Characterization of the Cardiolipin Synthase from *Arabidopsis thaliana*

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Summary

Cardiolipin (CL) is an anionic membrane phospholipid of a unique tetraacyl structure, consisting of two phosphatidyl moieties linked by a glycerol bridge. It constitutes the cytoplasmic membrane of eubacteria and represents the marker lipid of mitochondria in eukaryotes, where CL is predominantly found in the inner membrane. In all eukaryotes, CL biosynthesis occurs in the inner mitochondrial membrane via phosphatidylglycerol (PG) as conserved intermediate and the CL synthase (CLS) catalyzes the transfer of the phosphatidyl motiety from cytidinediphosphate-diacylglycerol onto PG. The analysis of yeast and mammalian mutants deficient in CL or PG biosynthesis provided evidence that these anionic lipids are required for proper biogenesis and function of mitochondria and, thus, for maintenance of cell integrity. In contrast to yeast and mammalian cells, little is known about the CL biosynthesis and functions in plants.

To gain access to the CLS protein of plants, the respective gene from Arabidopsis thaliana that encodes a hydrophobic protein of 38 kDa with a cleavable transit peptide for the import into mitochondria was cloned and its identity was confirmed by functional expression studies in Escherichia coli and yeast. The plant CLS was shown to exhibit an alkaline pH optimum, a strict requirement for divalent cations, and a distinctly lower Km value for CDP-DAG than for PG. It displayed a preference for both its substrates esterified with unsaturated acyl groups and the activity of the enzyme increased concomitantly with the unsaturation level of the substrates. Similarly to the mammalian enzyme, but unlike the yeast one, the plant CLS was highly sensitive towards detergents. Solubilization and purification experiments revealed that the protein requires a defined phospholipid environment, particularly the presence of CL, to acquire its catalytically active conformation.

To investigate the functional role of CL in plant mitochondria, A. thaliana mutants carrying a T-DNA insertion in four different positions of the CLS gene were analysed. Plants homozygous with respect to the insertion in either the CLS promoter region or the intron 7 of the CLS gene showed no significant alterations in the CL levels. On the other hand, only heterozygous insertional mutants were obtained for plants with a T-DNA insertion in exons 1 and 5, respectively, of the CLS gene. These data suggest that the T-DNA insertion in the exons represent loss-of-function mutations, and that these mutations are lethal for A. thaliana plants.

Zusammenfassung:

Cardiolipin (CL) ist ein anionisches Membran-Phospholipid mit einer einzigartigen Tetraacyl-Struktur, die aus zwei über Glycerin verknüpfte Phosphatidyl-Einheiten besteht. Es ist Bestandteil der zytoplasmatischen Membran von Eubakterien und ein Markerlipid der Mitochondrien von Eukaryoten, bei denen es vorwiegend in der inneren Membran vorhanden ist. In allen Eukaryoten findet die CL-Biosynthese über Phosphatidylglycerol (PG) als konserviertes Intermediat in der inneren mitochondrialen Membran statt, wo die CL-Synthase (CLS) den Transfer des Phosphatidyl-Rests von Cytidindiphosphat-Diacylglycerol (CDP-DAG) auf PG katalysiert. Die Analyse von Hefe- und Säuger-Mutanten mit Defekten in der CL- oder PG-Biosynthese deutete darauf hin, dass diese anionischen Lipide für Biogenese und Funktion der Mitochondrien und somit für die Vitalität der Zellen benötigt werden. Im Gegensatz zu Hefen und Zellen von Säugern ist wenig über die Biosynthese von CL und dessen Funktion in Pflanzen bekannt.

Um Zugang zu einem pflanzlichen CLS-Protein zu erhalten, wurde das entsprechende Gen aus Arabidopsis thaliana kloniert, das ein hydrophobes 38 kDa Protein mit abtrennbarem Transitpeptid für den Import in die Mitochondrien kodiert, und seine Identität durch Studien zur funktionellen Expression in Escherichia coli und Hefen bestätigt. Dabei konnte gezeigt werden, dass die pflanzliche CLS ein alkalisches pH-Optimum besitzt, bivalente Kationen benötigt und einen deutlich niedrigeren Km-Wert für CDP-DAG als für PG aufweist. Die CLS bevorzugte bei beiden Substraten solche, die mit ungesättigten Fettsäuregruppen verestert waren, und die Aktivität des Enzyms nahm mit der Anzahl an Doppelbindungen in den Substraten zu. Vergleichbar mit dem Enzym der Säugetiere, aber anders als das der Hefen ist die pflanzliche CLS höchst empfindlich gegenüber Detergenzien. Experimente zur Solubilisierung und Reinigung zeigten, dass das Protein eine definierte Phospholipid-Umgebung, vor allem die Anwesenheit von CL benötigt, um seine katalytisch aktive Konformation auszubilden.

Zur Analyse der Funktionen von CL in pflanzlichen Mitochondrien wurden A. thaliana-Mutanten mit einer T-DNA-Insertion in vier unterschiedlichen Positionen des CLS-Gens untersucht. Pflanzen, die bezüglich der Insertion in die CLS-Promotor-Region oder im Intron 7 des CLS-Gens homozygot waren, zeigten keine signifikanten Veränderungen in der Gesamtmenge von CL. Im Gegensatz dazu konnten für Pflanzen mit T-DNA-Insertionen in den Exons 1 und 5 des CLS-Gens nur heterozygote Insertions-Mutanten erzeugt werden. Diese Daten legen nahe, dass die T-DNA-Insertion in diesen Exons Mutationen mit Funktionsverlust darstellen und dass diese Mutationen letal für A. thaliana-Pflanzen sind.

Table of Contents

1 Introduction 1

1.1 Structure and acyl species of mitochondrial cardiolipin 1

1.2 Biosynthesis of cardiolipin 5

1.3 Remodelling of cardiolipin 9

1.4 Eukaryotic cardiolipin synthases and their properties 10

1.5 Genetic regulation of cardiolipin synthesis 13

1.6 The functional role of cardiolipin 17

1.7 Goals of the research 22

2 Materials and Methods 23

2.1 Biological Materials 23

2.2 Manipulation and analysis of nucleic acids and proteins 24

2.3 Protein concentration measurement 25

2.4 Gel electrophoresis of macromolecules 25

2.5 Protein immunoblot 28

2.6 Cloning and expression in *Escherichia coli* 29

2.7 Cloning and expression in *Saccharomyces cerevisiae* 34

2.8 Analyses and manipulations of *Arabidopsis thaliana* 38

2.9 PCR methods 48

2.10 Enzymic assays 53

2.11 Partial purification of CLS 55

3 Results and Discussion 60

3.1 Identification of a putative *A. thaliana CLS* gene 60

3.2 Cloning and expression of plant cardiolipin synthase 62

3.3 CLS expression studies in *Saccharomyces cerevisiae* 66

3.4 Characterization of the *A. thaliana* cardiolipin synthase 69

3.5 Solubilization and reconstitution of the cardiolipin synthase activity 75

3.6 Analyses of the *A. thaliana* insertional mutants 78

4 Literature 86

5 Appendix 97

5.1 Primers 97

5.2 Chimeric construct maps 99

5.3 Abbreviations 101

List of the figures:

Figure 2-1: Structure of cardiolipin....2
Figure 1-2-2: CL and PG contents in eukaryotic mitochondria....3
Figure 2-3: Fatty acid composition of mitochondrial phospholipids....4
Figure 2-4: The biosynthetic pathway of cardiolipin in eukaryotes and prokaryotes....7
Figure 4-1: Alignment of the amino acid sequences of eukaryotic phosphatidyltransferases....61
Figure 4-2: Homology tree of phosphatidyltransferases....62
Figure 4-3: Western blot analysis of subcellular fractions from *E. coli* cells expressing the pCLS_C-His construct....63
Figure 4-4: Time course of expression of the recombinant plant CLS in *E. coli* cells cultivated in different media....64
Figure 4-5: Functional expression of the *CLS* cDNA from *A. thaliana* in *E. coli*....64
Figure 4-6: CLS activites displayed by induced *E. coli* carrying respective constructs....65
Figure 4-7: Complementation analysis by CL profile analysis....66
Figure 4-8: Estimation of CL and PG levels....67
Figure 4-9: Functional expression of *A. thaliana CLS* cDNA in *Saccharomyces cerevisiae*....68
Figure 4-10: Dependance of the *A. thaliana* CLS activation on dialent cations....69
Figure 4-11: The pH optimum of the *A. thaliana* CLS....70
Figure 4-12: Influence of temperature on plant CLS activity and stability....70
Figure 4-13: Inhibition of the activity of the *A. thaliana* CLS by detergents....71
Figure 4-14: Substrates dependency of the *A. thaliana* CLS activity....72
Figure 4-15: PG species specificity of the *A. thaliana* CLS....73
Figure 4-16: CDP-DAG species selectivity of the *A. thaliana* CLS activity....74
Figure 4-17: The optimization of plant CLS solubilization....76
Figure 4-18: Reactivation of the solubilized *A. thaliana* CLS by phospholipids....76
Figure 4-19: Mutants carrying a T-DNA insertion at dfferent positions of the CLS gene....78
Figure 4-20: PCR screening of the *cls-4* mutant plants....79
Figure 4-21: The abundance of the plant *CLS* and *Actin2* transcripts....80
Figure 4-22: Activities of the CLS and PGS in mitochondria....81
Figure 4-23: ^{33}P phospholipid labelling pattern of wild type and *cls-1* mutant....82

