Functional Specificity of Cardiolipin Synthase Revealed by the Identification of a Cardiolipin Synthase CrCLS1 in *Chlamydomonas reinhardtii*

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Phosphatidylglycerol (PG) and cardiolipin (CL) are two essential classes of phospholipid in plants and algae. Phosphatidylglycerophosphate synthase (PGPS) and cardiolipin synthase (CLS) involved in the biosynthesis of PG and CL belong to CDP-alcohol phosphotransferase and share overall amino acid sequence homology. However, it remains elusive whether PGPS and CLS are functionally distinct *in vivo*. Here, we report identification of a gene encoding CLS in *Chlamydomonas reinhardtii*, CrCLS1, and its functional compatibility. Whereas CrCLS1 did not complement the growth phenotype of a PGPS mutant of *Synechocystis* sp. PCC 6803, it rescued the temperature-sensitive growth phenotype, growth profile with different carbon sources, phospholipid composition and enzyme activity of Δcrd1, a CLS mutant of *Saccharomyces cerevisiae*. These results suggest that CrCLS1 encodes a functional CLS of *C. reinhardtii* as the first identified algal CLS, whose enzyme function is distinct from that of PGPSs from *C. reinhardtii*. Comparison of CDP-alcohol phosphotransferase motif between PGPS and CLS among different species revealed a possible additional motif that might define the substrate specificity of these closely related enzymes.

**Keywords:** CDP-alcohol phosphotransferase, cardiolipin, cardiolipin synthase, phosphatidylglycerol, PGPS, *Chlamydomonas reinhardtii*, *Synechocystis* sp. PCC 6803, *Saccharomyces cerevisiae*

**INTRODUCTION**

Functional specificity of an enzyme is crucial in keeping metabolic reactions in order. This largely relies on the substrate specificity defined by the catalytic motif. Thus, enzymes are often categorized into groups according to the existence of common catalytic motif(s). In phospholipid metabolism, a number of important reaction steps are catalyzed by CDP-alcohol phosphotransferases (Li-Beisson et al., 2013, 2015). These include CLS, PGPS, phosphatidylinositol (PI) synthase, phosphatidylserine (PS) synthase, phosphatidylcholine (PC)

**Abbreviations:** CDP, cytidine 5′-diphosphate; CL, cardiolipin; CLS, cardiolipin synthase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate; PGPP, phosphatidylglycero-phosphate phosphatase; PGPS, phosphatidylglycerophosphate synthase; PI, phosphatidylinositol; PS, phosphatidylserine.
synthase, and aminoalcohol phosphotransferase for the biosynthesis of CL, PG, PI, PS, PC, and PE, respectively. Because these are the major phospholipid classes found in diverse organisms from bacteria to mammals and seed plants, it can be stated that CDP-alcohol phosphotransferases are crucial in the entire phospholipid metabolism.

