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# The biosynthetic pathway for lipoic acid is present in plastids and mitochondria in *Arabidopsis thaliana*[1]

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**Abstract In eukaryotes, the biosynthetic pathway for lipoic acid is present in mitochondria. However, it has been hypothesized that, in plants, the biosynthetic pathway is present in plastids in addition to mitochondria. In this study, *Arabidopsis thaliana LIP1p* cDNA for a plastidial form of lipoic acid synthase has been identified. We show that it encodes a lipoic acid synthase by demonstrating its ability to complement an *Escherichia coli* mutant lacking lipoic acid synthase activity. We also show that LIP1p is targeted to chloroplasts. These findings suggest that the biosynthetic pathway for lipoic acid is present not only in mitochondria but also in plastids.** 

*Key words:* Lipoic acid; Lipoic acid synthase; 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 the decarboxylation of α-ketoacids [1–3] and in the glycine cleavage system [4–6]. These enzyme complexes play important roles in the central metabolism. It is known that five enzyme complexes, pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH) and branched chain 2-oxo acid dehydrogenase complexes, and the glycine cleavage system require lipoic acid as an essential cofactor [1–6]. In animal cells, these complexes are located in mitochondria [1–4]. In plant cells, the enzyme complexes are also located in mitochondria, although the PDH complex is present in mitochondria and also in plastids [7,8]. Lipoic acid is covalently bound to the dihydrolipoamide *S*-acetyltransferase (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 that are conserved in the E2 subunits and H protein of the complexes [9]. The attached lipoyl moiety functions as a carrier of reaction intermediates among the active sites of the subunits of the complexes [10,11].

In *Escherichia coli*, lipoic acid is synthesized from octanoic acid by the addition of two sulfur atoms into the octanoyl group bound to acyl carrier protein (ACP). This reaction is catalyzed by lipoic acid synthase encoded by *lipA* as demonstrated by Miller et al. [12]. In eukaryotic cells, it was reported that a major part of the fatty acids synthesized in the mitochondria might be used for the biosynthesis of lipoic acid [13,14], and that a lipoic acid synthase [15,16] and a lipoyltransferase [17] capable of transferring a lipoyl group from lipoyl-ACP to apoproteins are located in the mitochondria [14]. These findings suggested that lipoic acid is synthesized in the mitochondria and utilized for the lipoylation of the mitochondrial enzyme complexes that require a lipoic acid as a cofactor. However, in plant cells, the PDH complex that is one of the enzyme complexes that require lipoic acid as a cofactor is localized not only in mitochondria but also in plastids [7,8]. A lipoyltransferase responsible for the transfer of a lipoyl group from lipoyl-ACP to an apoprotein in the plastids was recently identified in *Arabidopsis thaliana* [18]. These findings raise the possibility that, in plants, lipoic acid is synthesized not only in mitochondria but also in plastids, or it is synthesized only in mitochondria, then transported into plastids and used for the lipoylation of the E2 subunit of the PDH complex in plastids.

In this study, we have identified an *A. thaliana* cDNA, designated *LIP1p*, for a lipoic acid synthase and shown that the LIP1p is localized in plastids. These findings provide the first evidence that the biosynthetic pathway for lipoic acid is present not only in mitochondria but also in plastids.

## 2. Materials and methods

### 2.1. Plant material

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

### 2.2. cDNA cloning and analysis

A fragment of the *A. thaliana LIP1p* 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′-CATGCCATGGAGGATTCGTCGTCC-3′ (F2 primer) and 5′-AGCTCTTAGATCAGCCATTGC-3′, which anneal to *LIP1p* cDNA, were used for the PCR. The 5′-terminal region of *A. thaliana LIP1p* cDNA was amplified by the

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[1] The nucleotide sequence data of the *Arabidopsis thaliana LIP1p* cDNA for a lipoic acid synthase located in plastids was deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB073745.

