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Identification of an *Arabidopsis* cDNA encoding a lipoyltransferase located in plastids[1]

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Abstract In plant cells, the pyruvate dehydrogenase (PDH) complex that requires lipoic acid as an essential coenzyme is located in plastids and mitochondria. The enzyme complex has to be lipoylated in both organelles. However, the lipoyltransferase located in plastids has not been reported. In this study, an *Arabidopsis thaliana LIP2p* cDNA for a lipoyltransferase located in plastids has been identified. We have shown that this cDNA encodes a lipoyltransferase by demonstrating its ability to complement an *Escherichia coli* mutant lacking lipoyltransferase activity, and that LIP2p is targeted into chloroplasts. These findings suggest that LIP2p is located in plastids and responsible for lipoylation of the plastidial PDH complex.

Key words: Lipoic acid; Lipoylation; Lipoyltransferase; Plastid; Pyruvate dehydrogenase; *Arabidopsis thaliana*

1. Introduction

Lipoic acid (6,8-thioctic acid or 1,2-dithiolane-3-pentanoic acid) is a sulfur-containing coenzyme that is required for the activity of enzyme complexes involved in central metabolism. Pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH) and branched chain 2-oxo acid dehydrogenase complexes, and the glycine cleavage system are known as lipoic acid-requiring enzyme complexes [1–6]. Lipoic acid is covalently bound to the dihydrolipoamide acyltransferase (E2) subunits of PDH, OGDH and branched chain 2-oxo acid dehydrogenase complexes and the H-protein of the glycine cleavage system via an amide linkage to the ε-amino group of specific lysine residues of a conserved protein domain [7]. The attached lipoyl moiety functions as a carrier of reaction intermediates among the active sites of the components of the complexes [8,9].

Despite the importance of the lipoyl prosthetic group in the function of the enzyme complexes involved in central metabolism, little is known about the enzymes responsible for the transfer of lipoic acid in higher plants. We have recently cloned a *LIP2* cDNA of *Arabidopsis thaliana* encoding a lipoyltransferase located in the mitochondria [10]. The lipoyltransferase encoded by the *LIP2* gene is functionally and structurally homologous to the lipoyltransferase (LipB) of *Escherichia coli* [11]. In non-plant eukaryotes, all of the known lipoic acid-containing proteins are located in the mitochondria [1–6]. In plants, the PDH complex, a lipoyl enzyme complex, is present both in mitochondria and plastids [12]. Thus, it is reasonable to assume that also lipoyltransferases are located in mitochondria as well as in plastids. However, neither the gene nor enzyme responsible for lipoylation of the E2 subunit of the plastidial PDH complex has been identified. In this study, we report on an *A. thaliana* cDNA, designated as *LIP2p*, encoding a lipoyltransferase that is located in plastids. To our knowledge, this is the first report to describe the lipoate attachment enzyme from higher plants that is located in plastids.

2. Materials and methods

2.1. Plant materials

A. thaliana (Columbia ecotype) was grown on vermiculite in trays under continuous light (40 μmol/m^2/s) at 25°C.

2.2. cDNA cloning and analysis

A fragment of the *A. thaliana LIP2p* cDNA was amplified by polymerase chain reaction (PCR). An *A. thaliana* cDNA library was constructed with λgt11 and cDNA synthesized from poly(A)$^+$ RNAs of leaves. Lambda phage DNAs from the library were used for the PCR as templates. The primers, 5′-CTGGAAAGCTTCAGAGAATTCCTTT-3′ and 5′-GTTCCATACAAGAAAGCTTGGTCA-3′, which anneal to *LIP2p* cDNA, were used for the PCR. The 5′-terminus region of *A. thaliana LIP2p* cDNA was amplified by the 5′-rapid amplification of cDNA ends method (5′-Full RACE Core Set; Takara, Shiga, Japan). The 3′-terminus region of *LIP2p* was also amplified by PCR with lambda DNAs from the *A. thaliana* cDNA library as templates. The amplified cDNAs were subcloned into pCR2.1 (Original TA Cloning kit; Invitrogen, Carlsbad, CA, USA) and their nucleotide sequences were determined. The sequences of 5′-, internal, and 3′-regions were combined and the total sequence (1045 bp) of *A. thaliana LIP2p* cDNA was obtained.