Figure 4-24: The T-DNA region of the pB6 CLS Cre construct integrated into plant genome.....84
Figure 6-1: Chimeric constructs for expression studies in *E. coli* 99
Figure 6-2: Chimeric construct maps for *S. cerevisiae* expression.....100
Figure 6-3: Plant transformation construct.....100

List of the tables:

Table 1-1: Properties of CLSs from different mitochondria 12
Table 1-2: Cardiolipin-dependent proteins in mitochondria 18
Table 3-1: Phenotypes of the different insertional mutants of *A. thaliana*.....79
Table 3-2: Labelling pattern of phospholipids from the cell suspension cultures of wild type and the *cls-1* and *cls-4* mutants 83
Table 3-3: Comparison of seed germination rates 84

1 Introduction

Cardiolipin (bis-(1,2-diacyl-*sn*-glycero-3-phospho)-1',3'-*sn*-glycerol, diphosphatidyl-glycerol, CL) is an anionic phospholipid with a unique tetraacyl structure. It gained its name because it was for the first time isolated and purified from the heart tissue, where it is highly abundant (Pangborn 1942). Subsequently, cardiolipin was shown to be the marker lipid of mitochondria of all eukaryotes (Hoch 1992), but it is also present in the energy transducing membranes of eubacteria as well (Hoch 1992, Robinson 1993).
In the following, the biochemistry and functions of CL will be discussed, with special emphasis on mitochondrial CL.

1.1 Structure and acyl species of mitochondrial cardiolipin

Cardiolipin possesses a unique tetra-acyl structure, in which two phosphatidyl moieties are linked by a central glycerol bridge (Figure 1-1). Thus, unlike the other phospholipids, CL has four acyl chains and two phosphate groups, making it highly hydrophobic and acidic. Each phosphate group of CL contains one acidic proton, having very different levels of acidity (pK_1=2.8 and pK_2>7.5, Kates et al. 1993). The weak acidity of the second phosphate is probably due to the formation of a stable intramolecular hydrogen bond with the central 2'-hydroxy group at the glycerol bridge. The resulting cyclic hydrogen-bonded monoprotonated form of CL may function as a reservoir of protons at the relatively high pH. This assumption was supported by the analyses of the chemically synthesized 2'-deoxy CL analogue, for which the large pK gap was abolished (Kates et al. 1993).
The structure of CL depicted in Figure 1-1 suggests symmetry in the structure of the lipid. Closer investigations, however, revealed the two phosphatidyl moieties to be chemically distinct (Powell and Jacobus 1974). There are two diastereoisomeric carbon atoms present, one in each outer glycerol group of CL. Hence, four different isomers of CL are possible, only two of them having symmetrical conformation, in which the outer glycerol groups are mirror images of each other. In the natural CL, both outer glycerol moieties carry chiral centres in *R* configuration (Figure 1-1), but one of the *R*-glycerols is in pro-*R* position and the other one in pro-*S* position with respect to the central glycerol. Thus, the two phosphate groups have different chemical environments as

observed by distinct ^{31}P–NMR spectra and by enzymatic analyses (Powell and Jacobus 1974). The phosphate groups were correspondingly designated as 1'- and 3'-phosphate, with respect to the central glycerol (Figure 1-1). Consequently, each acyl ester bond has a unique chemical environment as a result of the steric relationship between the three glycerols (Powell and Jacobus 1974).

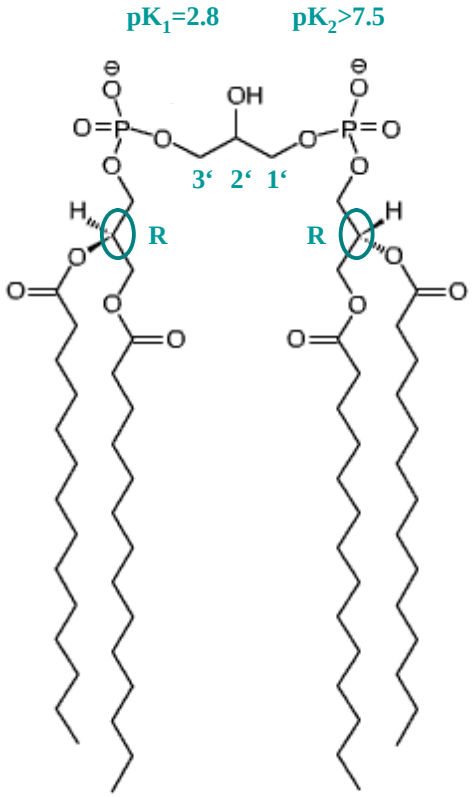


Figure 1-1: Structure of cardiolipin
Depicted is the 1,1',2,2'-tetramyristoyl cardiolipin in the dissociated form (adopted from Avanti Polar Lipids Inc., Alabaster, USA). The acidity of the phosphate groups, as well as the numbering of the carbon atoms of central glycerol bridge is indicated. The stereoisomery of the respective chiral centres is given.

The presence of four distinct acyl positions gives rise to a large number of permutations, when multiple fatty acids are attached to CL. For instance, in the presence of five fatty acids, only 4% of the calculated CL species is symmetric and only 1% in the presence of 10 fatty acid species, assuming random distribution of the acyl groups (Schlame et al. 2005). Analyses of the most abundant CL species from various eukaryotic organisms and tissues, such as human heart, human lymphoblasts, rat liver, *Drosophila*, sea urchin sperm, yeast, and mung bean hypocotyls showed that these species contained one or two different acyl groups. This feature generated a big proportion of the CL molecules with high degree of structural uniformity and symmetric fatty acid distribution, i.e. identical

fatty acids found in both *sn*-1 and in both *sn*-2 positions were present at 20-90% in the various organisms and tissues (Schlame et al. 2005). Even at the low end of this spectrum, the proportion of symmetric CL was too high to be accounted for by random acyl distribution.

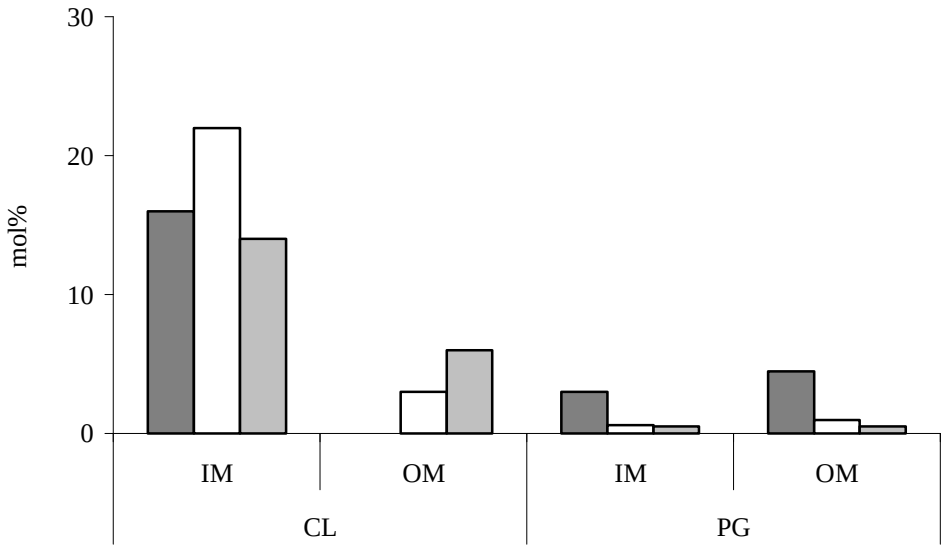


Figure 1-1-2: CL and PG contents in eukaryotic mitochondria
CL and PG contents are expressed as % of total phospholipids in inner (IM) and outer (OM) mitochondrial membrane of plants (dark grey bars), mammals (white bars), and yeast (light grey bars). Data obtained from Daum 1985.