*Abbreviations:* ACP, acyl carrier protein; GFP, green fluorescent protein; PDH, pyruvate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction

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5′-rapid amplification of the cDNA ends method (5′-Full RACE Core Set; Takara, Shiga, Japan). The 3′-terminal region of *LIP1p* was also amplified by PCR with λ 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 (1371 bp) of *A. thaliana LIP1p* cDNA was obtained.

DNA sequence reactions were performed using a DNA sequencing kit (Applied Biosystems, Tokyo, Japan). DNA was sequenced using a DNA sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystems). Double-stranded DNAs were used as templates, and the sequences of both strands were determined. Nucleotide and deduced amino acid sequences were analyzed with GENETYX-MAC Software (Software Development, Tokyo, Japan).

### 2.3. Nucleic acid extraction and analyses

Poly(A)$^+$ RNAs used for Northern blot analysis were extracted from leaves of 2-week-old *A. thaliana* plants by an mRNA extraction kit (mRNA purification kit; Pharmacia Biotech). The extracted poly(A)$^+$ RNAs from leaves were separated by electrophoresis in an 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). The total RNAs used for reverse transcription (RT)-PCR analysis were prepared from the leaves, roots and flowers of 4-week-old *A. thaliana* plants by an RNA extraction kit (RNeasy plant kit, Qiagen, Chatsworth, CA, USA). RT-PCR was performed with a primer set, 5′-CATGCCATGGGGATGCATCATTGCTCAATTACGAAAC-3′ and 5′-AACTGCAGGTTATTAAGACAAGGATTTAGAGTAGC-3′ (R3 primer). As a control, the expression of the *tubA* gene for α-tubulin was also checked with the same RNA preparations using another primer set, 5′-CTACTGAGAGAAGATGCGAG-3′ and 5′-CAACATCTCCTCGGTACATC-3′.

### 2.4. Functional complementation of an E. coli lipA mutant strain

The coding region of *LIP1p* cDNA was amplified by PCR with a primer set, F2 primer and R3 primer. The nucleotide sequences 5′-CATGCCATGG-3′ and 5′-AACTGCAG-3′ including an *Nco*I site and a *Pst*I site, respectively, were added to the 5′ end of each primer. The PCR product was digested with *Nco*I and *Pst*I, and ligated into the *Nco*I–*Pst*I site of expression vector pKK233-2 (Clontech, Palo Alto, CA, USA) to give the desired in-frame product. This plasmid was designated pKK-LIP1p and was used to transform the *E. coli lipA* strain KER176 [19]. Complementation of the growth of the transformants was checked as described previously [15,16]. Transformants of strain KER176 with the vector (pKK233-2) and pLIP1-Δ18 [15] were used as negative and positive controls, respectively, in the complementation experiments.

### 2.5. Intracellular localization of LIP1p protein

A part of *LIP1p* cDNA encoding the N-terminal region of LIP1p including a putative transit peptide was amplified by PCR with a primer set, 5′-ACGCGTCGACATGCATCATTGCTCAATTACG-3′ and 5′-CATGCCATGGCTCCTCCTGGGTAAGGCT-3′. The nucleotide sequences 5′-ACGCGTCGAC-3′ and 5′-CATGCCATGGC-3′ including a *Sal*I site and a *Nco*I site, respectively, were added to the 5′ end of each primer. The 261 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′ [20] in order to form the expression construct for LIP1p transit fused to green fluorescent protein (GFP). As a control, pLIP1-GFP encoding a GFP fused to the N-terminal region of LIP1 [15] including a transit peptide for mitochondria was also constructed. A part of the cDNA encoding the N-terminal region of LIP1 was amplified by PCR with a primer set, 5′-ACGCGTCGACATGC-ATCCGCGCTCCGC-3′ and 5′-CATGCCATGGGAGATTGCGT-TACGGTG-3′. The sequences 5′-ACGCGTCGAC-3′ and 5′-CA-TGCCATGG-3′ including a *Sal*I site and a *Nco*I site, respectively, were added to the 5′ end of each primer. The 120 bp PCR product was digested with *Sal*I and *Nco*I, and ligated into the same site of CaMV35SΩ-sGFP(S65T)-nos3′. The obtained plasmids, pLIP1p-GFP and pLIP1-GFP, were 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 scanning microscope (LSM410, Carl Zeiss). Excitation wavelengths were set at 488 nm for GFP and at 543 nm for chlorophyll. Fluorescent images were collected through the FITC and TRITC filters for fluorescence from GFP and from chlorophyll, respectively. Incorporated images were processed using Adobe Photoshop software.