DNA sequence reactions were performed using a DNA sequencing kit (Thermo Sequenase Sequencing kit; Amersham, Buckinghamshire, UK). DNA was sequenced using a DNA sequencer (ALF Red DNA Sequencer; Pharmacia Biotech, Tokyo, Japan). Double-stranded DNAs were used as templates and the sequence of both strands was determined. Nucleotide and deduced amino acid sequences were analyzed with GENETYX-MAC software (Software Development; Tokyo, Japan).

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[1] The nucleotide sequence data of the *Arabidopsis thaliana LIP2p* cDNA for a lipoyltransferase located in plastids were deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB048535.

Abbreviations: ACP, acyl carrier protein; GFP, green fluorescent protein; ORF, open reading frame; OGDH, 2-oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR

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2.3. Nucleic acid extraction and analyses

Genomic DNA used for Southern blot analysis was extracted from the leaves of *A. thaliana* using a DNA extraction kit (ISOPLANT; Nippon Gene, Tokyo, Japan). Genomic DNA was digested with appropriate restriction enzymes, separated by electrophoresis in a 0.8% (w/v) agarose gel, and transferred to a nylon membrane (Hybond-N+; Amersham). Hybridization and detection were performed using a DNA labeling and detection system (ECL kit; Amersham).

Poly(A)$^+$ RNAs used for Northern blot analysis and reverse transcription-PCR (RT-PCR) were extracted from leaves, roots and flowers of 4-week-old *A. thaliana* plants by an RNA extraction kit (mRNA purification kit; Pharmacia Biotech). RT-PCR was performed with a primer set, 5′-ACTCTCCTGTTTACACAATGGGAAC-3′ and 5′-GTCTCTAATCCCACATGGAACTATC-3′. 2 μg of poly(A)$^+$ RNAs from each organ was used for the RT-PCR analysis. As a control, the expression of *LIP1* gene for lipoic acid synthase was also checked with the same RNA preparations using another primer set, 5′-TTGGGGATACATGTACACGC-3′ and 5′-CTACGGGGATGTAGAAGG-3′.

2.4. Functional complementation of an E. coli lplA lipB strain

The coding region of *LIP2p* cDNA was amplified by PCR with a primer set, 5′-CGCTGCAGAGTTGCTGAATGGAGTA-3′ and 5′-CGCTGCAGAGACTCAGCTTACAGAA-3′. An eight-nucleotide sequence 5′-CGCTGCAG-3′ including a *Pst*I site was added to the 5′ end of each primer. The PCR product was digested with *Pst*I and ligated into the *Pst*I site of an expression vector pKK233-2 (Clontech, Palo Alto, CA, USA) to give the desired in-frame product. This plasmid was designated as pKK-LIP2p and was used to transform the *E. coli lplA lipB* strain TM136 [13]. Complementation of the growth of the transformants was checked as described previously [10]. Transformants of strain TM136 with the vector (pKK233-2) were used as negative controls in the complementation experiments.