In plants and algae, PG is an indispensable phospholipid class in photosynthetic function (Hagio et al., 2000, 2002; Sato et al., 2000; Babychuk et al., 2003; Yu and Benning, 2003). Moreover, CL, which is an anionic phospholipid class widely distributed in different kingdom and found exclusively at the inner membrane of mitochondria (Lewis and McElhaney, 2009), has an essential role in mitochondrial function and thus plant growth (Katayama et al., 2004; Pineau et al., 2013). The biosynthesis of these lipid classes begins with the conversion of phosphatidic acid (PA) into CDP-diacylglycerol (CDP-DAG) by CDP-DAG synthase (CDS; Sato et al., 2000; Babiychuk et al., 2003; Yu and Benning, 2009), has an essential role in mitochondrial function and thus plant growth (Katayama et al., 2004; Pineau et al., 2013). The biosynthesis of these lipid classes begins with the conversion of phosphatidic acid (PA) into CDP-diacylglycerol (CDP-DAG) by CDP-DAG synthase (CDS; Sato et al., 2000; Hasler et al., 2010; Zhou et al., 2013). Next, PGPS converts CDP-DAG to phosphatidylglycerol phosphate (PGP), which is dephosphorylated by PGP phosphatase (PGPP) to produce PG (Muller and Frentzen, 2001; Hagio et al., 2000, 2002; Wu et al., 2006; Osman et al., 2010; Hung et al., 2015b). Furthermore, PG is converted to CL by CLS in mitochondria (Kadenbach et al., 1982; Jiang et al., 1997, 1999). Initially, 3 PGPSs were proposed in Arabidopsis thaliana (PGP1, PGP2, and PGP3) based on the amino acid sequence similarity (Xu et al., 2002). However, the third isoform (PGP3) was later shown not to be a functional PGPS but instead functions as CLS (Katayama et al., 2004). Subsequent gene knockout studies defined distinct in vivo function of CLS associated with mitochondrial function (Pineau et al., 2013). Thus, PGPS and CLS are functionally independent, although they are homologous and belong to the same CDP-alcohol phosphotransferase family in A. thaliana. Recently, we identified and characterized genes for PGPS of Chlamydomonas reinhardtii strain CC-503 (cw92 mt+) with the primers CH227 and CH228, and cloned into pENTR/D-TOPO. Then, to construct pCH178, the open reading frame (ORF) of CrCLS1 was amplified from pCH069 with the primers CH831 and CH832, and inserted into XbaI and EcoR1 sites of pCH078 (Hung et al., 2013). To construct pCH158, the open reading frame (ORF) of CrCLS1 was amplified from pCH069 with the primers CH831 and CH832, and inserted into XbaI and EcoR1 sites of pCH078 (Hung et al., 2013). To construct pCH158, the open reading frame (ORF) of CrCLS1 was amplified from pCH069 with the primers CH831 and CH832, and inserted into XbaI and EcoR1 sites of pCH078 (Hung et al., 2013).

MATERIALS AND METHODS

Strains
The strains produced in this work are listed in Supplementary Table S1.

Protein Sequence Analysis
The multiple alignment of protein sequences was performed by use of CLUSTALW. The mitochondrial targeting sequence was predicted by use of the subcellular localization program MitoProtII (Claro and Vicens, 1996).

Cloning of Plasmid Vectors
CrCLS1 (Cre13.g604700): To construct pCH069, a 1,060-bp fragment was amplified from the cDNA template of C. reinhardtii strain CC-503 (cw92 mt+) with the primers CH227 and CH228, and cloned into pENTR/D-TOPO. Then, to construct pCH178, the open reading frame (ORF) of CrCLS1 was amplified from pCH069 with the primers CH831 and CH832, and inserted into XbaI and EcoR1 sites of pCH078 (Hung et al., 2013). To construct pCH158, the ORF of CrCLS1 was amplified from pCH069 with the primers CH831 and CH832, and inserted into XbaI and EcoR1 sites of pCH078 (Hung et al., 2013).

Complementation Assay of the Synechocystis sp. PCC 6803 pgsA Mutant by CrCLS1
Complementation assay of the Synechocystis sp. PCC 6803 pgsA mutant by CrCLS1 (pCH158) was performed as described previously (Hung et al., 2015a).

Complementation Assay of the S. cerevisiae Δcrd1 Mutant by CrCLS1
Complementation assay of the S. cerevisiae Δcrd1 mutant by CrCLS1 (pCH178) was performed as described previously (Hung et al., 2015a).

Lipid Extraction and Analysis
Lipid extraction and analysis were performed as previously described (Hung et al., 2013) except that 2D thin-layer chromatography (TLC) was used to separate phospholipid classes with the solvent system of chloroform/methanol/7 N ammonia 120:80:8 (by vol) for the first dimension and chloroform/methanol/acetic acid/water 170:20:15:3 (by vol) for the second dimension (Nakamura et al., 2003).