## 3. Results

### 3.1. Identification of the cDNA encoding a plastidial form of lipoic acid synthase

A BLAST search [21] of the GenBank database using the amino acid sequence of a mitochondrial form of *A. thaliana* lipoic acid synthase (LIP1) detected a gene of *A. thaliana* encoding a homologous polypeptide. The gene was present on chromosome 5 (accession number AL392174). The nucleotide sequence of the gene was used to synthesize the primers, and the *LIP1p* cDNA corresponding to the gene was amplified by PCR. The cloned *LIP1p* cDNA contained an open reading frame of 1182 bp encoding a polypeptide of 394 amino acids (molecular mass, 43 621 Da).

The deduced amino acid sequence of the LIP1p protein was compared with the sequences of the lipoic acid synthases of *E. coli* LipA [19], yeast LIP5 [22], mouse mLIP1 [16] and *A. thaliana* LIP1 [15] (Fig. 1). It appears that the amino acid sequence of *A. thaliana* LIP1p is homologous to those of the lipoic acid synthases of the organisms. The amino acid sequence identities between the *A. thaliana* LIP1p and the lipoic acid synthases of *E. coli*, yeast, mouse and *A. thaliana* LIP1 were 42%, 51%, 55% and 52%, respectively. These findings suggest that the *A. thaliana LIP1p* cDNA encodes a lipoic acid synthase. A comparison of the N-terminal region of the sequences clearly indicated that *A. thaliana* LIP1p contains a 60 residue extension relative to the lipoic acid synthase of *E. coli*. The amino acid sequence of this region has some characteristics in common with transit peptides that target into chloroplasts [23]. These findings suggest that this N-terminal region of LIP1p functions as a transit peptide required for targeting into chloroplasts and that LIP1p is localized in chloroplasts.

### 3.2. Complementation of the E. coli lipA mutant

To confirm that *LIP1p* cDNA encodes a lipoic acid synthase, it was expressed in an *E. coli lipA* mutant defective in lipoic acid synthase [19]. As shown in Fig. 2, when the *E. coli* mutant was transformed with pKK233-2 (control vector) and plated on lipoic acid-free medium, no growth of the transformant was observed, although the transformant was able to grow on a plate containing lipoic acid. In contrast, the transformant with the plasmid (pKK-LIP1p) carrying the *LIP1p* cDNA was able to grow on a plate without lipoic acid nearly as well as on a plate containing lipoic acid. The transformant with pLIP1-Δ18 used as a positive control was also able to grow on a plate without lipoic acid. These findings clearly demonstrate that *A. thaliana LIP1p* cDNA functionally complements the *E. coli lipA* mutant and that *LIP1p* cDNA encodes a lipoic acid synthase.

### 3.3. Expression and organization of A. thaliana LIP1p

The size of the transcript of the *LIP1p* gene was checked by Northern blot analysis using poly(A)$^+$ RNA prepared from the leaves of 2-week-old *A. thaliana* plants. A single hybridizing signal was detected at the position of about 1400 nucleotides, which was close to the size of the cloned cDNA (Fig.