2.5. Intracellular localization of LIP2p protein

A part of *LIP2p* cDNA encoding an N-terminal region of LIP2p including a putative transit peptide was amplified by PCR with a primer set, 5′-TAGTCGACTATGGAGTTGCTGAATG-3′ and 5′-TGTACCATGGTCACTGTATCAGCGC-3′. The sequences 5′-TAGTCGAC-3′ and 5′-TGTACCATGG-3′ including a *Sal*I site and a *Nco*I site, respectively, were added to the 5′ end of each primer. The 277 bp PCR product was digested with *Sal*I and *Nco*I, and ligated into the *Sal*I–*Nco*I site of CaMV35SΩ-sGFP(S65T)-nos3′ [14] in order to form the expression construct for LIP2p transit fused to green fluorescent protein (GFP). The obtained plasmid, pPreLIP2p::GFP, was introduced into guard cells of *Vicia faba* with a particle bombardment device (PDS-1000/He Biolistic® Particle Delivery System, Bio-Rad). The guard cells were observed by a confocal Laser Scan Microscope (LSM410, Carl Zeiss). Excitation wavelengths were set at 488 nm for GFP and at 543 nm for chlorophyll. Fluorescent images were collected through FITC and TRITC filters for fluorescence from chlorophyll and that from GFP, respectively. Incorporated images were processed using Adobe Photoshop software.

3. Results

3.1. Identification of the cDNA encoding a plastidial form of lipoyltransferase

A BLAST search [15] of the GenBank database using the amino acid sequence of a mitochondrial form of *A. thaliana* lipoyltransferase (LIP2) detected two genes of *A. thaliana* encoding homologous polypeptides. The first gene was present on chromosome 4 (accession numbers AC2980760 and AC2980761). In the database, it was predicted that the genes were present tandemly as two separated genes, and that the two genes encoded polypeptides of 259 and 205 amino acid residues; these polypeptides were homologous to the N-terminal and C-terminal regions of LIP2, respectively. These findings suggest that the computer predicted the wrong splice donor sites. As described below, the predicted two genes were not separated and instead were present as one gene. The nucleotide sequence of the gene was used to synthesize the primers and the *LIP2p* cDNA corresponding to the gene was amplified by PCR. The cloned *LIP2p* cDNA contained an open reading frame (ORF) of 834 bp encoding a polypeptide of 278 amino acids (molecular mass, 31 858 Da). The other gene was located on chromosome 1 (accession number AC007519, F16N3.14 gene). Although several sets of primers specific to this gene were synthesized and used for amplification of the cDNA by PCR, the cDNA corresponding to the gene was not amplified. This result suggests that the expression level of the gene was very low or that the gene was not

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Arabi-LIP2p   1:MELLNGVETLVSGIHHHHRTNAKRNRLVRSVKILNSGNHEIPRKCLCFDLYDKLVPYKKA
Arabi-LIP2    1:                                        MRSPRTLEVWKL-GT-VNYLKS
E. coli       1:                                                    LYQDKILVRQL
                                                                    *

Arabi-LIP2p  61:WSWQKSIVEE-KKTLID-RNQDCADTVILLQHSPVYTMGTASTEDYLNFDIKD-APFNV-
Arabi-LIP2   21:LKLQEKLVSE-RKA-----HQ-IPDTLLSLQHPPTYTLGKRRTDHNLLIPESELTKIGAE
E. coli      12:GLQPYEPISQAMHEFTDTRDDSTLDEIWLVEHYPVFTQGQAGKAEHI-LMPGDI--PVIQ
                                          *      * *  * *

Arabi-LIP2p 117:YR-TERGGEVTYHGPGQLVMYPIINLRNHEMDLHWYLRMLEEIVIRVLSSTFSIKASRLD
Arabi-LIP2   74:LHYTQRGGDITFHGPHQAILYPIISLRSIGFGARNYVETLERSMIE-FASIYGVKARAGN
E. coli      69:---SDRGGQVTYHGPGQQVMYVLLNLKRRKLGVRELVTLLEQTVVN-TLAELGIEAHPRA
                     ***  * *** *   *    *              **              *

Arabi-LIP2p 176:G-LTGVWVGNQKVAAIGIRVSKWITYHGLALNVTTDLTPFNWIVPCGIRDRKVGNIKGLL
Arabi-LIP2  133:KCETGVWVGDRKIGAIGVRISSGITSHGLALNIDPDMKYFEHIVPCGIAD-K--EVTSLR
E. coli     125:D-APGVYVGEKKICSLGLRIRRGCSFHGLALNVNMDLSPFLRINPCGYAGMEMAKISQWK
                    ** **  *    * *       ******   *   *  * ***