Radiolabeling Assay of CLS Activity
Logarithmically growing cells were resuspended in 5 ml SC-Ura medium at the cell density (OD600 of 5) with 30 µCi KHP32PO4 (PerkinElmer). After shaking incubation for 8 h at room temperature, lipids were extracted from cells by the method

1http://www.genome.jp/tools/clustalw/
2http://ihg.gsf.de/ihg/mitoprot.html
FIGURE 1 | Multiple amino acid sequence alignment of Chlamydomonas reinhardtii CrCLS1 (CrCLS1) with other known CLSs, Saccharomyces cerevisiae CRD1 (ScCRD1), Homo sapiens CLS1 (HsCLS1), Arabidopsis thaliana CLS (AtCLS), and Drosophila melanogaster CLS (DmCLS). The region conserved among proteins containing a CDP-OH-P motif (PF01066.9) is underlined. Asterisks indicate the amino acid residues conserved in all sequences of proteins with the CDP-OH-P motif. Square frames indicate the terminal amino acid residues of the predicted cleavage site of putative N-terminal mitochondrial targeting sequence.

RNA Extraction and cDNA Synthesis
RNA extraction and cDNA synthesis were performed as previously described (Hung et al., 2013).

Quantitative RT-PCR
Quantitative RT-PCR analysis involved the ABI 7500 Real Time PCR System (Applied Biosystems) with the specific oligonucleotide primer sets, CH955 and CH956, CH957 and CH958, and CH531 and CH532, for CrCLS1, CRD1, and ACT1, respectively. Gene expression was normalized to that of ACT1. Data were averaged by three technical replicates in the same run and three biological replicates in separate runs. The primer sequences are described in Supplementary Table S2.

RESULTS
Sequence Analysis of CrCLS1
To compare the amino acid sequence similarity of the putative CrCLS1 with other known CLSs in different organisms, the deduced amino acid sequence of CrCLS1 was compared with those of S. cerevisiae CRD1, Homo sapiens CLS1,
A. thaliana CLS, and Drosophila melanogaster CLS, which are functionally characterized CLS (Figure 1) (Tuller et al., 1998; Katayama et al., 2004; Chen et al., 2006; Acehan et al., 2011). In Figure 1, the region containing the CDP-OH-P motif D(X)2DG(X)2AR(X)8−9G(X)3D(X)3D is underlined and asterisks indicate the conserved eight amino acid residues. All eight amino acids were conserved in CrCLS1, which suggests that CrCLS1 encodes a functional CLS. In addition, CrCLS1 contained a putative N-terminal mitochondrial targeting sequence predicted by the subcellular localization program MitoProtII, suggesting a possible localization of CrCLS1 in mitochondria, where CL is exclusively localized.

Complementation of pgsA by CrCLS1

To examine whether CrCLS1 functions as PGPS, we transformed CrCLS1 into the pgsA mutant of Synechocystis sp. PCC 6803, which abolishes PGPS activity and thus requires exogenous supplementation of PG for growth (Hagio et al., 2000). As shown in Figure 2, whereas the CrPGP1 and CrPGP2 functionally complemented the lethal phenotype of the pgsA mutant as reported previously (Hung et al., 2015a), CrCLS1 failed to
complement the growth phenotype, showing the rescued growth only in the presence of PG. Therefore, CrCLS1 does not function as a PGPS in vivo in Synechocystis sp. PCC 6803.

Recovery of Growth Defect in the Δcrd1 Mutant Complemented by CrCLS1

To investigate whether CrCLS1 encodes a functional CLS, we performed a heterologous complementation assay with the S. cerevisiae Δcrd1 mutant, because Synechocystis sp. PCC 6803 does not contain CL and no other CLS mutant is known in algae. As previously reported, Crd1p has CLS activity and Δcrd1 mutant cells show a temperature-sensitive growth defect, severe at 37°C but not at 30°C (Jiang et al., 1999). The temperature-sensitive phenotype of Δcrd1 mutant cells was rescued by heterologous complementation of HsCLS1 (Houtkooper et al., 2006), so we used this approach to investigate the function of CrCLS1. We cloned the ORF of CrCLS1 into a yeast shuttle
CrCLS complemented cardiolipin synthesis in Δcrd1 cells. Wild type (WT), Δcrd1 or Δcrd1 CrCLS cells were grown in the presence of [32P]-phosphate for 8 h at room temperature. Radioactivity incorporated into phospholipids was determined by TLC analysis and autoradiography, with unlabeled PG and CL visualized by primuline staining as standards. Two biologically independent experiments (Experiments 1 and 2) were shown. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid.

