Identification of an *Arabidopsis thaliana* gene for cardiolipin synthase located in mitochondria

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Abstract Cardiolipin (CL) is an anionic phospholipid with a dimeric structure. In eukaryotes, it is primarily localized in the inner membranes of mitochondria. Although the biosynthetic pathway of CL is well known, the gene for CL synthase has not been identified in any higher organisms. In this study, the *CLS* gene for a CL synthase has been identified in a higher plant, *Arabidopsis thaliana*. We have shown that the *CLS* gene encodes a CL synthase by demonstrating its ability to catalyze the reaction of CL synthesis from CDP-diacylglycerol and phosphatidylglycerol, and that CLS is targeted into mitochondria. These findings demonstrate that CLS is a CL synthase located in mitochondria.

Keywords: Cardiolipin; Cardiolipin synthase; CDP-alcohol phosphotransferase motif; *CLS* gene; Mitochondria; *Arabidopsis thaliana*

1. Introduction

Cardiolipin (CL) is a unique phospholipid with dimeric structure, carrying four acyl groups and two negative charges [1]. It is widely distributed in various prokaryotes and mitochondria of eukaryotes as a membrane phospholipid [2,3]. CL is synthesized from phosphatidic acid (PA), which is a common intermediate in the biosynthesis of phospholipids by the following four sequential reactions [1]. The first step of the sequential reactions is the synthesis of CDP-diacylglycerol (CDP-DG) from PA and CTP by CDP-DG synthase. CDP-DG is then converted with glycerol 3-phosphate to phosphatidylglycerolphosphate (PGP) by PGP synthase. PGP is further dephosphorylated to phosphatidylglycerol (PG) by PGP phosphatase. The last step of the sequential reactions, which is specific for CL biosynthesis, is the synthesis of CL catalyzed by CL synthase. CL synthases of bacteria use PG as a substrate to synthesize CL [4], whereas those of eukaryotes use PG and CDP-DG [5,6]. In eukaryotes, CL is synthesized in mitochondria and predominantly localized in the inner membranes of mitochondria [7]. Although the precise physiological role of CL is unknown, a growing body of evidence suggests that it plays an important role in mitochondria by interacting with several protein complexes in the inner membranes required for respiratory electron transport and oxidative phosphorylation [1].

The genes for bacterial (prokaryotic) CL synthases have been identified in *Escherichia coli* [8] and *Bacillus subtilis* [9], and a gene encoding a eukaryotic CL synthase has also been identified in the yeast, *Saccharomyces cerevisiae* [10,11]. Because the reaction catalyzed by CL synthase is specific for CL synthesis, the identified gene could be a useful target for manipulation of CL content. In *E. coli*, the *clsA* gene for CL synthase was disrupted and the obtained disruptant was used for investigation of CL function [8,12]. The results obtained with the mutant demonstrated that CL is not essential for growth of *E. coli*. A mutant of the yeast was also made and it was shown that the mutant is viable on fermentable and non-fermentable carbon sources at 16–30 °C, but cannot form colonies at elevated temperature, even on fermentable medium [10,13]. These findings suggest that CL is required for some essential cellular functions at elevated temperature, even under non-respiratory growth conditions. Identification of the genes for CL synthases and disruption of the identified gene have provided us with powerful molecular tools to understand the function of CL. However, the gene for CL synthase has not been identified in higher organisms and molecular genetical studies have not been reported.

In the present study, we have identified an *A. thaliana* gene, designated *CLS*, for a CL synthase located in mitochondria. This is the first identification of the gene for CL synthase in higher organisms.

2. Materials and methods

2.1. Plant materials

A. thaliana (Columbia ecotype) and broad bean (*Visia faba* cv. Ryosai Issun) were grown on vermiculite in trays under a continuous light condition (40 μmol/m^2/s) at 25 °C. Onions were obtained from the market.

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Abbreviations: CDP-DG, CDP-diacylglycerol; CDP-OH-P, CDP-alcohol phosphotransferase; Chl, chlorophyll; CL, cardiolipin; DG, diacylglycerol; GFP, green fluorescent protein; ORF, open-reading frame; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PI, phosphatidylinositol; PCR, polymerase chain reaction; PS, phosphatidylserine; RFP, red fluorescent protein; RT-PCR, reverse transcription-PCR

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2.2. Phylogenetic analysis and searching of A. thaliana gene for CL synthase

To collect protein sequences containing a CDP-alcohol phosphotransferase (CDP-OH-P) motif and to remove the motif parts from these sequences, we applied the criteria of the CDP-OH-P motif (PF01066.9) at Pfam (http://www.sanger.ac.uk/Software/Pfam/) [14] using the program HMMER (http://hmmer.wustl.edu/). The alignment and the unrooted phylogenetic tree of the sequences were constructed with the program CLUSTAL W (http://www.ddbj.nig.ac.jp/search/clustalw-j.html/) [15].

2.3. cDNA cloning

A fragment of the *CLS* cDNA corresponding to the *At4g04870* gene in the *A. thaliana* genome was amplified by reverse transcription-polymerase chain reaction (RT-PCR). The nucleotide sequence of the gene was used to synthesize the primers. The total RNAs used for the RT-PCR were prepared from the leaves of 3-week-old *A. thaliana* plants by an RNA extraction kit (RNeasy plant kit, Qiagen, Chatsworth, CA) and used as templates. RT-PCR was performed with a primer set, 5′-CATGCCATGGCGATTTACAGATCTCTAAGAAAG-3′ and 5′-CCCAAGCTTCTATGATCTCTTAATCATAGATATAGGTCT-3′. The amplified cDNA was subcloned into pCR2.1 (Original TA Cloning Kit; Invitrogen, Carlsbad, CA) and its nucleotide sequence was determined. DNA sequence reactions were performed using a DNA sequencing kit (Applied Biosystems, Tokyo). DNA was sequenced using a DNA sequencer (ABI PRISM 310 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).