Arabi-LIP2p 235:EDGEHGMVDDLRLIDIVHESLLKEFS-EAFQLQIEKQTVSDPNIL
Arabi-LIP2  190:RETDTLLPSEEVIHEQLVSCLAKAFSYDDVVWKEDPSLILDTQDKE
E. coli     184:PEATTNNIAPRLLENILALLNNPDFEYITA
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Fig. 1. Comparison of the amino acid sequences of lipoyltransferases. The deduced amino acid sequence of the lipoyltransferase encoded by *A. thaliana LIP2p* cDNA is compared to those of lipoyltransferases of *A. thaliana* LIP2 (mitochondrial form) [10] and *E. coli* LipB [13]. The amino acid residues conserved in all sequences are indicated by asterisks. Hyphens represent gaps to maximize the alignment of the sequences.

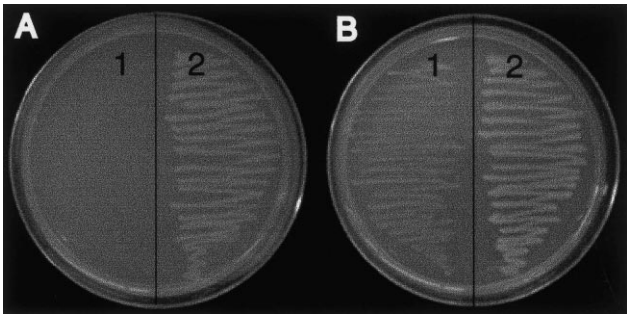

Fig. 2. Complementation of an *E. coli lplA lipB* mutant by expression of *A. thaliana LIP2p* cDNA. The *E. coli lplA lipB* strain (TM136), which is completely defective in lipoic acid attachment [13], was transformed with either plasmid pKK233-2 (control) or plasmid pKK-LIP2p. Colonies of each transformant with pKK233-2 (1) and pKK-LIP2p (2) were streaked onto a plate containing only glucose (plate A), or onto another plate containing glucose, acetate, and succinate (plate B). The plates were then incubated at 37°C for 2 days.

expressed in leaves. Therefore, only the *LIP2p* cDNA was further characterized in this study.

The deduced amino acid sequence of the LIP2p protein was compared with those of lipoyltransferases of *E. coli* LipB [13] and *A. thaliana* LIP2 [10] (Fig. 1). The amino acid sequence identities between the *A. thaliana* LIP2p and the lipoyltransferases of *E. coli* and *A. thaliana* LIP2 were 34.1% and 35.3%, respectively. These findings suggest that the *A. thaliana LIP2p* cDNA encodes a lipoyltransferase. As discussed below, we expect that part of the N-terminal region of *A. thaliana* LIP2p represents a transit peptide required for targeting the protein to the plastids.

3.2. Complementation of the E. coli lipB lplA mutant

To confirm that *A. thaliana LIP2p* cDNA encodes a lipoyltransferase, *LIP2p* cDNA containing an ORF was expressed in an *E. coli lplA lipB* mutant strain (TM136) lacking both lipoyltransferase and lipoate ligase [13]. The strain TM136 lacks the activity of all lipoate-dependent enzyme complexes due to the lack of a lipoate attachment [11]. Due to the lack of active PDH and OGDH complexes, growth of the strain TM136 requires acetate (to bypass the PDH deficiency), succinate (to bypass the OGDH deficiency), and glucose (as an energy source). When the strain TM136 was transformed with the vector plasmid pKK233-2 and plated on media A and B, growth of the transformants was observed only on the plate with medium B containing glucose, acetate, and succinate (Fig. 2). In contrast, the transformants with pKK-LIP2p carrying the *A. thaliana LIP2p* cDNA grew on medium A, which contained only glucose. Thus *A. thaliana LIP2p* cDNA complemented the acetate and succinate requirements and allowed for the growth of the *E. coli lplA lipB* strain on medium A. These findings indicate that PDH and OGDH activities were recovered in the presence of pKK-LIP2p by LIP2p-catalyzed lipoylation of the E2 subunits of the PDH and OGDH complexes, and thus *LIP2p* cDNA encodes a lipoyltransferase.