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LIP1p        1:                          MMHHCSITKPTFSISISTQKLHHHSSKFLNLGFRIRC
LIP5         1: MYRRSVGVLFVGRNTRWISSTIRCGTSATRPIRSNALNTDSDNASVRVPVGNSTEVENAT
mLIP1        1:                                                MALRCWDTARSLGSR
LIP1         1:                                                  MHSRSALLYRFLR

LIP1p       38: ESGDVSSPLRTKAVSLSSEMEDSSSLKKSLMELEGKKSEPYPGGMPKMGPFTGRDPNVKK
LipA         1:                          MSKPIVMERGVKYRDADKMALIPVKNVATEREALVRK
LIP5        61: SQLTGTSGKRRKGNRKRITEFKDALNLGPSFADFVSGKASKMILDPLEKARQNTEEAKKL
mLIP1       16: IFGRYAFTVRALSSLPDKKKEFLHNGPDLQDFVSGDLADKSTWDEYKGNLKRQKGERLRL
LIP1        14: PASRCFSSSSAVTPVTVTQSPKSLEALRARLANESPSLTDFIHGDTYSVEVGTKKKPLPK

LIP1p       98: PAWLRQKAPQG-ERFQEVKESLSRLNLNTVCEEAQCPNIGECWNGGGDGVATATIMVLGD
LipA        38: PEWMKIKLPADSTRIQGIKAAMRKNGLHSVCEEASCPNLAECFNHG-----TATFMILGA
LIP5       121: PRWLKVPIPKG-TNYHKLKGDVKELGLSTVCEEARCPNIGECWGGKDKSKATATIMLLGD
mLIP1       76: PPWLKTKIPMG-KNYNKLKNTLRNLSLHTVCEEARCPNIGECWGGGEYATATATIMLMGD
LIP1        74: PKWMKESIPGG-ERYVQIKKKLRDLKLHTVCEEAKCPNLGECWSGGETGTATATIMILGD
                * *     *         *       *  ***** ***  **         *** *  *

LIP1p      157: TCTRGCRFCAVKTSRNPPPPDPMEPENTAKAIASWGVDYIVITSVDRDDIPDGGSGHFAQ
LipA        93: ICTRRCPFCDVAHGR-PVAPDANEPVKLAQTIADMALRYVVITSVDRDDLRDGGAQHFAD
LIP5       180: TCTRGCRFCSVKTNRTPSKPDPMEPENTAEAIKRWGLGYVVLTTVDRDDLVDGGANHLAE
mLIP1      135: TCTRGCRFCSVKTARNPPPLDANEPDNTAKAIAEWGLDYVVLTSVDRDDVADGGAEHIAK
LIP1       133: TCTRGCRFCNVKTSRTPPPPDPNEPNNVAEAIASWGVDYVVITSVDRDDLPDQGSGHFAE
                 *** * ** *   * *   *  **   *  *      * * * *****  * *  * *

LIP1p      217: TVKAMKRHKPDIMIECLTSDFRGDLE-AVDTLVHSGLDVFAHNVETVKRLQRLVRDPRAG
LipA       152: CITAIREKSPQIKIETLVPDFRGRMDRALDILTATPPDVFNHNLENVPRIYRQVR-PGAD
LIP5       240: TVRKIKQKAPNTLVETLSGDFRGDLK-MVDIMAQCGLDVYAHNLETVESLTPHVRDRRAT
mLIP1      195: TVSCLKERNPKILVECLTPDFRGDLR-AVEKVALSGLDVYAHNVETVPELQRKVRDPRAN
LIP1       193: TVQRLKFLKPEMLIEALVPDFRGDGG-CVEKVSKSGLDVLAHNIETVEELQSFVRDHRAN
                        *    * *  ****             **  ** * *     **   *