2.4. Functional complementation of the E. coli mutant defective in CL synthase activity

The region of *CLS* cDNA encoding a protein with a deletion of 100 amino acid residues at the N-terminus was amplified by PCR with a primer set, 5′-CATGCCATGGCACGAATTAGAAGCAGAACTAGG-3′ and 5′-CCCAAGCTTCTATGATCTCTTAATCATAGATATAGGTCT-3′. The cDNA cloned by RT-PCR was used for the PCR as a template. The nucleotide sequences 5′-CATGCCATGG-3′ and 5′-CCCAAGCTT-3′, including an *Nco*I site and a *Hin*dIII site, respectively, were added to the 5′ end of each primer. The PCR product was digested with *Nco*I and *Hin*dIII, and ligated into the *Nco*I–*Hin*dIII site of expression vector pBAD/Myc-His B (pBAD) (Invitrogen, Carlsbad, CA) to give the desired in-frame product. This plasmid was designated as pBAD-*AtCLS* and was used to transform the *E. coli* SD9 [12]. The obtained transformants with pBAD-*AtCLS* were grown at 37 °C in LB medium. When the optical density at 600 nm reached 0.4, 0.002% arabinose was added to the growth medium to induce *CLS* expression and then incubated further for 2 h. After incubation, the cells were collected and used for lipid analysis. Lipids were extracted from the cells and analyzed as described previously [16]. Transformants of SD9 with pBAD were used as negative controls.

For the CL synthase assay, the transformants of SD9 with pBAD and pBAD-*AtCLS* were grown in 50 ml of LB medium supplemented with 20 mM $MgCl_2$ and 400 mM sucrose at 30 °C. When the optical density at 600 nm reached 0.8, 0.002% arabinose was added to the growth medium and further incubated at 30 °C for 1.5 h. After incubation, crude membrane fractions were prepared according to the method of Sankaran et al. [17] and used for the assay of CL synthase. The assay for CL synthase was performed as described by Frentzen and Griebau [5]. Membrane fractions corresponding to 400 μg protein, 50 μM [^{14}C]-labeled PG (62 000 cpm), and 50 μM CDP-DG (C-27; Doosan Serdary Research Laboratories, Tronto) were used for each assay in a total volume of 50 μl. In some cases, CDP-DG was omitted from the assay mixture. Reaction products were separated on a TLC plate (No. 5721; Merck, Darmstadt) with a developing solution of chloroform:methanol:glacial acetic acid (65:25:5, v/v) and radioactivity on the TLC plate was detected by autoradiography. [^{14}C]-Labeled PG used for the assay was prepared from *E. coli* cells incubated with [1-^{14}C]palmitic acid, as described by Shibuya et al. [12].

2.5. Intracellular localization of CLS protein

A portion of the *CLS* cDNA encoding an N-terminal region of CLS protein, including a presequence presumably required for targeting of the protein into mitochondria, was amplified by PCR with a primer set, 5′-ACGCGTCGACATGGCGATTTACAGATC-3′ and 5′-CATGCCATGGCCATTGATATCATATTC-3′. The nucleotide sequences 5′-ACGCGTCGAC-3′ and 5′-CATGCCATGGC-3′, including a *Sal*I site and an *Nco*I site, respectively, were added to the 5′ end of each primer. The amplified PCR product (471 bp) was digested with *Sal*I and *Nco*I and ligated into the *Sal*I–*Nco*I site of CaMV35SΩ-sGFP(S65T)-nos3′ [18] to form the expression construct for the CLS presequence fused to green fluorescent protein (GFP). pMt-RFP (kindly provided by S. Arimura and N. Tsutsumi, University of Tokyo) encoding a red fluorescent protein (RFP) fused to the N-terminal region of ATPase δ subunit, including a presequence for mitochondria [19], was also used as a mitochondrial control. The plasmids were introduced into leaves of broad bean and epidermal tissues of onion with a particle bombardment device (PDS-1000/He Biolistic Particle Delivery System, Bio Rad) according to the instructions from the manufacturer. The applied conditions were 1350 p.s.i. (onion) and 1550 p.s.i. (broad bean) of helium gas pressure, a distance of 9 cm from macrocarrier to sample, and a decompression vacuum of 28 in. Hg. Tungsten particles (1 μm) were used as a carrier of plasmid DNAs. The bombarded samples were incubated at room temperature in the dark overnight. In the case of broad bean, the bombarded epidermis was stripped from the leaves after the incubation. The epidermal cells were observed by a confocal Laser Scan Microscope (LSM510, Carl Zeiss). Excitation wavelengths were set at 488 nm for GFP and at 543 nm for chlorophyll (Chl) and RFP. Fluorescent images were collected through the FITC and Rhodamine filters for fluorescence from GFP and from Chl and RFP, respectively. A fluorescent microscope (BX60; Olympus, Tokyo) was also used for observation of epidermal cells with filter sets for GFP and Rhodamine. Incorporated images were processed using Adobe Photoshop software.