Expression of CrCLS1
To investigate whether CrCLS1 is appropriately expressed in the Δcrd1 mutant, we analyzed the gene expression of CrCLS1 in the Δcrd1 mutant harboring CrCLS1. The relative gene expression of CrCLS1 was 12.6-fold higher in the Δcrd1 mutant harboring CrCLS1 than CRD1 in the wild type (Figure 3B). Thus, CrCLS1 is sufficiently expressed in Δcrd1 mutant cells, which supports the functional complementation shown in Figure 3A.

Effect of Different Carbon Sources on the Growth of Δcrd1 Mutant Complemented by CrCLS1
A previous study showed that the growth of the Δcrd1 mutant under aerobic conditions was affected with ethanol used as the sole carbon source (Tuller et al., 1998). To investigate whether the Δcrd1 mutant harboring CrCLS1 rescued the growth defect under this condition, cells were grown in synthetic complete medium supplemented with 2% glucose or 2% ethanol as the sole carbon source. The growth rates of both the Δcrd1 mutant and Δcrd1 harboring CrCLS1 were indistinguishable from that of wild type in 2% glucose medium (Figure 4A). However, with 2% ethanol medium, the Δcrd1 mutant harboring CrCLS1 fully restored the growth phenotype to that of the wild type, whereas the Δcrd1 mutant showed growth retardation, as reported (Tuller et al., 1998) (Figure 4B). Therefore, CrCLS1 could complement the growth defect of the Δcrd1 mutant with ethanol supplementation as the carbon source.

Lipid Contents of the Δcrd1 Mutant Complemented by CrCLS1
The phospholipid profiles of the Δcrd1 mutant were previously analyzed by radiolabeling (Tuller et al., 1998) and transformed it into Δcrd1 mutant cells. The Δcrd1 mutant harboring CrCLS1 fully recovered cell growth at 37°C, whereas the Δcrd1 mutant alone showed a growth defect at this temperature (Figure 3A). Therefore, CrCLS1 complemented the temperature-sensitive phenotype of Δcrd1, which suggests that CrCLS1 encodes a functional CLS of C. reinhardtii.
or mass spectrometry (Zhang et al., 2003). However, whether the \textit{acr1d} mutant alters the composition of major membrane phospholipid classes remained unclear. To investigate whether the complementation of the growth defect observed in Figures 3 and 4 is associated with lipid compositional change, we analyzed the major phospholipid composition of these strains. The \textit{acr1d} mutant showed an increase in PC content and decrease in PS and PI contents as compared with the wild type (Figure 5A). In the \textit{acr1d} mutant harboring \textit{CrCLS1}, phospholipid composition was restored to a level similar to that of the wild type. The fatty acid composition of PE, PC, and PI was similar among the three strains (Figure 5B). Thus, \textit{CrCLS1} encodes a functional CLS that complements lipid compositional changes in the \textit{acr1d} mutant.

**Enzyme Activity of \textit{CrCLS1} Expressed in the \textit{acr1d}**

To investigate whether \textit{CrCLS1} encodes a functional CLS to restore the CL synthesis defect in the \textit{acr1d}, we performed radiolabeling assay to analyze CLS activity. As shown in Figure 6, the \textit{acr1d} mutant harboring \textit{CrCLS1} recovered radiolabeled spot that co-migrates with the commercial standard of CL, which is present in wild type but absent in the \textit{acr1d} mutant, demonstrating that the activity of CLS was recovered in the mutant harboring \textit{CrCLS1}. Thus, \textit{CrCLS1} encodes a functional CLS that complements CL synthesis defect of \textit{acr1d} mutant.