3.3. Expression and organization of A. thaliana LIP2p

Fig. 3A shows the genomic Southern blot analysis of the *A. thaliana LIP2p* gene using *LIP2p* cDNA as a probe. When the genomic DNA was digested with *Bam*HI, *Eco*RI, or *Eco*RV, a single hybridizing band was detected in all cases. This result indicates that this lipoyltransferase LIP2p is encoded by a single-copy gene on the *A. thaliana* genome.

The size of the transcript of the *LIP2p* gene was checked by Northern blot analysis using poly(A)$^+$ RNA prepared from the leaves of 4-week-old *A. thaliana* plants. A single hybridizing signal was detected at the position of 1060 nucleotides, which was close to the size of the cloned cDNA (Fig. 3B). This result suggests that the *LIP2p* gene is transcribed as an

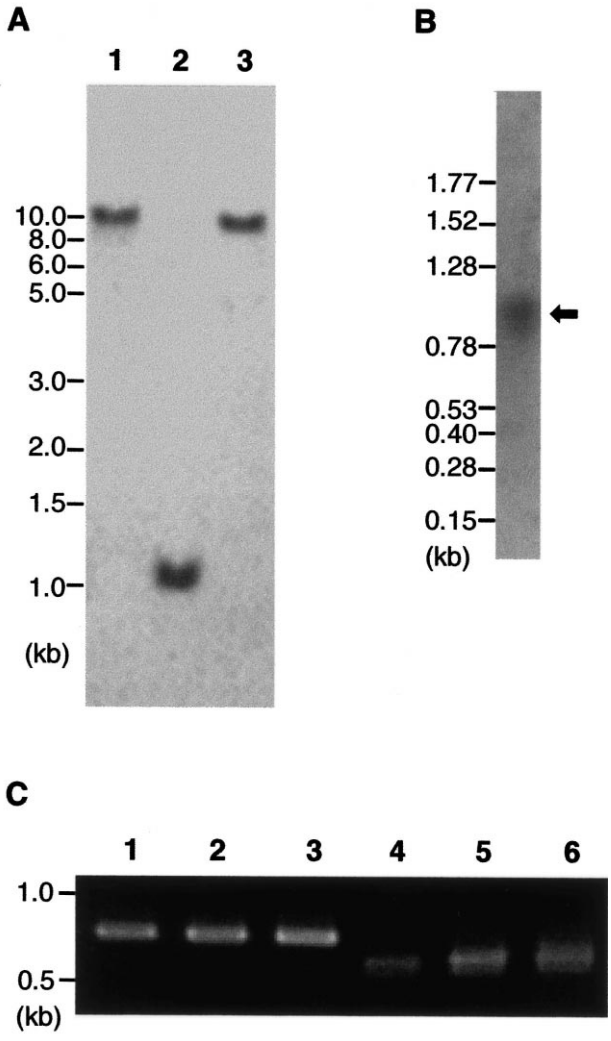

Fig. 3. Southern and Northern hybridization analyses and RT-PCR analysis of the *A. thaliana LIP2p* gene. A: Southern hybridization analysis. Genomic DNA was extracted from *A. thaliana* leaves and digested with *Bam*HI (lane 1), *Eco*RI (lane 2), or *Eco*RV (lane 3). 3 μg of DNA was applied to each lane. The 732 bp coding region of the *LIP2p* cDNA was used as a probe. The positions of DNA size markers are indicated on the left. B: Northern hybridization analysis. Poly(A)$^+$ RNA was prepared from *A. thaliana* leaves and used for the analysis. About 4 μg of poly(A)$^+$ RNA was applied to the gel. The probe was identical to that used for the Southern blot analysis. An arrow indicates the position of mRNA for *LIP2p*. The positions of RNA size markers are indicated on the left. C: RT-PCR analysis of organ-specific expression of the *LIP2p* gene. The expression of *LIP1* for lipoic acid synthase as a control (lanes 1–3) and that of *LIP2p* (lanes 4–6) were analyzed. Poly(A)$^+$ RNAs extracted from roots (lanes 1 and 4), leaves (lanes 2 and 5), and flowers (lanes 3 and 6) were used for the analysis.