3. Results

3.1. Identification of the gene encoding a CL synthase

CL synthase of eukaryotes uses CDP-DG and PG as substrates to form CL [5,6]. Because, it is known that in general enzymes requiring CDP-DG or CDP-choline/CDP-ethanolamine as one of the substrates have a CDP-OH-P motif in their protein sequences [20] and the CL synthase of *S. cerevisiae* has this motif [21], genes encoding polypeptides containing the CDP-OH-P motif were searched using a profile hidden Markov model (http://hmmer.wustl.edu/) in databases to find the candidate genes for CL synthases. Phylogenetic analysis of the proteins encoded by the obtained genes clearly showed a distinct classification of their amino acid sequences into six groups (A–F group), as shown in Fig. 1. The gene for a eukaryotic type of CL synthase, which has been identified only in *S. cerevisiae* [10,11], was included in group A. The proteins including PGP synthases and those including phosphatidylinositol (PI) synthases were classified into groups B and C, respectively. Phosphatidylcholine (PC) synthases and phosphatidylethanolamine (PE) synthases in the Kennedy pathway use diacylglycerol (DG) and CDP-choline or DG and CDP-ethanolamine, respectively, as substrates [22]. The proteins including those enzymes were classified into group D. The enzymes requiring CDP-DG and serine or CDP-DG and choline for synthesis of phosphatidylserine (PS) and PC, respectively, were included in groups E and F. These findings clearly indicate that enzymes containing a CDP-OH-P motif in their protein sequences are clearly classified into the six functionally different groups. According to this analysis, it could be expected that those encoding proteins included in group A are the genes for CL synthases. To confirm this possibility, we chose the *A. thaliana* gene *At4g04870* encoding a protein (*At*CLS) included in the group A and investigated whether this gene encodes a CL synthase. As demonstrated below, the gene

was found to encode a CL synthase of *A. thaliana*. We therefore designated this gene as the *CLS* gene. The *CLS* gene is composed of 8 exons and 7 introns, and is present on chromosome 4 as a single copy gene. The cDNA corresponding to the *CLS* gene was searched in the full-length cDNA library [23]. The cDNA clone RAFL08-16-C14 (R11367) was found to correspond to the *CLS* gene and to contain an open-reading frame (ORF) of 1023 bp encoding a polypeptide of 341 amino acids (molecular mass, 38 042 Da).

As shown in Fig. 2, the deduced amino acid sequence of the CLS protein of *A. thaliana* was compared with the sequence of the CL synthase of yeast CRD1 [21] and those of proteins classified as members of group A in Fig. 1 (putative CL synthases). Although the N-terminal region of the sequences that could include the presequences required for targeting proteins into mitochondria is not conserved among the sequences, the remaining C-terminal region is conserved. The region conserved among proteins containing a CDP-OH-P motif was included in the conserved C-terminal region (underlined in Fig. 2). According to the hydropathy [24] and amphiphilicity [25] profiles (data not shown), six putative membrane-binding regions were predicted in the conserved C-terminal region and were divided into six domains, I to VI. In domains II and III, a sequence $D(X)_2DG(X)_2AR(X)_{8–9}G(X)_3D(X)_3D$, which was conserved in the sequences of proteins with CDP-OH-P motif, was found (shaded by black in Fig. 2). Extensions of the sequence that are not present in the proteins of other groups with the CDP-OH-P motif were found between domains IV and V of CL synthases (shaded by gray in Fig. 2). The amino acid sequences specific for CL synthases or CL synthases and PGP synthases that are indicated by # and =, respectively, were primarily found in domains IV, V, and VI. The amino acid sequence of the N-terminal region of CL synthases has some characteristics in common with presequences that target the proteins into mitochondria [26]. This finding is consistent with previously reported findings that CL synthase of eukaryotes is located in the inner membranes of mitochondria [27]. These findings suggest that the *A. thaliana CLS* encodes a CL synthase and that the N-terminal region of the CLS protein functions as a presequence required for targeting into mitochondria.