**DISCUSSION**

Present study reported identification of a CLS gene in \textit{C. reinhardtii}, \textit{CrCLS1}, and examined its \textit{in vivo} function by heterologous complementation of \textit{pgsA}, a PGPS mutant of \textit{Synechocystis} sp. PCC 6803, and \textit{acr1d}, a CLS mutant of \textit{S. cerevisiae}. Whereas \textit{CrCLS1} did not complement the growth phenotype of \textit{pgsA}, it rescued the temperature-sensitive growth phenotype, growth profile with different carbon sources, phospholipid composition and enzyme activity of \textit{acr1d} of \textit{S. cerevisiae}. These results suggest that \textit{CrCLS1} is a functional gene for CLS of \textit{C. reinhardtii} as the first identified algal CLS, which is functionally incompatible with PGPS despite their sequence homology.

Physiological roles of \textit{CrCLS1} in \textit{C. reinhardtii} are not reported yet; however, several transcriptomic studies have shown gene expression profiles in response to environmental stresses. For example, expression of \textit{CrCLS1} is down-regulated in response to the deprivation of iron (Urzica et al., 2013) and nitrogen (Goodenough et al., 2014). Conversely, an upregulation is seen by copper deficiency (Castruita et al., 2011) and singlet oxygen stress (Wakao et al., 2014). These data suggest possible roles of \textit{CrCLS1} in adaptation to circumvent environmental stresses.

Given that both PGPS and CLS belong to the CDP-alcohol phosphotransferase family and the relevant CDP-OH-P motifs are closely related (Katayama et al., 2004), what defines substrate specificity of these enzymes?

Recently, structural basis for catalysis in a CDP-alcohol phosphotransferase was revealed by crystallographic analysis (Sciara et al., 2014). According to this structure, conserved amino acid residues in the CDP-OH-P motif are associated with CDP-DAG. Since CDP-DAG is the common substrate between CLS and PGPS, this study suggests that an additional motif recognizes the other substrate (PG for CLS; glycerol 3-phosphate for PGPS). We aligned the amino acid sequences of core CDP-OH-P motif among three CLSs (\textit{C. reinhardtii} cardiolipin synthase 1, \textit{CrCLS1}; \textit{S. cerevisiae} CRD1, ScCRD1; \textit{A. thaliana} CLS, AtCLS) and five PGPSs (\textit{C. reinhardtii} PGP1, CrPGP1; \textit{C. reinhardtii} PGP2, CrPGP2; \textit{A. thaliana} PGP1, AtPGP1; \textit{A. thaliana} PGP2, AtPGP2; \textit{Synechocystis} sp. PCC 6803 PgsA, SynPgsA) (Figure 7). While the eight amino acid residues of the core CDP-OH-P motif D(X)2DG(X)2AR(X)8−9G(X)3D indicated by asterisks in Figure 7 were conserved between the PGPS and CLS, we noted that seven amino acids (FxxAxxT) immediately before the core CDP-OH-P motif were highly conserved among PGPSs but not CLSs (underlined in Figure 7). In addition, we found additional four amino acid residues that were conserved among PGPS but not in CLS (indicated by dots in Figure 7). It is possible that these additional residues may define the substrate specificity between PGPS and CLS. Detailed structural analysis as well as enzymatic characterization of these residues are anticipated to experimentally validate this proposal.

**CONCLUSION**

We suggest functional specificity of CLS by the identification and characterization of a CLS, \textit{CrCLS1}, in \textit{C. reinhardtii}.

**AUTHOR CONTRIBUTIONS**

KK, HW, and YN conceived research. KK and C-HH performed experiments and analyzed data. All authors wrote and commented on the manuscript and approved the contents.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01542
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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