The majority of CL is located in the inner mitochondrial membrane, where it comprises 10 – 20% of the total phospholipids (Figure 1-2). The level of phosphatidylglycerol (1,2-diacyl-*sn*-glycero-3-phospho-*rac*-[1-glycerol]; PG), on the other hand, is distinctly lower and shows no preference for neither of the mitochondrial membranes. The CL of complex eukaryotes has been noted for the unique fatty acid pattern, which is essentially restricted to C_{18} chains. This rule applies to most mammalian tissues and higher plants (Figure 1-3; Hoch 1992). Mammalian heart and liver tissues membrane lipids contain approximately 50% unsaturated fatty acids, linoleic acid (18:2) and arachidonic acid (20:4) being the most prominent ones (Figure 1-3). On the other hand, linoleic and oleic fatty acids contribute to more than 85% of the CL fatty acids. The PG fatty acid composition differs significantly, since palmitoyl, oleoyl and stearyl fatty acids are present at high levels (Figure 1-3).

As compared with the membrane lipids of mammalian mitochondria, those of plant mitochondria contain a lower proportion of stearic acid, but higher levels of linoleic and

linolenic acid (Figure 1-3). Almost 90% of the fatty acid contents in plant CL are covered by the unsaturated C_{18} residues, mainly linoleoyl and linoleonyl groups. This remains in striking difference with the fatty acid composition of plant PG, where the palmitoyl residue constitutes almost 50% of the fatty acid contents (Figure 1-3).

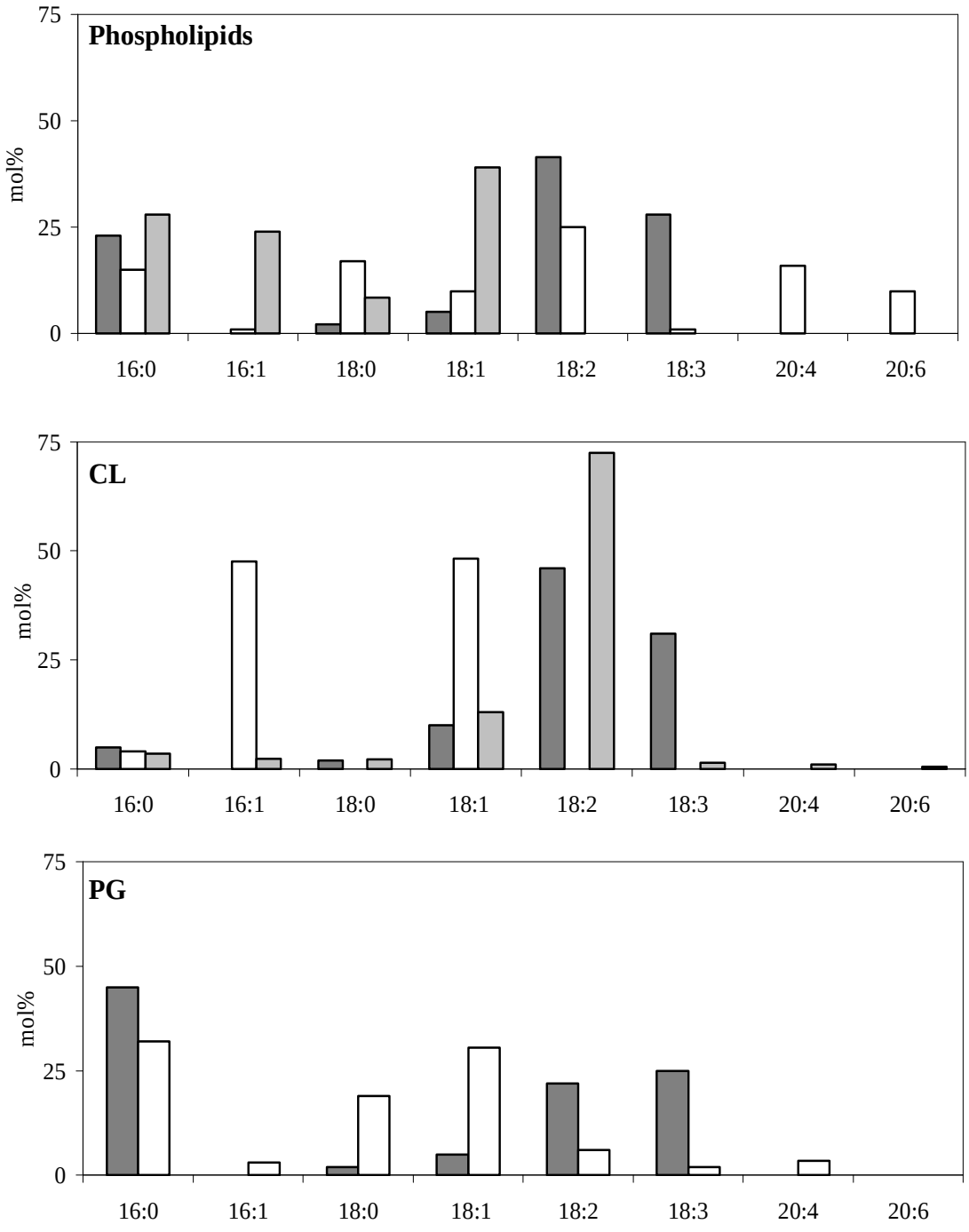


Figure 1-3: Fatty acid composition of mitochondrial phospholipids
Plant (dark grey bars), mammalian (white bars) and yeast (light grey bars) fatty acid composition of mitochondria, CL and PG are expressed as % of total fatty acid. Data applied from Daum 1985, Hoch 1992, Bligny and Douce 1980.

As *S. cerevisiae* does not synthesize remarkable amounts of the polyunsaturated fatty acids (Daum 1985), the fatty acid composition of yeast mitochondrial lipids is restricted to the palmitoyl, palmitoleoyl, stearyl and oleoyl species. About 65% of the total fatty acids of yeast mitochondria are unsaturated, while the fatty acid composition of CL almost exclusively consists of about equal amounts of the oleoic and palmitoleic acids (Figure 1-3).

Hence, CL from various eukaryotes possesses a typical restricted fatty acid pattern that differs from these of the other mitochondrial membrane lipids. Moreover, the major species of CL are assembled from a limited assortment of fatty acids. Although the exact type of fatty acids varies between organisms and tissues (Daum 1985, Hoch 1992, Schlame et al. 1993, Schlame et al. 2005), the structural uniformity and molecular symmetry of CL emerged as characteristic consequences of the restrictive fatty acid pattern. The portion of symmetrical molecular CL species, i.e. species with two identical diacylglycerol moieties, was 20-90% in various eukaryotes (Schlame et al. 1993, Schlame et al. 2005). In addition to the high degree of symmetric fatty acids distribution, in most molecular species, the four acyl groups were relatively similar, i.e. they differed by no more than one double bond or two carbon atoms.

1.2 Biosynthesis of cardiolipin

The widespread distribution of CL among eubacteria and eukaryotes (Hoch 1992) suggests that it evolved early before these two kingdoms branched in the phylogenetic tree. The biosynthetic routes of CL are similar in those two clusters, as they pass through common intermediates, namely phosphatidic acid (PA), CDP-diacylglycerol (1,2-diacyl-*sn*-glycerol-3-[5′-diphosphocytidine]; CDP-DAG), phosphatidylglycerol-phosphate (3-*sn*-phosphatidyl-1’-*sn*-glycerol-3’-phosphate; PGP), and phosphatidyl-glycerol (PG). However, eukaryotes and bacteria employ different reactions to convert PG into CL (Figure 1-4).

The bacterial PG comprises 10-35% of the total phospholipids and in some organisms of this cluster it is the major phospholipid, reaching 50-80% total phospholipids (Hostetler 1982). Thus, bacteria synthesize CL mainly in the state of growth recession from the vast source of PG residing in their membranes. In eubacteria, CL biosynthesis is catalyzed by a transesterification reaction: a phosphatidyl group (*sn*-1,2-

diacylglycerol-3-phosphate) of one PG molecule is transferred to the free 3'-hydroxyl group of another PG (Hirschberg and Kennedy 1972). CLS of bacteria, thus, catalyze a near-equilibrium reaction, proceeding in the presence of a relatively high substrate concentration (Finnerty 1978, Hostetler 1982). The CL contents in bacterial membranes differs significantly, ranging from about 15% of total phospholipids (*M. lysodeikticus*, *S. thyphimurium*, *B. cereus*), to 40% (*Actinomycetes*), or up to 80% in a form of *S. aureus* (Hoch 1992).