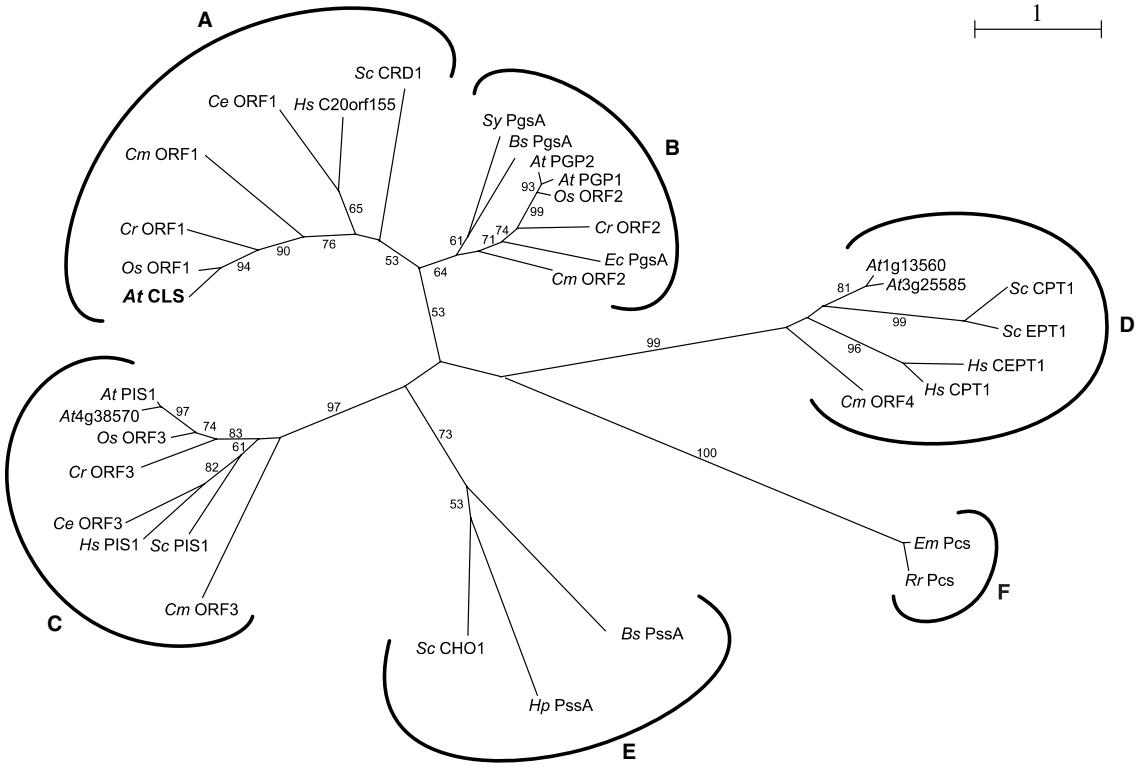

Fig. 1. Unrooted phylogenetic tree of proteins with a CDP-OH-P motif. Parts of protein sequences including a CDP-OH-P motif were used in this figure. The sequences used were *At* CLS (Accession No. NP_567273), *At* PGP1 (BAB39133) [28], *At*PGP2 (NP_191063) [28] and *At*PIS1 (CAA04172) [31] from *A. thaliana*, *Sc*CRD1 (NP_010139.1) [21], *Sc*PIS1 (NP_015438) [32], *Sc*CHO1 (NP_010943) [33], *Sc*CPT1 (NP_014269) [34] and *Sc*EPT1 (NP_011991) [35] from *S. cerevisiae*, *Hs*PIS1 (AF014807) [36], *Hs*CEPT1 (NP_006081) [37] and *Hs*CPT1 (NP_064629) [38] from *Homo sapiens*, *Sy*PgsA (NP_441788) [16] from *Synechocystis* sp. PCC6803, *Bs*PgsA (BAA46041) [39] and *Bs*PssA (P39823) [40] from *B. subtilis*, *Ec*PgsA (P06978) [41] from *E. coli*, *Em*Pcs (AAF27310) [42] from *Ensifer meliloti*, *Rr*Pcs (AF410774) [43] from *Rhizobium radiobacter* strain C58, *Hp*PssA (AAD08116) [44] from *Helicobacter pylori* 26695, and the sequence of ORF from the genome of *H. sapiens*, *Hs*C20orf155 (NP_061968), ORFs from the genome of *Caenorhabditis elegans*, *Ce*ORF1 (NP_497290) and *Ce*ORF3 (NP_496711), ORFs from the genome of *A. thaliana*, *At*PIS2 (CAB80521), *At*1g13560 (NP_973817) and *At*3g25585 (NP_850744), ORFs from the unfinished genome of *Oryza sativa* (japonica cultivar-group) at http://rgp.dna.affrc.go.jp/, *Os*ORF1 (NP_915744), ORF2 (AAN64500) and ORF3 (BAD07954), ORFs from the unfinished genome of *Chlamydomonas reinhardtii* at http://genome.jgi-psf.org/chlre2.home.html, *Cr*ORF1 (gene number C_2150006), *Cr*ORF2 (C_140036) and *Cr*ORF3 (C_260084), ORFs from the genome of *Cyanidioschyzon merolae* 10D [45] at http://merolae.biol.s.u-tokyo.ac.jp/, *Cm*ORF1 (Accession No. AP006496, gene number CMN196C), *Cm*ORF2 (AP006492, CMJ134C), *Cm*ORF3 (AP006495, CMM125C) and *Cm*ORF4 (AP006488, CMF133C). Percentage bootstrap values of 1000 replicates higher than 50% are shown for each clade. Distances between sequences are expressed as changes per amino acid residue.