LIP1p      276: YEQSMSVLKHAKISKPG-MITKTSIMLGLGETDEELKEAMADLRAIDVDILTLGQYLQPT
LipA       211: YNWSLKLLERFKEAHPE-IPTKSGLMVGLGETNEEIIEVMRDLRRHGVTMLTLGQYLQPS
LIP5       299: YRQSLSVLERAKATVPS-LITKTSIMLGLGETDEQITQTLKDLRNIQCDVVTFGQYMRPT
mLIP1      254: FDQSLRVLRHAKEVQPD-VVSKTSIMLGLGETDEQVYATLKALRAADVDCLTLGQYMQPT
LIP1       252: FKQSLDVLRMAKEYAPAGTLTKTSVMLGCGETPDQVVKTMEKVRAAGVDVMTFGQYMRPS
                   *   *   *   *     *   * * ***           *       * ***  *

LIP1p      335: PLHLTVKEYVTPEKFDFWKTYGESIGFRYVASGPLVRSSYRAGELFVKTMVKESYSKSLS
LipA       270: RHHLPVQRYVSPDEFDEMKAEALAMGFTHAACGPFVRSSYHADLQAKGMEVK
LIP5       358: KRHMKVVEYVKPEKFDYWKERALEMGFLYCASGPLVRSSYKAGEAFIENVLKKRNMK
mLIP1      313: KRHLKVEEYVTPEKFKYWEKVGNELGFLYTASGPLVRSSYKAGEFFLKNLVARRKTKASK
LIP1       312: KRHMPVAEYVTPDAFERYRLLGMEMGFRYVASGPMVRSSYKAGEYYIKSMIEADRVASPS
                  *  *  ** *  *          **   * ** ***** *