Since the eukaryotic CLS is a mitochondrial enzyme and mitochondria are phylogenetic derivatives of ancient prokaryotes, it appears that the transition from bacteria to mitochondria also involved a transition from the prokaryotic to the eukaryotic reaction mechanism. The prokaryotic pathway changed concomitantly with the genesis of mitochondria most likely because of different functions of CL in the respective structures. In mitochondria, the PG levels are very low, but can be efficiently converted to CL because its biosynthesis is performed by an irreversible reaction that involves cleavage of a high-energy bond, such as the one in CDP-DAG. Thus, in contrast to the bacterial enzyme, the eukaryotic CL synthases (CLS, CDP-DAG : PG phosphatidyltransferase, E.C. 2.7.8.*) catalyze the anhydride bond split and transfer of the phosphatidyl moiety from CDP-diacylglycerol (CDP-DAG) on the hydroxyl group in the *sn*-3' position of PG (Hostetler 1972, Stanacev et al. 1973, Schlame et al. 2000). The CL biosynthesis from CDP-DAG and PG was demonstrated in mitochondria from diverse eukaryotes strongly suggesting that this pathway exists in the entire eukaryotic kingdom (Schlame et al. 1993). Additionally, the reaction takes place at low substrate levels and is mainly regulated by the activity of the mitochondrial CLS (Daum 1985).

In accordance with the distinct mechanisms of CL synthesis displayed by the prokaryotic and eukaryotic enzymes, the amino acid sequences of respective proteins also showed striking differences. Analysis of the CLS sequences revealed that they are members of two distinct protein families. The bacterial CLS belong to the HKD protein family, as do the PGS from yeast and mammals, as well as other proteins (Dzugasova et al. 1998, Stone and Vance 1999, Chang et al. 1998,Kawasaki et al. 1999, Exton 2000, Roper and Moss 1999, Ponting and Kerr 1996). These proteins have a molecular mass of about 60 kDa and contain the typical **H**x_2**K**x_{4-6}**D** motif.

On the other hand, the mitochondrial CLS belong to the DGAR family. To the same cluster belong the PGS from bacteria and plants, as well as further CDP-alcohol dependent enzymes. The proteins of this family have a mass of about 36 kDa and

contain the characteristic CDP-alcohol binding motif Dx_2**DG**x_2**AR**xGx_3Dx_3D (Ohta et al. 1981, Ohta 1985, Müller and Frentzen 2001, Jiang et al. 1997, Tuller et al. 1998, Chang et al. 1998, Houtkooper et al. 2006).

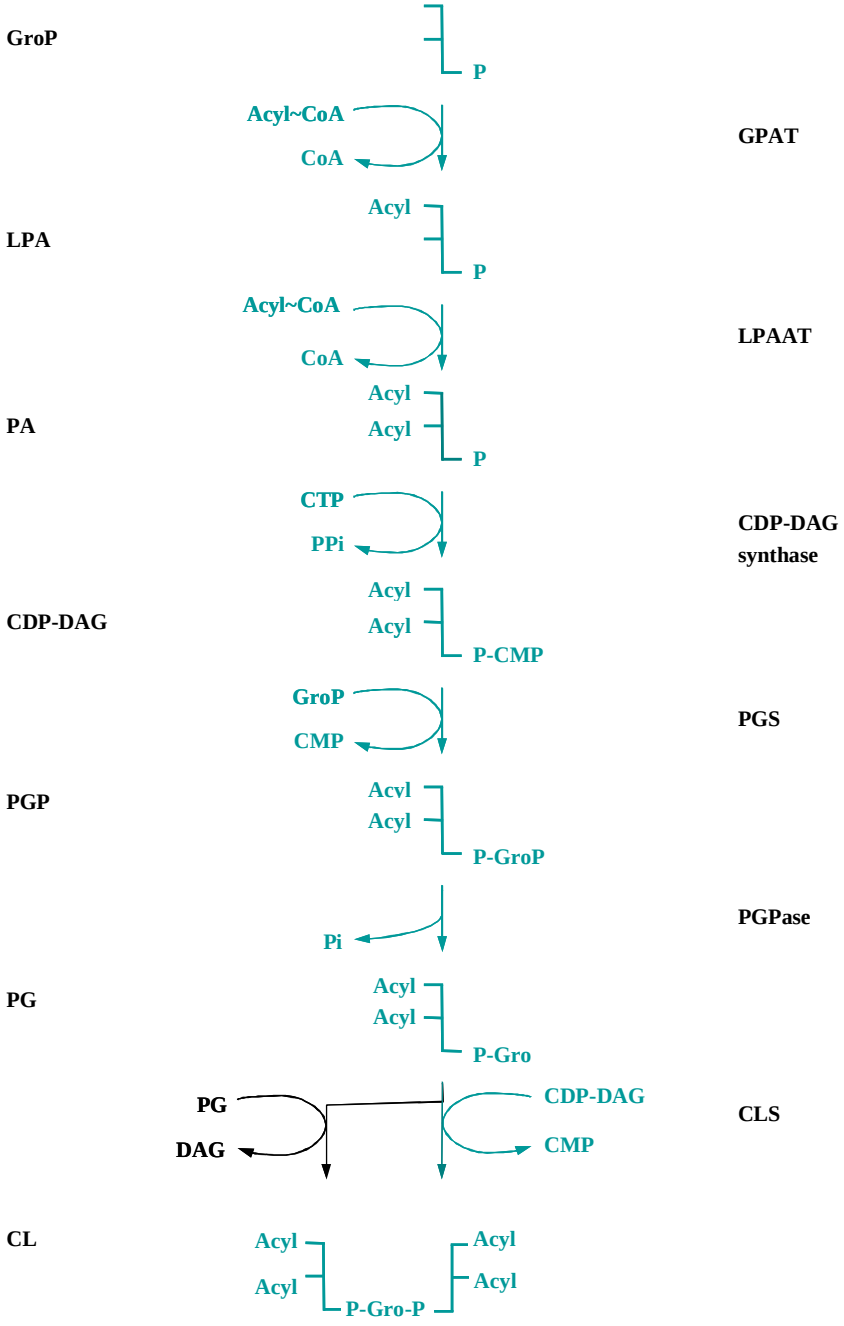


Figure 1-4: The biosynthetic pathway of cardiolipin in eukaryotes and prokaryotes
GroP: glycerol-3-phosphate; Acyl-ACP: Acylated Acyl Carrier Protein; Acyl-CoA: Acyl-Coenzyme A; LPA: lysophosphatidic acid (*sn*-1-acylglycerol-3-phosphate); PA: phosphatidic acid (*sn*-1,2-diacylglycerol-3-phosphate); PGP: phosphatidylglycerol-phosphate (1,2-[diacyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol-3-phosphate]); PG : phosphatidylglycerol (1,2-diacyl-*sn*-glycero-3-phospho-*rac*-[1-glycerol]); CL: cardiolipin (bis-(1,2-diacyl-*sn*-glycero-3-phospho)-1',3'-*sn*-glycerol, diphosphatidyl-glycerol); GPAT: Acyl-CoA / ACP : glycerol 3-phosphate acyltransferase; LPAAT: Acyl-CoA / ACP : LPA acyltransferase; CDS: CTP : PA cytidyltransferase (CDP-DAG synthase); PGS: CDP-DAG : glycerol 3-phosphate cytidyltransferase (phosphatidylglycerolphosphate synthase); PGPase: phosphatidyl-glycerolphosphate phosphatase. The CL synthesis reaction characteristic for eubacteria is marked in black.

The mitochondria are largely dependent on the import of proteins and bulk phospholipids, but the plant organelles are able to synthesize CL *de novo* from acylated Co-A and glycerol phosphate (Sparace and Moore 1979, Kuchler et al. 1986, Frentzen et al. 1990, Frentzen and Griebau 1994). Thus, the CL synthesis represents an example of limited autonomy in the mitochondrial membrane biogenesis. The first step of the CL pathway takes place in the outer membrane where glycerol phosphate is acylated to form phosphatidic acid (Hesler et al. 1985, Frentzen et al. 1990). Mitochondria of yeast and animal cells lack the lysophosphatidic acid acyltransferase (LPAAT; Figure 1-4) activity and, therefore, produce the CL mainly from the PA imported from the ER. This intermediate is able to travel from the outer to the inner membrane (Wojtczak et al. 1990, Schlame and Haldar1993) in order to react with CTP.