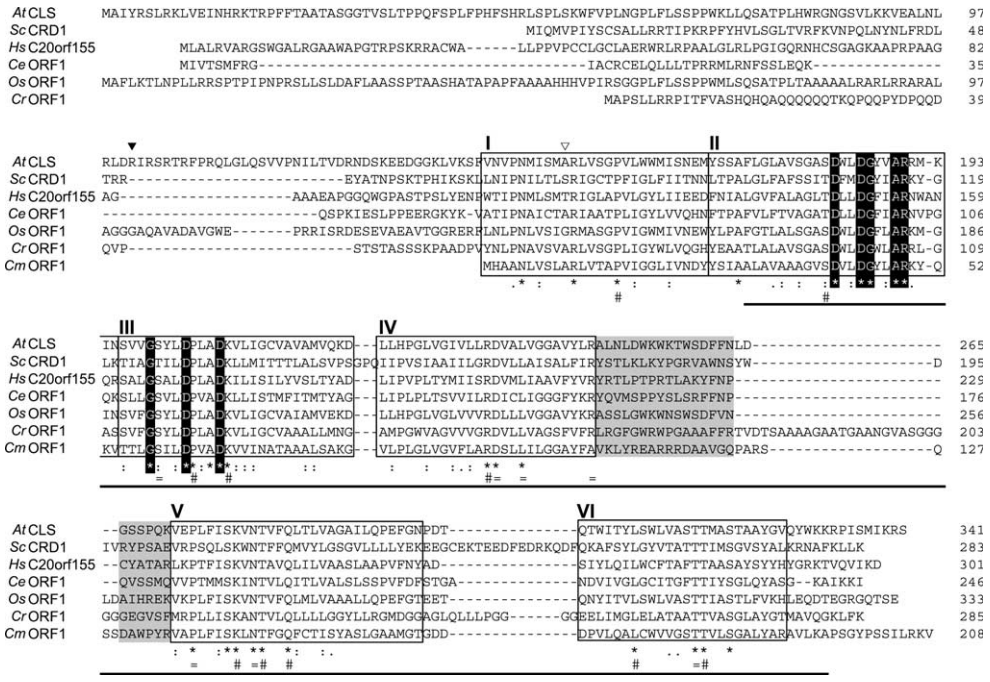

Fig. 2. Comparison of the amino acid sequences of proteins that belong to a group of putative CL synthases. The deduced amino acid sequence of the polypeptide encoded by *A. thaliana CLS* (*At*CLS) is compared to that of the CL synthase of *S. cerevisiae* CRD1 [21], and those of putative CL synthases of *H. sapiens* (*Hs*C20orf155, NP_061968), *C. elegans* (*Ce*ORF1, NP_497290), *O. sativa* (*Os*ORF1, NP_915744), *C. reinhardtii* (*Cr*ORF1, C_2150006), and *C. merolae* (*Cr*ORF1, AP006496, CMN196C). Membrane-binding regions are shown as boxes I to VI. Hyphens represent gaps to maximize alignment of the sequences. The amino acid residues conserved in all sequences are indicated by asterisks. The region conserved among proteins containing a CDP-OH-P motif (PF01066.9) is underlined. The amino acid residues having conserved substitutions and semi-conserved substitutions are marked by "." and ":", respectively, as defined in the program CLUSTAL W [15]. The amino acid residues conserved in all sequences of proteins with the CDP-OH-P motif are shaded in black. Extensions of the sequence that are not present in the proteins of other groups with CDP-OH-P motif are shaded in gray. # and = represent amino acid residues that are specific for CL synthases, and CL synthases and PGP synthases, respectively. The closed and open arrow heads represent N-terminal residues removed from *At*CLS in the complementation of the *E. coli* mutant and those used for the targeting of *At*CLS to mitochondria, respectively.

3.2. *Complementation of the* E. coli *mutant defective in CL synthase activity*

To confirm that the *CLS* gene of *A. thaliana* encodes a CL synthase, its cDNA encoding a protein with a deletion of 100 amino acid residues at the N-terminus was expressed under the arabinose-inducible promoter in an *E. coli* mutant (SD9) defective in CL synthase activity [12]. As shown in Fig. 3A, when the *E. coli* mutant was transformed with pBAD (control vector), CL content was below the detection limit like in the original mutant SD9 [12]. By contrast, the transformant with the plasmid (pBAD-*AtCLS*) carrying the *CLS* cDNA produced high amounts of CL (16%). The CL content in the transformant of the wild-type W3110 with pBAD-*AtCLS* was also slightly higher than that in the transformant of the wild type with pBAD. In addition, when the *CLS* cDNA of *A. thaliana* encoding a precursor form of CLS having a presequence was expressed in the mutant SD9, CL content was below the detection limit (data not shown), suggesting that the precursor form of CLS is not active in *E. coli* cells because of the presence of presequence.

Eukaryotic CL synthases use CDP-DG and PG for CL biosynthesis. To determine whether *A. thaliana* CLS also uses CDP-DG and PG as substrates, the enzyme assay for CL synthase was performed using crude membrane extracts prepared from transformants of the *E. coli* mutant with pBAD and pBAD-*AtCLS*. As shown in Fig. 3B, CL was not detected in the presence of PG (lane 1) or PG and CDP-DG (lane 2), with the crude membrane fractions prepared from the transformant with pBAD. By contrast, with the crude membrane fractions prepared from the transformant with pBAD-*AtCLS*, a significant amount of CL was detected in the presence of

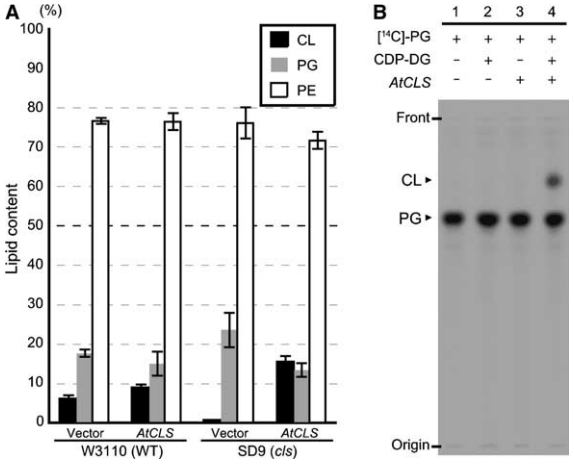

Fig. 3. Expression of *A. thaliana CLS* cDNA in the *E. coli* mutant induces production of CL. (A) Lipid composition of intact cells of the transformants of the wild type (W3110) and the mutant (SD9) defective in CL synthase activity [12] with pBAD (negative control) or pBAD-*AtCLS*. (B) Production of CL in the crude membrane fractions prepared from transformants of the mutant with pBAD (lanes 1 and 2) or pBAD-*AtCLS* (lanes 3 and 4). Crude membrane fractions were incubated with [^{14}C]PG in the absence (lanes 1 and 3) or presence of CDP-DG, and reaction products were analyzed on a TLC plate.

