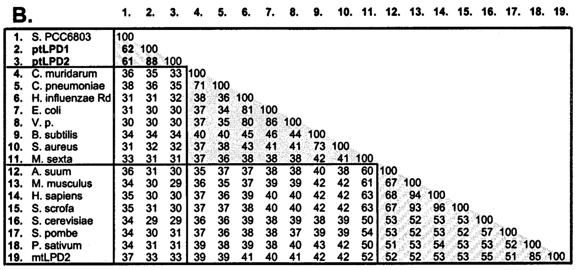
To make sure that ptLPD is indeed targeted into the chloroplasts, we performed a chloroplast import assay. Pea chloroplasts were isolated via a Percoll gradient. Radiolabeled ptLPD1 was prepared by in vitro transcription/translation. Thermolysin treatment was performed after the import step to remove unincorporated proteins and the plastids were separated into stromal, thylakoid, and envelope fractions after uptake. Fig. 4 shows the resulting fluorograph. A preprotein of about 60 kDa can be found in the lane labeled translation product. The mature protein of about 52 kDa accumulates inside the chloroplasts. After thermolysin treatment, only the mature form could be found and the preprotein had been digested. Further it can be concluded that the protein is targeted to the stroma, as no band can be found in the envelope and only a faint one in the thylakoid fraction, which most likely just represents contamination.

The location of the multienzyme complexes which have LPD as a subunit is in the matrix of mitochondria and the stroma of plastids. In *Synechocystis*, it has been reported that LPD is located in the extracellular periplasm between the cytoplasmic membrane and the peptidoglycan layer [27]. Its function in the periplasm is unknown and may indicate a different function for this LPD other than just the one associated with the multienzyme complexes. *Synechocystis* has PDC and GDC so the LPD must also function in these complexes. LPD in vitro is well known to show transhydrogenase, diaphorase [28], as well as nitroreductase activities, but none of them has been proved to play a role in vivo [2]. Therefore, another role for LPD other than in the multienzyme complexes is uncertain.

3.5. *Northern analysis shows no apparent differences in expression levels between the two ptlpds*

RNA from different tissues was isolated from mature *A. thaliana*. Northern blot was hybridized with 3′ UTR from *ptlpd1*, stripped and rehybridized with *ptlpd2*. The gel was stained with EtBr as an equal loading control (Fig. 5). Both autoradiograms show increased RNA expression mainly in flower buds and immature siliques, and to a lesser extend in flowers. Some expression could be found in all other examined tissues. The RNA expression pattern is similar in both genes, except that the second gene (*ptlpd2*) is less strongly expressed.





B.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.
1. S. PCC6803	100																		
2. ptLPD1	62	100																	
3. ptLPD2	61	88	100																
4. C. muridarum	36	35	33	100															
5. C. pneumoniae	38	36	35	71	100														
6. H. influenzae Rd	31	31	32	38	36	100													
7. E. coli	31	30	30	37	34	81	100												
8. V. p.	30	30	30	37	35	80	86	100											
9. B. subtilis	34	34	34	40	40	45	46	44	100										
10. S. aureus	31	32	32	37	38	43	41	41	73	100									
11. M. sexta	33	31	31	37	36	38	38	38	42	41	100								
12. A. suum	36	31	30	35	37	37	38	38	40	38	60	100							
13. M. musculus	34	30	29	36	35	37	39	39	42	42	61	67	100						
14. H. sapiens	35	30	30	37	36	39	40	40	42	42	63	68	94	100					
15. S. scrofa	35	31	30	37	37	38	40	40	42	42	63	67	93	96	100				
16. S. cerevisiae	34	29	29	36	36	39	38	39	38	39	50	53	52	53	53	100			
17. S. pombe	34	30	31	37	36	38	38	37	39	39	54	53	52	53	52	57	100		
18. P. sativum	34	31	31	39	38	39	38	40	43	42	50	51	53	54	53	53	52	100	
19. mtLPD2	37	33	33	39	39	41	40	41	42	42	52	52	52	53	53	55	51	85	100

Fig. 6. Phylogenetic analysis of LPD. The phylogenetic analysis was performed using the default program of ClustalW from the EBI. The guide tree was then viewed using TreeView and the amino acids identity matrix was created using the identities given by the ClustalW results.

The location of the stronger RNA expression in buds and immature siliques likely results because these proteins are part of the PDC in the plastids and since ptPDC is the major source of acetyl-CoA for de novo fatty acid synthesis. PDC levels should be elevated during embryo development (immature siliques) and tissue development such as in buds. Those results correlate nicely with the mRNA pattern for ptE1β published by Ke at al. [22]. These authors show an increased level of ptE1β in buds and flowers compared to leaves and that there is an increase in mRNA expression in siliques development from 3 days after flowering (DAF) with a peak at 6 DAF, followed by a hypobolic decrease to 15 DAF. This correlates with the expected pattern of de novo fatty acids in siliques development. In young tissue, the demand for de novo fatty acid synthesis is very high, as it is needed to support the newly grown membrane structures.

3.6. Phylogenetic analysis shows the closer relationship of ptlpds to those of cyanobacteria than to their mitochondrial counterparts

Fig. 6A shows LPDs grouping into cyanobacteria (plastid), prokaryotes (bacteria) and eukaryotes (mitochondria). The mitochondrial group is closely related (see Fig. 6B) ranging from 50% upward, whereas the prokaryotic group ranges from 34% upwards. It can be seen that the *A. thaliana* ptLPDs are more closely related to the *Synechocystis* (cyanobacteria) with 61% identities than to their mitochondrial counterpart mtLPDs (33%). This is another example that supports the endosymbiotic theory of plastid evolution from ancestral cyanobacteria.

This report ends the debate whether one LPD serves all α-ketoacid dehydrogenase multienzyme complexes in both organelles as well as the GDC. There is indeed a unique ptLPD.

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