The inner mitochondrial membrane, in turn, contains all the enzymes of the CL pathway downstream from PA (Hostetler 1972, Kelly and Greenberg 1990, Frentzen and Griebau 1994). The CDP-DAG synthase was shown to be localized in the inner membrane by the submitochondrial fractionation with digitonin (Hostetler 1972) and by probing the enzyme with proteases (Schlame 1993). Conversion of CDP-DAG into PG was also shown in isolated inner mitochondrial membranes (Hostetler 1972, Frentzen and Griebau 1994), suggesting the presence of both phosphatidylglycerolphosphate synthase (CDP-DAG : glycerol 3-phosphate cytidyltransferase; PGS) and phosphatidylglycerol-phosphate phosphatase (PGPase). The cloned plant PGS isozymes were indeed dual-targeted to mitochondria and chloroplasts or localized to the ER (Müller and Frentzen 2001, Babiychuk et al. 2003). Finally, CLS, catalyzing the last step of the pathway, was found in the inner membrane fraction of mitochondria (Frentzen and Griebau 1994, Tamai and Greenberg 1990, Hostetler 1972). The submitochondrial localization of CLS was further investigated with enzyme inhibitors and proteases (Schlame 1993). These data suggested that the catalytic site of the enzyme faces the matrix side of the inner membrane, implying that CL is synthesized in the inner leaflet of the mitochondrial inner membrane.

1.3 Remodelling of cardiolipin

As outlined before, CL from various eukaryotes possesses a typical restricted fatty acid pattern and a high proportion of species with symmetric fatty acid distribution. It is possible that fatty acid symmetry of CL is caused by the substrate species preference of the enzymes involved in the *de novo* CL synthesis, or by further remodelling of newly synthesized CL. The earlier appears unlikely, since the molecular acyl species of mitochondrial PG differ from the diacyl species of the phosphatidyl moiety in CL from mammalian and plant tissue (Figure 1-3; Rustow et al. 1989, Dorne and Heinz 1988, Schlame et al. 1986). Moreover, CLS of mammals lacks appropriate substrate specificity that could explain a preferential formation of molecular species with linoleoyl residues (Hostetler 1975, Houtkooper et al. 2006). Thus, remodelling mechanisms following CL synthesis were proposed.

While acyl remodelling is a common phenomenon in mammalian phospholipid metabolism (MacDonald and Sprecher 1991), the mechanism of CL remodelling still remains not fully clarified. Two mechanisms have been reported that can modify the newly synthesized CL to obtain its specific species pattern. The deacylation - reacylation cycle, first described several decades ago for the lecithin system (Lands 1960), was proved to utilize CL in rat liver mitochondria (Schlame and Rüstow 1990). In this model, the newly synthesized CL was deacylated, possibly by phospholipase A, to form monolyso-CL (MLCL), which is then condensed with oleoyl- and linoleoyl-CoA to form mature CL (Schlame and Rüstow 1990, Ma et al. 1999). Surprisingly, [^{14}C]linoleoyl incorporation into CL was stimulated not only by MLCL but also by CL. Regardless of the substrate, [^{14}C]linoleoyl was recovered in both CL and MLCL. Since [^{14}C]linoleoyl was present in the *sn*-1 and the *sn*-2 position, the exchange must either involve hydrolysis by phospholipase A_1 and A_2, or an intramolecular acyl migration within MLCL.

MLCL acyltransferase (MLCL AT) activities were observed in mitochondria prepared from various other rat tissues (Ma et al. 1999) and the purified 74-kDa MLCL AT protein from rat liver mitochondria protein catalyzed acylation of MLCL to CL with [^{14}C]linoleoyl CoA, but did not utilize other lysophospholipids as substrate. The acyl CoA dependence of MLCL AT was supported by the photoaffinity labelling with 12-[(4-[^{125}I]azidosalicyl)amino]dodecanoyl CoA (Taylor and Hatch 2003). In addition,

the MLCL AT activity was shown to be regulated in concert with the biosynthesis and the level of CL in the heart (Taylor et al. 2002).

It was recently reported, that rat liver and human lymphoblast CL can also be efficiently remodelled into linoleoyl-containing CL species (predominantly tetralinoleoyl and trilinoleoyl-CL) in the presence of labelled linoleoyl-PC by transacylation mechanism (Xu et al. 2003). In rat liver, where linoleic acid was the most abundant residue of CL (Figure 1-3), the linoleoyl groups were specifically transferred into CL, but not oleoyl or arachidonoyl groups. Thus, the complete remodelling of tetraoleoyl-CL to tetralinoleoyl-CL in rat liver mitochondria was demonstrated and the intermediates linoleoyl-trioleoyl-CL, dilinoleoyl-dioleoyl-CL, and trilinoleoyl-oleoyl-CL were identified by HPLC (Xu et al. 2003). A primary defect in human CL remodelling leads to the Barth syndrome, a severe X-linked human disease characterized by cardioskeletal myopathy, neutropenia, and respiratory chain defects (Vreken et al. 2000). The Barth syndrome, which is caused by a defect in the taffazine gene coding for a phospholipid acyltransferase, underlines the importance of CL remodelling mechanisms with regard to the fundamental role of this lipid in the mitochondria.

1.4 Eukaryotic cardiolipin synthases and their properties

In 1971, CL biosynthesis in mammalian mitochondria was demonstrated to require PG and CDP-DAG as substrates in a Mg^{2+}-dependent reaction (Hostetler et al. 1971). The reaction mechanism was demonstrated with a doubly labelled [^{3}H]phosphatidyl / [^{14}C]glycerol. In the presence of CDP-DAG, CL was formed, which had a ^{14}C / ^{3}H ratio identical to that of the PG substrate (Hostetler 1972). It was later demonstrated that the radiolabel from CDP-diacyl[^{14}C]glycerol was incorporated into CL (Stanacev et al. 1973). The properties of the isolated CLS suggest potential mechanisms of regulation on the cellular level (Table 1-1).

Eukaryotic CLS showed striking similarities with respect to their catalytic properties. The enzymes of mammals, yeast and plants required divalent cations for maximal activity, and the optimal cation concentrations were in close range (4-20 mM, Table 1-1). The enzymes displayed, however, different preferences towards cation species. Among the most active cations, only Mg^{2+} seems to be of physiological importance, due to its appropriately high intercellular concentrations.

Furthermore, the basic pH optimum and distinctly lower K_m value towards CDP-DAG than K_m towards PG rounded the properties of eukaryotic CLS. The sensitivity to detergents was one of the interesting differences displayed by the mitochondrial CLS. While the yeast enzyme was stimulated by addition of 0.6 mM Triton X-100, (Tamai and Greenberg 1990), the CLS of mammalian and plant origin where severely inhibited by the detergents. By employing the zwitterionic detergent, however, the mammalian CLS was successfully solubilized and characterized (Schlame and Hostetler 1991).

Table 1-1: Properties of CLSs from different mitochondria

Property	Source of mitochondria			
	Plants [a)]	**Rat liver** [b)]	**Yeast** [c)]	**Other** [d)]
Molecular mass		50 kDa	28 kDa [d)]	
Isoelectric point		pH 4-5		
pH optimum	pH 9.0	pH 8.0	pH 9.0	
Temperature optimum		45 °C	45 °C	
K_m for PG	5.0×10^{-6} M	4.5×10^{-5} M	1.0×10^{-3} M	
K_m for CDP-DAG	8.0×10^{-7} M	1.6×10^{-6} M	3.6×10^{-5} M	
Metal ion selectivity	$Mn^{2+}>Mg^{2+}>Co^{2+}$	$Co^{2+}>Mg^{2+}>Mn^{2+}$	$Mg^{2+}>Co^{2+}$	*N. crassa*, $Co^{2+}>Mg^{2+}$
Optimal cation	16 mM Mn^{2+}	4 mM Co^{2+}	20 mM Mg^{2+}	
Metal inhibitors [f)]		$Cd^{2+}>Zn^{2+}>Ca^{2+}>Ba_{2+}>Cu^{2+}>Hg^{2+}>Ni^{2+}$		
Lipid inhibitors [d), g)]		LPG (K_i 10^{-5} M), LPE (K_i 10^{-4} M), LPC (K_i $10^{-3.5}$ M)	LPG	LPG (in mussels, mung bean, *N. crassa*)
Sensitivity to detergents	Sensitive	Sensitive [h)]	Insensitive	
Sensitivity to SH-reagent *N*-ethylmaleimide [d)]		Insensitive	Insensitive	Insensitive (in mussels, mung bean, *N. crassa*)

a) Frentzen and Griebau 1994, b) Schlame and Hostetler 1991, c) Tamai and Greenberg 1990, d) Schlame 1993, e) Zhao et al. 1998, f) McMurray and Jarvis 1980, g) Schlame 1993, h) Hostetler 1972. LPG, lysophosphatidylglycerol; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine

1.5 Genetic regulation of cardiolipin synthesis

In the mammalian heart, CL is one of the principle phospholipids, comprising as much as 15-20% of the entire phospholipid phosphorus mass of that organ (Figure 1-1-2). Several regulatory mechanisms, that govern CL formation in the heart, have been uncovered. The rate-limiting steps of the CL pathway are catalyzed by the CDP-DAG synthase (Hatch 1994) and PGP synthase (Gaynor et al. 1991). Furthermore, some evidence suggests that the lipid biosynthesis is regulated by the energy status (ATP and CTP levels) of the heart, because phospholipid biosynthesis rates, including those of CL, were attenuated under CTP reduction in the cardiac myoblast. Simultaneously, the re-routing of lipid biosynthesis from PG, CL and phosphatidylinositol (1,2-diacyl-sn-glycero--3-phospho-(1'-myo-inositol), PI) towards diacylglycerol (1,2-diacyl-sn-glycerol; DAG) and triacylglycerol (1,2,3-triacyl-sn-glycerol; TAG) took place. Thus, the cellular CTP concentration may be a switch in the regulation between the phospholipids and TAG biosynthesis (Hatch and McClarty 1996).