CDP-DG and PG (lane 4), but not in the absence of CDP-DG (lane 3). These findings clearly demonstrate that *A. thaliana CLS* cDNA functionally complements the *E. coli* mutant, and that *CLS* cDNA encodes a CL synthase that uses CDP-DG and PG as substrates.

3.3. Intracellular localization of CL synthase

It is known that CL synthase is located in the inner membranes of mitochondria [27]. We therefore predicted that the N-terminal region of the CLS protein includes a presequence required for targeting the protein into mitochondria. To investigate this possibility, we prepared a plasmid (pCLS-GFP) that encodes a GFP fused to the CLS N-terminal region (150 amino acid residues) and bombarded the epidermal tissues of onion with the plasmid to express the CLS-GFP (Fig. 4). When CLS-GFP was transiently expressed, the green fluorescence from GFP was observed (panel D), with the pattern of fluorescence being similar to that observed in cells expressing Mt-RFP (mitochondrial control, panel F), suggesting that CLS-GFP was targeted into mitochondria. By contrast, when CaMV35SΩ-sGFP(S65T)-nos3′ [18] was bombarded as a control, the fluorescence of GFP was observed in the cytoplasm (panel B). To confirm that CLS-GFP was targeted into mitochondria, CLS-GFP was coexpressed with Mt-RFP in epidermal cells of onion. As shown in panels I and H, the red and green fluorescence were observed in the same cells that expressed both CLS-GFP and Mt-RFP. In the merged image that combined the red fluorescence from RFP and the green fluorescence from GFP, both areas of fluorescence overlapped well and displayed a yellowish-green color (panel J). These results demonstrate that CLS-GFP was targeted into mitochondria. To further confirm that CLS-GFP was targeted into mitochondria, CLS-GFP and Mt-RFP were also coexpressed in epidermal cells of *Visia faba*. Panels L to N show a guard cell that expresses both CLS-GFP and Mt-RFP. In the cells in which green fluorescence from GFP was observed in mitochondria-like organelles (panel L), the red fluorescence from RFP and Chls was observed in two distinct organelles, mitochondria (a smaller-sized organelle) and chloroplasts (a larger-sized organelle, compared to chloroplasts in another guard cell) (panel M). In the merged image that combined the red fluorescence from RFP and Chls and the green fluorescence from GFP, the red fluorescence from RFP but not that from Chl overlapped well with green fluorescence from GFP and displayed an orange color (panel N). These results also demonstrate that CLS-GFP was targeted into mitochondria, suggesting that the CL synthase encoded by the *CLS* gene is located in mitochondria.

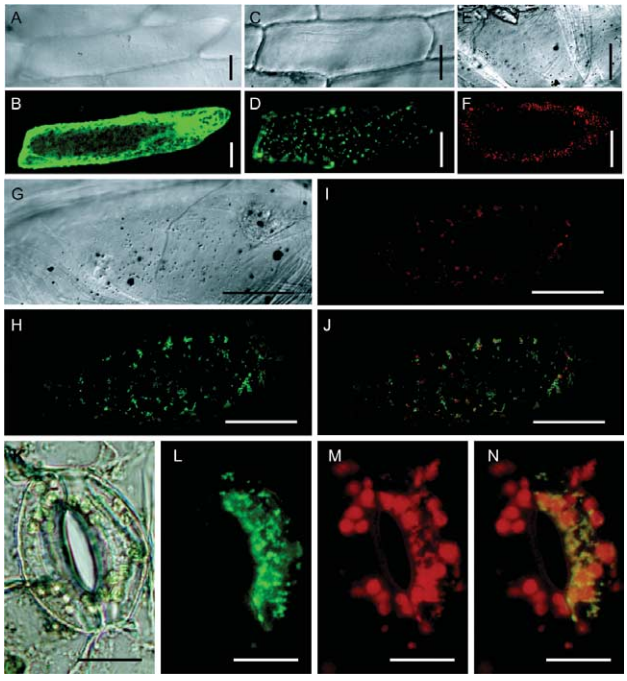

Fig. 4. Targeting of CLS-GFP into mitochondria in epidermal cells of onion and guard cells of broad bean. In panels A–F, pCLS-GFP (C and D) encoding a GFP fused with the N-terminal region of CLS and controls CaMV35SΩ-sGFP(S65T)-nos3′ (A and B) and pMt-RFP (E and F) were introduced into epidermal cells of onion. In panels G–J, both pCLS-GFP and pMT-RFP were introduced into epidermal cells of onion. Images of GFP and RFP fluorescence were taken with a laser-scanning confocal microscope, and an epidermal cell is shown in the center of the figures. Confocal pictures (A, C, E, and G), green fluorescence from GFP (B, D and H), red fluorescence from RFP (F and I), and a combined image of both the red fluorescence from RFP and the green fluorescence from GFP (J) are shown. In panels K–N, both pCLS-GFP and pMt-RFP were introduced into guard cells of broad bean. Images of GFP, RFP, and Chl fluorescence were taken with a fluorescent microscope, and a couple of guard cells are shown in the center of the figures. A picture of guard cells (K), green fluorescence from GFP (L), red fluorescence from RFP and Chls (M), and a combined image of both the green and red fluorescence from RFP and Chls (N) is shown. The scale bar represents 50 μm in A to J and 10 μm in K to N.