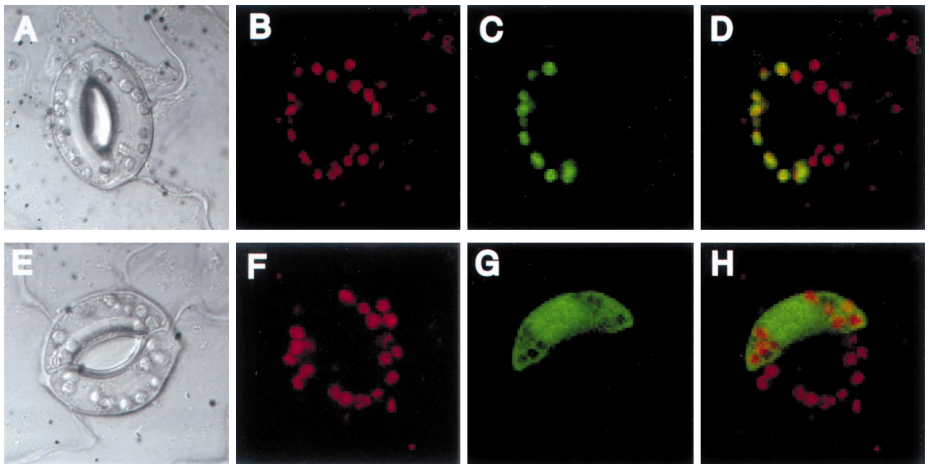

Fig. 4. Targeting of LIP2p::GFP into chloroplasts in guard cells of *V. faba*. pPre-LIP2p::GFP encoding a GFP fused with the N-terminal region of LIP2p, including a transit peptide, was introduced into guard cells (A–D). As a control, CaMV35SΩ-sGFP(S65T)-nos3′ was also introduced into guard cells (E–H). Images of GFP and chlorophyll fluorescence were taken with a laser scanning confocal microscope. A couple of guard cells are shown in the center of this figure. GFP or LIP2p::GFP is expressed in one of the guard cells. Confocal pictures (A and E), red fluorescence from chlorophylls (B and F), green fluorescence from GFP (C and G), and a combined image of both red fluorescence from chlorophylls and green fluorescence from GFP (D and H) are shown.

mRNA of 1060 nucleotides and the cloned cDNA is nearly full-length.

To estimate the level of expression of the *LIP2p* gene in organs, RT-PCR analysis was carried out using poly(A)$^+$ RNAs prepared from roots, leaves, and flowers. Fig. 3C shows the RT-PCR results. In all tested organs, a 585 bp DNA fragment corresponding to *LIP2p* cDNA was detected in relatively consistent levels (lanes 4–6); the levels of amplified cDNA in leaves and flowers appear to be slightly higher than that in roots. As a control, the level of expression of *LIP1* gene for lipoic acid synthase, which is expressed at the same level in all organs, was also checked by RT-PCR with the same RNA samples (lanes 1–3). In all tested organs, a 745 bp fragment, which corresponded to *LIP1* cDNA, was detected at the same level. These results suggest that the *LIP2p* gene is expressed in all tested organs and that the expression levels in leaves and flowers are slightly higher than that in roots.