Thyroxin is a well-known stimulator of mitochondrial biogenesis. It not only increases the number of mitochondria per cell, but also influences the structural and functional changes in mitochondria, designed to enhance their performance (Paradies and Ruggiero 1990). Upon thyroxin treatment, the increase in mitochondrial CL contents is accompanied by an increased enzymic activities involved in CL synthesis. In rat liver mitochondria, the activities of CLS and glycerolphosphate acyltransferase; GPAT) were elevated about 1.5 fold (Hostetler 1991), while in the heart mitochondria, a three-fold increase in these enzymic activities were observed (Cao et al. 1995). On the other hand, in hypothyroid rats, cardiac CL content was decreased by 29% and this was associated with a 32% decrease in CLS activity (Taylor et al. 2002).

The genetic regulation of the CL biosynthesis was extensively investigated in yeast. The structural genes coding for most of the phospholipid biosynthetic enzymes have been identified and characterized (Greenberg and Lopes 1996, Carman and Henry 1999). Synthesis of the major membrane lipids PI, phosphatidylcholine (1,2-diacyl-*sn*-glycero-3-phosphocholine; *PC*), phosphatidylserine (1,2-diacyl-*sn*-glycero-3-phosphoserine; PS), and phosphatidylethanolamine (1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PE), is regulated by the level of intracellular inositol and can be modified by the exogenous supply of this component. The transcriptional activation of respective genes takes place in the absence of inositol by binding the heterodimers of the transcriptional

regulators INO2 and INO4 to a consensus sequence element located in the promoter region of the inositol-responsive genes. In the presence of inositol, however, the *OPI1* gene product prevents transcription activation (Schlame et al. 2000).

The cross-pathway control of CL biosynthesis exerted by inositol occurs at the PGS step. The inositol-mediated regulation of does not influence the PGS transcript level, nor does it require the INO2-INO4-OPI1 circuit (Zhong and Greenberg 2003). Additionally, the growth in the presence of inositol-depleting drug valporte led to increased enzyme activity, independent of an increased PGS mRNA abundance (Zhong and Greenberg 2003). In accordance with these results, more recent experiments showed that the decreased CL contents and PGS activity in response to inositol are associated with increased phosphorylation of PGS, but not with degradation or mislocalization of the protein (He and Greenberg 2004). Thus, phosphorylation of PGS in response to inositol exhibits a new mechanism of inositol-mediated regulation.

On the other hand, the expression of *CRD1* gene encoding yeast CLS and the CLS activity are nor affected by the presence of exogenous inositol (Greeneberg et al. 1988, Tamai and Greenberg 1990, Karkhoff-Schweizer et al. 1991), and no inositol-responsive consensus sequence is present in the putative promoter region of *CRD1* (Jiang et al. 1999). In the most recent experiments, analyses of the expression level of *lacZ* under *CRD1* promoter (P_{CRD1}-*lacZ*) showed elevated expression in *ino4* but not in *ino2* mutant cells. These data suggest multiple and separate functions for the inositol-responsive *INO2*/*INO4* gene products, which normally operate as a dimmer in regulating the gene function (Su and Dowhan 2006). It seems therefore that the CL pathway is regulated in a different way as the PC pathway, in which all the structural genes are regulated by inositol. One possible explanation is that inositol regulation helps to maintain balance of charged phospholipids in the mitochondrial membrane. This may be achieved through the regulation of PG synthesis. Since PGS catalyzes one of the rate-limiting steps in CL synthesis, there may be no need to regulate the *CLS* in the same manner. Consistent with this hypothesis is the observation that in *S. pombe*, the activity of PI synthase increases, while PGS activity decreases upon addition of inositol to the growth media (Gaynor and Grenneberg 1992). An increase in the PI synthase activity leads to elevated contents of PI, which was shown to inhibit activity of the purified PGS (Jiang et al. 1998).

The data of the *cls* null mutant (Jiang et al. 1999) suggest that *CRD1* gene expression might be subject to regulation by either the level of PG or CL, or CLS protein. Most recent data highlight the regulatory role of PG in the *CRD1* expression (Su and Dowhan 2006). The translation of the *CRD1* gene product was up-regulated with increased PG levels and co-ordinately regulated with the expression of the *PGS* gene. Additionally, expression of CLS was found to be independent of the capacity of yeast cells to synthesize its common substrate, CDP-DAG (Su and Dowhan 2006).

The CL pathway is also regulated by factors which affect mitochondrial development (non-fermentable carbon source, aerobic growth, rho^+ cells), although PGS and CLS are controlled differently (Gaynor et al. 1991, Jiang et al. 1999, Zhao et al. 1998). Yeast is a facultative anaerobe organism, which allows for its growth under conditions requiring functional mitochondria (respiratory growth), but also under conditions in which fully functional mitochondria are not needed (fermentative growth). Yeast cells grown in the presence of fermentable carbon sources displayed distinctly lower *PGS* and *CLS* expression levels and decreased activities of both enzymes as compared with the respiratory growth. Consequently, contrary to cells grown in the presence of non-fermentable carbon sources CL levels were decreased and PG was remained undetectable during fermentative growth (Jiang et al. 1999, Tuller et al. 1998, Gaynor et al. 1991). When grown on non-fermentable carbon sources, the yeast *PGS* derepression takes place already during the logarithmic phase (Zhong and Greenebrg 2003). In rho^0 mutants, that lack the mitochondrial genome, both *CRD1* expression and PGS activity were reduced, but *PGS* expression was not affected (Zhong and Greenberg 2003). Therefore, mitochondrial lipids composition can be altered under circumstances that are not lethal to the cell.

Another mechanism of CL synthesis regulation operates through the growth phase. Expression of *CRD1* is derepressed by a factor 7-10 in stationary cells grown on non-fermentable carbon sources by transcriptional regulation (Jiang et al. 1999), suggesting that CL may play an important role in cell survival. This derepression contrasts sharply with the expression of genes encoding CDP-DAG synthase and the CDP-DAG dependent PS synthase, which are maximally expressed in the exponential phase media lacking inositol, but reduced three- to four fold as the cells enter stationary phase (Hormann et al. 1987).

Finally, yeast CLS activity is regulated by its protein and lipid environment. Rho^0 mutants defective in the assembly of respiratory complex IV display 50% lower CLS

activity than wild-type cells, implying a regulatory relationship between cytochrome *c* oxidase and CLS activity (Zhao et al. 1998). The same mutants showed no decrease in *CRD1* mRNA levels (Jiang et al. 1999), thus suggesting modulation of protein activity in the membrane. CLS activity is also regulated by the membrane lipids, as CL stimulates activity of the purified CLS (Schlame et al.1995).

In comparison with yeast, little is known about the regulation of CL biosynthetic pathway in plants. Although the lipid is present in plant mitochondria in significant amounts (Figure 1-1-2), only singular isozymes catalyzing consecutive steps in the CL pathway in plants were cloned and characterized so far. Information with regard to their genetic control is still not available.

Plant mitochondria possess the potential to synthesize CL from acylated CoA and glycerol. The cloned *A. thaliana* isozyme of the enzyme catalyzing the first reaction in the pathway was detected in both, mitochondrial and ER membranes. It was shown to play a pivotal role in pollen development and male fertility, since a deficiency in *AtGPAT1* in the disruption mutants affected tapetal differentiation and caused most of the microspores to abort before reaching maturity (Zheng et al. 2003).