4. Discussion

The *CLS* gene identified in this study was previously designated as *PGP3* [28] because it encodes a polypeptide homologous to PGP synthases encoded by *PGP1* and *PGP2* genes [28–30]. However, in the present study the cDNA corresponding to this gene did not complement the *E. coli pgsA* mutant defective in PGP synthase activity (data not shown), and it was demonstrated that this gene encodes a CL synthase. We have therefore renamed it as *CLS*. According to the following findings, it was confirmed that *CLS* encodes a CL synthase located in mitochondria. (i) The protein encoded by the *CLS* gene is classified into a group of putative CL synthases in the phylogenetic analysis of proteins with a CDP-OH-P motif (group A in Fig. 1). (ii) Expression of *CLS* in the *E. coli* mutant defective in CL synthase activity induced an accumulation of CL (Fig. 3A). (iii) CL synthase activity was detected in the crude membrane fraction prepared from the transformant of the mutant with pBAD-*AtCLS* and the activity was dependent on CDP-DG (Fig. 3B). (iv) GFP fused to the N-terminal region of CLS was targeted into mitochondria (Fig. 4). Identification of the *A. thaliana CLS* gene for CL synthase will provide a powerful molecular tool to understand the function of CL in higher organisms.

Phylogenetic analysis of the protein with a CDP-OH-P motif indicated that their protein sequences are clearly classified into six functionally different groups (Fig. 1). Several proteins encoded by the genes of various organisms, including CLS of *A. thaliana*, were classified into group A. Although it remains to be proved, it is likely that all of the proteins belonging to this group are CL synthases. In other groups, several proteins encoded by genes whose functions have not been identified were

also found. In addition, our results suggest that these proteins have an enzymatic function expected for each group: eukaryotic CL synthases in group A, prokaryotic and eukaryotic PGP synthases in group B, PI synthases in group C, CDP-choline-dependent PC synthases or CDP-ethanolamine dependent PE synthases in group D, CDP-DG dependent PS synthases in group E, and CDP-DG-dependent PC synthases in group F. This result might be useful in predicting the enzymatic function of proteins with the CDP-OH-P motif, as we have identified *A. thaliana CLS* for CL synthase. It will be of interest to investigate if the proteins do indeed have the expected enzymatic function.