3.4. Intracellular localization of lipoyltransferase in A. thaliana

As described above, we expected that the part of the *A. thaliana* LIP2p N-terminal extension relative to *E. coli* LipB is a transit peptide that is required for targeting the protein into plastids. To investigate the intracellular localization of LIP2p, we prepared a plasmid (pPre-LIP2p::GFP), which encodes a GFP fused to the LIP2p N-terminal extension, and bombarded it into the guard cells of *V. faba*. As shown in Fig. 4, the red fluorescence originated from chlorophylls was observed in all guard cells (Fig. 4B) and the green fluorescence was observed in guard cells that expressed GFP (Fig. 4C). The merged image combining with the red fluorescence from the chlorophyll and the green fluorescence from GFP was well overlapped and displayed a yellowish–green color (Fig. 4D). By contrast, in the guard cells bombarded with CaMV35SΩ-sGFP(S65T)-nos3′ [14], the fluorescence of GFP was observed in cytoplasm (Fig. 4G). These results demonstrate that LIP2p::GFP was targeted into chloroplasts, suggesting that the lipoyltransferase encoded by the *LIP2p* gene is located in chloroplasts.

4. Discussion

The transfer of lipoic acid to apoproteins is catalyzed by two classes of enzymes in *E. coli* [11,16]. Lipoyl ligase, encoded by a *lplA* gene, is an ATP-requiring enzyme that forms lipoyl-AMP as an intermediate for lipoyl transfer. In contrast, lipoyl-acyl carrier protein (ACP), N^{ε}-lysine lipoyltransferase, encoded by a *lipB* gene, transfers a lipoyl group from lipoyl-ACP to apoproteins. The LplA ligase functions in the utilization of exogenously supplied lipoic acid for protein modification [11,16], whereas the LipB lipoyltransferase transfers lipoic acid synthesized de novo [17]. In this study, we have identified an *A. thaliana LIP2p* cDNA for a lipoyltransferase located in plastids. Since the *A. thaliana* LIP2p is homologous to LipB and complements the *lipB* mutant of *E. coli*, it seems clear that *LIP2p* encodes a lipoyl-ACP-dependent lipoyltransferase. In plants, all of the known lipoic acid-containing proteins are located in the mitochondria [1–6] and plastids [12]. Thus, it is reasonable to assume that both mitochondria and plastids contain lipoyltransferase activity. In a previous study, we investigated the intracellular localization of lipoyltransferase in *A. thaliana* cells with an antibody against lipoyltransferase (LIP2) and found that LIP2 is present only in the mitochondria [10]. This finding raised the question of how the plastidial form of the E2 subunit of the PDH complex is lipoylated in plant cells. The findings obtained in the present study suggest that LIP2p is a plastid-localized lipoyltransferase that lipoylates the E2 subunit of the PDH complex in plastids.

We previously showed that pea mitochondrial extracts are capable of synthesizing fatty acids and that a major portion of the de novo synthesized fatty acids might be used for biosynthesis of lipoic acid [18]. These findings were further supported by Gueguen et al. [19]. Furthermore, we have recently demonstrated that lipoic acid synthase, responsible for the biosynthesis of lipoic acid, is localized in the mitochondria [20,21]. These findings, together with the identification of lipoyltransferase located in plastids, suggest that the lipoic acid synthesized in mitochondria has to be transported to plastids and is used for lipoylation of the E2 subunit of the

PDH complex in plastids. However, we have recently searched a database of the *A. thaliana* genome and found that there is another gene encoding a polypeptide that has a putative transit peptide for plastids that is similar to the lipoic acid synthase (LIP1) localized in the mitochondria [20]. It will be of interest to investigate if the gene encodes a plastid-localized lipoic acid synthase and to reveal whether or not lipoic acid is synthesized in plastids in addition to in the mitochondria. Cloning and characterization of the cDNA corresponding to the gene is now in progress.

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