Although the activity of the LPAAT was unambiguously displayed by plant mitochondria (Frentzen and Griebau 1994), the role of LPA for mitochondrial functionality needs to be addressed.

Only scarce information is available on plant CDP-DAG synthases (Figure 1-4). CDP-DAG resides at the branch point of glycerolipid biosynthesis as the precursor of the phosphoinositides, as well as PG and CL. As a rate-limiting step in both pathways, it is a likely target for regulation. Up to date, only cDNAs of potato and *A. thaliana* encoding CDP-DAG synthases were cloned, and identities confirmed by heterologous expression and enzymic assays (Kopka et al. 1997).. The respective proteins belong to the eukaryotic type of CDP-DAG synthases and contain eight predicted transmembrane-spanning domains (Kopka et al. 1997).

Two *A. thaliana* genes were shown to encode PGS isozymes of different cellular localization: the ER, mitochondria and plastids (Müller and Frentzen 2001, Babiychuk et al. 2003). Intracellular targeting analysis demonstrated the dual targeting of the *PGP1* gene product *in vivo* to both chloroplasts and mitochondria. Accordingly, analysis of the lipid composition showed a dramatic diminution of the plastidial PG pool in the *pgp1* mutant. On the other hand, the PGP1 deficiency had no effects on CL accumulation, mitochondrial structure or function. Thus, the results suggest that in the mutant, PG

formed by the activity of PGP2 in the ER is transported into the mitochondria, and provides the CLS with its substrate (Babiychuk et al. 2003).

1.6 The functional role of cardiolipin

CL is the specific lipid component of mitochondria, and defects in the CL biosynthetic pathway or alteration of its cellular level resulted in mitochondrial dysfunction. Hence, past and ongoing research has tried to characterize the biological functions of CL in this organelle. Perhaps the most intriguing property of CL is its ability to interact with a large number of apparently unrelated proteins, most of which reside in the inner mitochondrial membrane (Table 1-2). It appears that some, if not all, mitochondrial protein complexes contain a distinct number of CL molecules firmly integrated in their quaternary structure. The key enzymes of oxidative phosphorylation founding these complexes are conserved throughout the eukaryotic kingdom, providing a rationale for the universal presence of CL in mitochondria (Schlame et al. 2000).

CL molecules are the essential elements of the interface between the complex and its microenvironment or between subunits within the complex, such that removal of the lipid leads to the disintegration. This interaction typically involves both non-chemical binding and functional activation. In many instances, binding of CL is associated with the functional changes of the protein *in vitro*. It is widely viewed as the key to understanding the biological role of CL in mitochondria. It is most puzzling that the protein binding by CL is relatively non-selective, while many proteins prefer CL over other phospholipids. Clearly, the origin of this one-sided specificity must either be found in the structure of CL or, in some common feature of hydrophobic protein domains (Hoch 1992). Thus, it is the promiscuity in protein interaction, rather than its specificity that is characteristic for CL.

Mitochondrial CL species, especially those with high linoleoyl acyl contents, strongly bind many carrier and enzyme proteins that are involved in oxidative phosphorylation (Table 1-2). The comparison of the yeast *crd1* strain lacking CL with the wild-type revealed that the mitochondrial membrane supercomplexes formed by the multisubunit homodimeric complexes III and IV, required for efficient electron transport, are tightly associated with and stabilized by CL (Schagger 2002, Zhang et al 2002), although the lipid was not essential for their biogenesis in the inner mitochondrial membrane

(Pfeiffer et al. 2003). Tight interactions were shown to take place between CL and ADP-ATP carrier (Hoffmann et al. 1994), cytochrome bc_1, and cytochrome *c* oxidase (Schager 2002). Dissociation of CL from the ADP-ATP carrier could be achieved only under the denaturing conditions (Beyer and Nutscher 1996). On the other hand, the loss of tightly bound cytochrome *c* oxidase did not lead to its complete inactivation (Robinson et al. 1990). Furthermore, CL was implicated in mitochondrial protein transport and may play a role in any of the steps of translocase assembly of mitochondrial proteins (Schlame et al. 2000).

Table 1-2: Cardiolipin-dependent proteins in mitochondria [a)]

Compartment	Protein
Inner membrane	ADP-ATP carrier
	Phosphate carrier
	Pyruvate carrier
	Carnitine carrier
	Complex I
	Complex III
	Complex IV (Cytochrome *c* oxidase)
	Complex V (ATP synthase)
	Cytochrome P450SCC
	CLS
Intermembrane space	Cytochrome *c*
	Creatine kinase

a) For details and references see text. Applied from Schlame et al. 2000.

The evidence suggested that the functional importance of CL for energy transformation strongly depends on the respiratory rate. CL prevents rate-dependent uncoupling of oxidative phosphorylation and ensures osmotic stability of yeast mitochondria in isotonic medium. Recent investigations of the interdependence of the mitochondrial respiratory chain and CL biosynthesis showed that CL biosynthesis *in vivo* was reduced in the respiratory chain assembly mutants (Gohil et al. 2004). Furthermore, disruption of the pH gradient, but not of the membrane potential or ATP synthesis resulted in decreased CL synthesis. These results support the hypothesis that the pathway for CL

biosynthesis in yeast is regulated by the transmembrane pH component of the proton-motive force, generated by the mitochondrial respiratory chain.

The Chinese hamster ovary mutant cells with thermo-labile PGS was defective in the biogenesis of both PG and CL (Ohtsuka et al. 1993) and displayed altered mitochondrial morphology, apparently due to swelling and disorganization of the cristae membrane arrangements (Ohtsuka et al. 1993). Over-expression of a chimeric gene encoding PGS in the mutant cells restored the wild-type phenotype and suggested that PG and CL are required for mitochondrial bioenergetics (Kawasaki et al. 1999). Recent identification of the human *CLS* gene (Houtkooper et al. 2006) opens the possibilities to study the functional role of CL in mammalian cells.

The disruption of yeast *PGS*, which results in a complete loss of both PG and CL (Janitor et al. 1996, Chang et al. 1998), causes a *petite* (respiratory incompetent) lethal phenotype. A translational defect of cytochrome *c* oxidase subunits I-IV and cytochrome *b* was shown to be the reason for this phenotype (Ostrander et al. 2001).

Growth defects of the *pgs* null mutant were more severe than that of the *crd1* null mutant lacking CL only (Chang et al. 1998). Whereas the *crd1* null mutant looses viability on both fermentable and non-fermentable carbon sources at elevated temperature and does not form colonies from single cells seeded on YPD plates (Jiang et al. 1999, Jiang et al. 2000, Zhong et al. 2004), the *pgs*Δ mutant cannot grow at all at 37 °C, even on glucose (Chang et al. 1998). Mutations in yeast *CRD1* encoding CLS resulted in a reduced respiratory rate, reduced ATPase and cytochrome *c* oxidase activity, protein import, and a decreased membrane potential (Jiang et al. 200, Zhong et al. 2004). The mitochondrial membrane potential is most decreased in glucose-grown yeast cells, which lack both PG and CL, but is less apparent in membranes lacking CL only (i.e. grown in non-fermentable carbon sources). Thus, CL is required for a number of mitochondrial functions, only some of which can be partly compensated for by PG.

To understand the essential functions of mitochondrial anionic lipids at elevated temperatures, a *pgs* suppressor mutant that grew on 37 °C was isolated (Zhong et al. 2005). The suppressor was identified as a loss of function allele of *KRE5*, which is involved in the cell wall biogenesis. The authors also suggested that inability of *pgs* null mutant to grow in the presence of ethidium bromide, which induces loss of mt DNA, was due to the defective cell wall integrity, not to *petite* lethality (Zhong et al. 2005). Moreover, the recent evidence suggested that the cross-talk pathways between

mitochondria and the nucleus can be controlled by the level of PG and CL in the mitochondrial membranes (Su and Dowhan 2006).

For the functionality of mitochondria, not only presence of CL *per se*, but also its molecular species is of critical importance. As mentioned before, the impaired remodelling of CL in humans caused by a defect in the human taffazin gene (*TAZ1*) encoding a transacylase is associated with the Barth syndrome (BTHS), a severe genetic disorder characterised by cardiomyopathy, skeletal myopathy and respiratory chain defects (Vreken et al. 2000, Xu et al. 2003). BTHS is associated with striking abnormalities in the fatty acid composition of CL and the presence of multiple molecular CL species, randomly esterified with various fatty acids (Schlame et al. 2002, Schlame et al. 2003, Valianpour et al. 2002, Schlame et al. 2005). Such aberrant acyl pattern is not found in CL form patients with other cardiac or skeletal myopathies (Schlame et al. 2003). Mitochondria from BTHS patients generate insufficient proton-motive force (Xu et al. 2005), presumably as a result of incorrect acyl configuration in CL. The fact that heart and skeletal muscles are very rich in symmetric CL (Schlame et al. 2005), express the *CLS* gene at high levels (Lu et al. 2006) and at the same time are tissues most affected in BTHS (Barth et al. 1999, Kelley et al. 1991) is another piece of circumstantial evidence to support the pivotal role of the CL molecular species.