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References

[1] Schlame, M., Rua, D. and Greenberg, M.L. (2000) Prog. Lipid Res. 39, 257–288.
[2] Raetz, C.R.H. and Dowhan, W. (1990) J. Biol. Chem., 1235–1238.
[3] Schlame, M., Brody, S. and Hostetler, K.Y. (1993) Eur. J. Biochem. 212, 727–735.
[4] Hirschberg, C.B. and Kennedy, E.P. (1972) Proc. Natl. Acad. Sci. USA 69, 648–651.
[5] Frentzen, M. and Griebau, R. (1994) Plant Physiol. 106, 1527–1532.
[6] Hostetler, K.Y. and van den Bosch, H. (1972) Biochim. Biophys. Acta 260, 380–386.
[7] Hoch, F.L. (1992) Biochim. Biophys. Acta 1113, 71–133.
[8] Ohta, A., Obara, T., Asami, Y. and Shibuya, I. (1985) J. Bacteriol. 163, 506–514.
[9] Kawai, F., Shoda, M., Harashima, R., Sadaie, Y., Hara, H. and Matsumoto, K. (2004) J. Bacteriol. 186, 1475–1483.
[10] Jiang, F., Rizavi, H.S. and Greenberg, M.L. (1997) Mol. Microbiol. 26, 481–491.
[11] Chang, S.C., Heacock, P.N., Mileykovskaya, E., Voelker, D.R. and Dowhan, W. (1998) J. Biol. Chem. 273, 14933–14941.
[12] Shibuya, I., Miyazaki, C. and Ohta, A. (1985) J. Bacteriol. 161, 1086–1092.
[13] Jiang, F., Gu, Z., Granger, J.M. and Greenberg, M.L. (1999) Mol. Microbiol. 31, 373–379.
[14] Bateman, A., Coin, L., Durbin, R., Finn, R.D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E.L.L., Studholme, D.J., Yeats, C. and Eddy, S.R. (2004) Nucleic Acids Res. 32, D138–D141.
[15] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucleic Acids Res. 22, 4673–4680.
[16] Hagio, M., Gombos, Z., Várkonyi, Z., Masamoto, K., Sato, N., Tsuzuki, M. and Wada, H. (2000) Plant Physiol. 124, 795–804.
[17] Sankaran, K., Gupta, S.D. and Wu, H.C. (1995) Methods Enzymol. 250, 683–697.
[18] Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M. and Kobayashi, H. (1999) Plant J. 18, 455–463.
[19] Arimura, S. and Tsutsumi, N. (2002) Proc. Natl. Acad. Sci. USA 99, 5727–5731.
[20] Williams, J.G. and McMaster, C.R. (1998) J. Biol. Chem. 273, 13482–13487.
[21] Tuller, G., Hrastnik, C., Achleitner, G., Schiefthaler, U., Klein, F. and Daum, G. (1998) FEBS Lett. 421, 15–18.
[22] Carman, G.M. and Henry, S.A. (1999) Prog. Lipid Res. 38, 361–399.
[23] Yamada, K., Lim, J., Dale, J.M., Chen, H., Shinn, P., Palm, C.J., Southwick, A.M., Wu, H.C., Kim, C., Nguyen, M., Pham, P., Cheuk, R., Karlin-Newmann, G., Liu, S.X., Lam, B., Sakano, H., Wu, T., Yu, G., Miranda, M., Quach, H.L., Tripp, M., Chang, C.H., Lee, J.M., Toriumi, M., Chan, M.M.H., Tang, C.C., Onodera, C.S., Deng, J.M., Akiyama, K., Ansari, Y., Arakawa, T., Banh, J., Banno, F., Bowser, L., Brooks, S., Carninci, P., Chao, Q., Choy, N., Enju, A., Goldsmith, A.D., Gurjal, M., Hansen, N.F., Hayashizaki, Y., Johnson-Hopson, C., Hsuan, V.W., Iida, K., Karnes, M., Khan, S., Koesema, E., Ishida, J., Jiang, P.X., Jones, T., Kawai, J., Kamiya, A., Meyers, C., Nakajima, M., Narusaka, M., Seki, M., Sakurai, T., Satou, M., Tamse, R., Vaysberg, M., Wallender, E.K., Wong, C., Yamamura, Y., Yuan, S., Shinozaki, K., Davis, R.W., Theologis, A. and Ecker, J.R. (2003) Science 302, 842–846.
[24] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[25] Mitaku, S., Hirokawa, T. and Tsuji, T. (2002) Bioinformatics 18, 608–616.
[26] von Heijne, G. (1992) Genet. Engin. 14, 1–11.
[27] Schlame, M. and Hostetler, K.Y. (1997) Biochim. Biophys. Acta 1348, 207–213.
[28] Xu, C., Härtel, H., Wada, H., Hagio, M., Yu, B., Eakin, C. and Benning, C. (2002) Plant Physiol. 129, 594–604.
[29] Babiychuk, E., Müller, F., Eubel, H., Braun, H.P., Frentzen, M. and Kushnir, S. (2003) Plant J. 33, 899–909.
[30] Hagio, M., Sakurai, I., Sato, S., Kato, T., Tabata, S. and Wada, H. (2002) Plant Cell Physiol. 43, 1456–1464.
[31] Collin, S., Justin, A.M., Cantrel, C., Arondel, V. and Kader, J.C. (1999) Eur. J. Biochem. 262, 652–658.
[32] Nikawa, J. and Yamashita, S. (1984) Eur. J. Biochem. 143, 251–256.
[33] Nikawa, J., Tsukagoshi, Y., Kodaki, T. and Yamashita, S. (1987) Eur. J. Biochem. 167, 7–12.
[34] Hjelmstad, R.H. and Bell, R.M. (1987) J. Biol. Chem. 262, 3909–3917.
[35] Hjelmstad, R.H. and Bell, R.M. (1988) J. Biol. Chem. 263, 19748–19757.
[36] Lykidis, A., Jackson, P.D., Rock, C.O. and Jackowski, S. (1997) J. Biol. Chem. 272, 33402–33409.
[37] Henneberry, A.L. and McMaster, C.R. (1999) Biochem. J. 339, 291–298.
[38] Henneberry, A.L., Wistow, G. and McMaster, C.R. (2000) J. Biol. Chem. 275, 29808–29815.
[39] Kontinen, V.P. and Tokuda, H. (1995) FEBS Lett. 364, 157–160.
[40] Okada, M., Matsuzaki, H., Shibuya, I. and Matsumoto, K. (1994) J. Bacteriol. 176, 7456–7461.
[41] Ohta, A., Waggoner, K., Radominska-Pyrek, A. and Dowhan, W. (1981) J. Bacteriol. 147, 552–562.
[42] Sohlenkamp, C., de Rudder, K.E.E., Röhrs, V., López-Lara, I.M. and Geiger, O. (2000) J. Biol. Chem. 275, 18919–18925.
[43] Martinez-Morales, F., Schobert, M., Lopez-Lara, I.M. and Geiger, O. (2003) Microbiology 149, 3461–3471.
[44] Ge, Z. and Taylor, D.E. (1997) J. Bacteriol. 179, 4970–4976.
[45] Matsuzaki, M., Misumi, O., Shin-I, T., Maruyama, S., Takahara, M., Miyagishima, S.Y., Mori, T., Nishida, K., Yagisawa, F., Nishida, K., Yoshida, Y., Nishimura, Y., Nakao, S., Kobayashi, T., Momoyama, Y., Higashiyama, T., Minoda, A., Sano, M., Nomoto, H., Oishi, K., Hayashi, H., Ohta, F., Nishizaka, S., Haga, S., Miura, S., Morishita, T., Kabeya, Y., Terasawa, K., Suzuki, Y., Ishii, Y., Asakawa, S., Takano, H., Ohta, N., Kuroiwa, H., Tanaka, K., Shimizu, N., Sugano, S., Sato, N., Nozaki, H., Ogasawara, N., Kohara, Y. and Kuroiwa, T. (2004) Nature 428, 653–657.