mLIP1      373: V
LIP1       372: TSP
```

Fig. 1. Comparison of the amino acid sequences of lipoic acid synthases. The deduced amino acid sequence of the polypeptide encoded by *A. thaliana LIP1p* cDNA is compared to those of the lipoic acid synthases of *E. coli* LipA [19], yeast LIP5 [22], mouse mLIP1 [16] and *A. thaliana* LIP1 (mitochondrial form) [15]. The amino acid residues conserved in all sequences are indicated by asterisks. Hyphens represent gaps to maximize the alignment of the sequences.

3A). This result suggests that the *LIP1p* gene is transcribed as mRNA of about 1400 nucleotides and that the cloned cDNA is nearly full-length.

To estimate the level of expression of the *LIP1p* gene in various organs, RT-PCR analysis was carried out using total RNAs prepared from roots, leaves, and flowers. Fig. 3B shows the RT-PCR results. In all of the tested organs, a 1206 bp DNA fragment corresponding to *LIP1p* cDNA was detected at the same level (lanes 4–6). As a control, the level of expression of the *tubA* gene for α-tubulin, which is expressed at the same level in all organs, was also checked by RT-PCR using the same RNA samples (lanes 1–3). In all of the tested organs, a 985 bp fragment, which corresponded to *tubA* cDNA, was detected at the same level. These results suggest that the *LIP1p* gene is expressed in all of the tested organs at the same level.

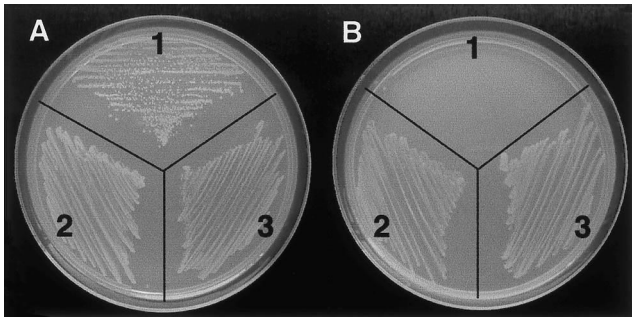


Fig. 2. Complementation of an *E. coli lipA* mutant by the expression of *A. thaliana LIP1p* cDNA. The *E. coli lipA* strain (KER176), which is completely deficient in lipoic acid synthase activity [19], was transformed with pKK233-2 (negative control), pLIP1-Δ18 (positive control) [15] or pKK-LIP1p. Colonies of each transformant with pKK233-2 (1), pLIP1-Δ18 (2) and pKK-LIP1p (3) were streaked onto a plate containing acetate and succinate (plate B), or onto another plate containing acetate, succinate and lipoic acid (plate A). The plates were then incubated at 37°C for 2 days.

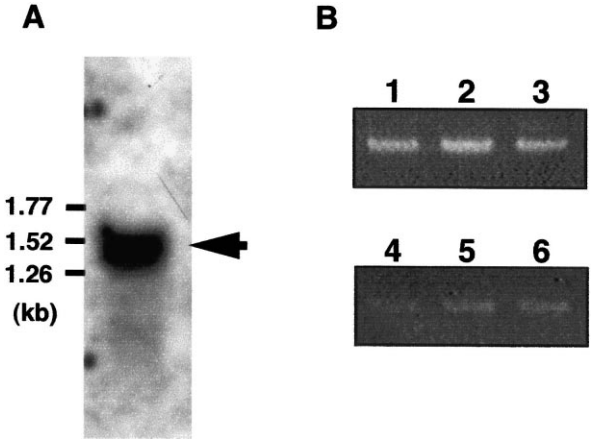


Fig. 3. Northern hybridization analysis and RT-PCR analysis of the *A. thaliana LIP1p* gene. A: Northern hybridization analysis. Poly-(A)$^+$ RNA was prepared from *A. thaliana* leaves and used for the analysis. About 2.5 μg of poly(A)$^+$ RNA was applied to the gel. The 1088 bp coding region of the *LIP1p* cDNA was used as a probe. An arrow indicates the position of mRNA for *LIP1p*. The positions of RNA size markers are indicated on the left. B: RT-PCR analysis of organ-specific expression of the *LIP1p* gene. The expression of *tubA* for α-tubulin as a control (lanes 1–3) and that of *LIP1p* (lanes 4–6) was analyzed. Total RNAs extracted from leaves (lanes 1 and 4), roots (lanes 2 and 5), and flowers (lanes 3 and 6) were used for the analysis.

### 3.4. Intracellular localization of lipoic acid synthase

As described above, we expected that the part of the *A. thaliana* LIP1p N-terminal extension relative to *E. coli* LipA is a transit peptide that is required for targeting the protein into chloroplasts. To investigate the intracellular localization of LIP1p, we prepared a plasmid (pLIP1p-GFP), which encodes a GFP fused to the LIP1p N-terminal extension, and bombarded the guard cells of *V. faba* with it. As shown in Fig. 4, the red fluorescence that originates from chlorophylls was observed in all guard cells (panel B), and the green fluorescence was observed in guard cells that expressed GFP (panel C). In the merged image that combined the red fluorescence from the chlorophyll and the green fluorescence from GFP, the two areas of fluorescence overlapped well and displayed a yellowish-green color (panel D). By contrast, in the guard cells bombarded with CaMV35SΩ-sGFP(S65T)-nos3′ [20], the fluorescence of GFP was observed in the cytoplasm (panel G). In the case of pLIP1-GFP (panel K), the green fluorescence was observed in the organelles, which are clearly distinct from the chloroplasts. Since LIP1 is localized in mitochondria as demonstrated previously [15], the organelles where the green fluorescence was observed must be mitochondria. These results demonstrate that LIP1p-GFP was targeted into chloroplasts, suggesting that the lipoic acid synthase encoded by the *LIP1p* gene is located in chloroplasts.

## 4. Discussion

In the present study we have cloned and characterized an *A. thaliana LIP1p* cDNA for lipoic acid synthase located in plastids. The cloned *LIP1p* functionally complemented the *E. coli* mutant that lacked lipoic acid synthase (LipA) activity, and the LIP1p was targeted into chloroplasts. These findings demonstrate that *A. thaliana LIP1p* encodes a lipoic acid synthase that is located in plastids and has the same activity as LipA. It was recently demonstrated that the *E. coli* LipA is capable of synthesizing lipoic acid by introducing sulfur atoms into an octanoyl group bound to ACP [12]. Since octanoyl-ACP is synthesized as an intermediate of fatty acid synthesis

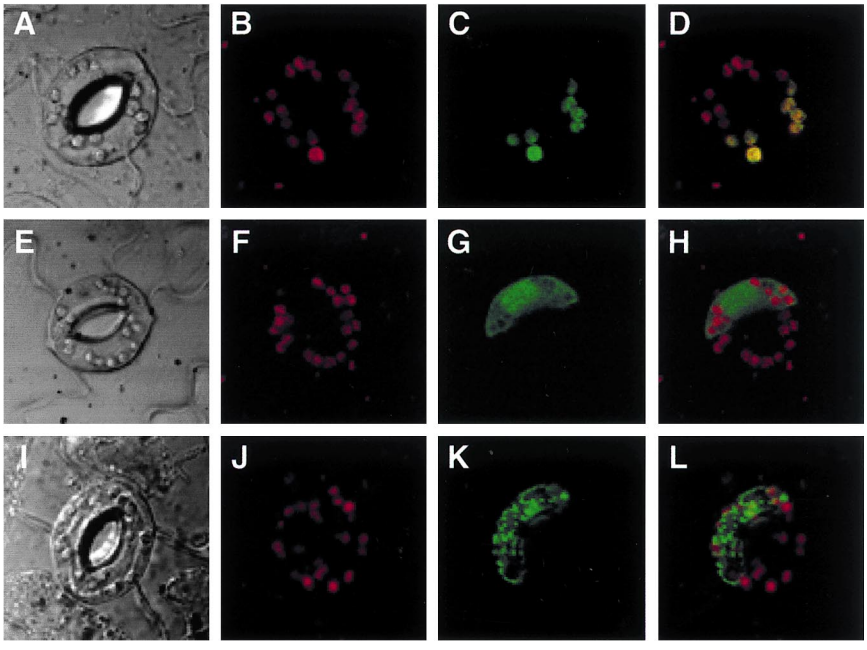


Fig. 4. Targeting of LIP1p-GFP into chloroplasts in guard cells of *V. faba*. pLIP1p-GFP encoding a GFP fused with the N-terminal region of LIP1p, including a transit peptide, was introduced into guard cells (A–D). As controls, CaMV35SΩ-sGFP(S65T)-nos3′ (E–H) and pLIP1-GFP (I–L) were also introduced into guard cells. Images of GFP and chlorophyll fluorescence were taken with a laser scanning confocal microscope. A pair of guard cells is shown in the center of these figures. GFP, LIP1-GFP or LIP1p-GFP is expressed in one of the guard cells. Confocal pictures (A, E and I), red fluorescence from chlorophylls (B, F and J), green fluorescence from GFP (C, G and K), and a combined image of both the red fluorescence from chlorophylls and green fluorescence from GFP (D, H and L) are shown.

in plastids [24], it is thus possible that lipoic acid is synthesized in plastids by the action of LIP1p; namely, the biosynthesis of lipoic acid is directly linked to the fatty acid biosynthesis in plastids. We have recently identified an *Arabidopsis* cDNA for lipoyltransferase located in plastids that transfers a lipoyl group from lipoyl-ACP to an apoprotein [18]. In plastids, the E2 subunit of the PDH complex is only known as a lipoic acid binding protein [8]. It is thus reasonable to assume that the synthesized lipoic acid in plastids is used for lipoylation of the E2 subunit of the plastidial PDH complex. These findings, together with those obtained in this study, clearly demonstrate that, in plant cells, a full set of enzymes involved in the biosynthesis of lipoic acid and the transfer of lipoic acid to a lipoic acid-dependent enzyme is present in plastids in addition to mitochondria.

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