Likewise, deletion of the yeast homolog of *TAZ1* leads to the temperature sensitivity of cells in ethanol as a sole carbon source, but viability on glucose or glycerol plus ethanol (Gu et al. 2004). The yeast Taz1p is exclusively localized in mitochondria (Ma et al. 2004). The mitochondria from *taz1*Δ mutant cells exhibited defective energy coupling at the elevated temperatures, because their rate of oxidative phosphorylation and their membrane stability was compromised at increased temperature and under hypotonic stress (Ma et al. 2004). The total CL content was reduced in *taz1*Δ, and MLCL accumulated. The predominant acyl species of CL found in the wild-type cells, oleoyl and palmitoleoyl (Figure 1-3), were markedly reduced in the mutant at the expense of species with saturated fatty acids. Interestingly, CL synthesis increased in the mutant, although expression of *CRD* and *PGS* genes did not. This result suggested a link between enzyme activities of the *de novo* CL biosynthesis and CL acylation (Gu et al. 2004).

In eukaryotic cells, the dissociation of cytochrome *c*, normally bound to the inner mitochondrial membrane by an association with CL, is a critical step for cytochrome *c* release into the cytosol and the induction of apoptosis. In particular, it was demonstrated

that the exposure of submitochondrial particles to reactive oxygen species produced by the mitochondrial electron transport chain results in a mobilization of cytochrome *c* and a concomitant loss of CL (Paradies et al. 2001). Similarly, other studies showed that lowering the mitochondrial CL contents correlates not only with a decrease in respiration, but also with a stoichiometric increase in cytochrome *c* release (Ostrander et al. 2001). Furthermore, a peroxidation of CL rather than permeabilization of the outer mitochondrial membrane by oligomeric Bax was shown to be the critical first step for the release of cytochrome *c* (Ott et al. 2002).

In summary, these findings indicate that CL plays an important role in the structure and function of the respiratory chain, as well as in the retention of cytochrome *c* within the intermembrane space. In yeast cells, the mitochondrial anionic lipids are also required for the cellular functions that are essential in cell wall biogenesis, the maintenance of the cell integrity, survival at elevated temperature, and appear to be essential to regulate the cross-talk pathways between mitochondria and the nucleus.

1.7 Goals of the research

Unlike for the yeast or mammalian CL biosynthesis, little is known about the respective pathway in the plant cells, the mechanisms that control the plant CL biosynthesis, or *in vivo* functions of the lipid in plant cells. As a first step to address these topics, the gene encoding *A. thaliana* CLS should be identified, by taking an advantage of the availability of the plant genome sequence. Subsequent over-expression of the *CLS* sequence in a suitable microorganism would gain access to the protein, in order to study its enzymic properties.

Additionally, the possession of the sequence information would raise the possibility to isolate and analyse the *A. thaliana* mutants carrying T-DNA insertion in the *CLS* gene. Cultivated as cell suspension cultures, the mutant plants would provide the material for the extraction and analyses of mitochondria, organelle of almost exclusive location of CL. This should facilitate the investigations on the plant CL biosynthesis and functions.

2 Materials and Methods

2.1 Biological Materials

The following organisms were used throughout the study:

Organisms	Strain (Reference)	Genotype / Comment
Escherichia coli	TOP10 (Invitrogen, Karlsruhe)	F^-, *mcrA*, Δ*(mrr-hsdRMS-mcrBC)*, ø80Δ*lacZΔM15*, Δ*lacX74*, *recA1*, *ara*Δ*139*, Δ*(ara-leu)7697*, *galU*, *galK*, *rpsL*, (Str^R), *endA1*, *nupG*
Escherichia coli	DH5α (Hanahan 1983)	F^-, ø80*lacZ*Δ*M15*, Δ*(lacZYA-argF)U169*, *deoR*, *recA1*, *endA1*, *hsdR17(*rK^-, mK^+*)*, *phoA*, *supE44*, λ–, *thi-1*, *gyrA96*, *relA1*
Agrobacterium tumefaciens	ATHV C58C1 (Hood et al. 1986)	strain carries chromosomal rifampicin resistance (Rif^R) and hosts the helper plasmid pEHA101
Saccharomyces cerevisiae	UTL7A (Warnecke et al. 1999)	MATα, *ura3-52*, *trp1*, *leu2-3,112*, *trp1*
	D2000 (Tuller et al. 1998)	MATα, *ura3-52*, *trp1Δ63*, *leu2Δ1*, *his3*Δ*200*, *cls1*::KanMX4
Arabidopsis thaliana	ecotype Columbia	CS60000, the sequenced genome.
	derivatives of ecotype Columbia (Alonso et al. 2003)	T-DNA insertional mutants were obtained from NASC (Loughborough, UK)

Vectors used throughout the study:

Plasmid	Selection marker	Source
pBAD-TOPO	Amp^R	Invitrogen
pYES2	Amp^R, Uracil selection	Invitrogen
pB6actin1[a)]	$Basta^R$	Kushnir, kind gift
pBScremtCM18[b)]	$Chloramphenicol^R$	Kushnir, kind gift

a) backbone of the plasmid is pCAMBIA vector, derivative of pPZP200 (Hajdukiewicz et al. 1994); b) pBluescript KS (MBI Fermentas, St. Leon - Rot) derivative, containing gene expression cassette of CRE under heat shock promoter (hsp-CRE); c) derivative of pBluescript (MBI Fermentas) vector, carrying a chloramphenicol resistance cassette.

The maps of respective vectors, as far as they are available, are presented in 5.1 (Appendix).

2.2 Manipulation and analysis of nucleic acids and proteins

2.2.1 Analysis of nucleic acids

Endonuclease restriction of nucleic acids was performed according to standard procedures (Sambrook et al. 1989) and under conditions recommended by the manufacturers (New England Biolabs, Frankfurt/Main; MBI Fermentas). Restriction analysis was performed by means of agarose gel electrophoresis (see 2.4).
DNA sequencing was performed at the sequencing unit of the Fraunhofer IME (Aachen) and Seqlab GmbH (Göttingen).

The sequence data were analysed using the following programmes:

Chromas	http://www.technelysium.com.au/chromas.html	
CLUSTAL X	http://www-igbmc.u-strasbg.fr/BioInfo/	Thompson et al. 1997
GeneDoc	http://www.psc.edu/biomed/genedoc/	
BLAST	http://www.ncbi.nlm.nih.gov/BLAST/	Altschul et al. 1990
TreeView	http://taxonomy.zoology.gla.ac.uk/rod/rod.html	

For extraction of DNA fragments from agarose gels, the Qiaex II Kit (Qiagen GmbH, Hilden) was employed according to the instructions of the manufacturer.

2.2.2 Modification of DNA fragments: creation of blunt end DNA fragments

Fill in of 5'-termini and removal of 3'-termini from DNA fragments in order to produce blunt ends was performed by using the polymerase and 3'→5' exonuclease activity of the Klenow (Large DNA Polymerase I) fragment (MBI Fermentas), according to the instructions of the manufacturer. The enzyme was inactivated by phenol/chloroform extraction of DNA fragments (2.8.3).

2.2.3 Modification of DNA fragments: dephosphorylation of DNA fragments

Calf intestine alkaline phosphatase (CIAP, MBI Fermentas) was used according to the protocol of manufacturer, to catalyse the release of 5'-phosphate groups from DNA fragments, to preserve self ligation of DNA termini during the reaction performed by DNA ligases.

2.2.4 Modification of DNA fragments: ligation of sticky and blunt DNA ends

The formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA (ligation) was catalysed by T4 DNA ligase (MBI Fermentas), as described by Sambrook et al. 1989, and following the instructions of manufacturer. A molar ratio of 3:1 (insert/dephosphorylated vector, approximately 100 ng) was applied, unless otherwise stated. To improve ligation reaction outcome, sticky end or blunt end ligation was conducted overnight at 16 °C. Blunt end ligation was conducted in presence of 5% PEG 4000 (v/v).

2.2.5 Quantification of nucleic acids

A spectrophotometric measurement at a wavelength of 260 nm was used as standard procedure for quantification of isolated nucleic acids (Sambrook et al. 1989).

The purity of isolated nucleic acids was estimated by the $OD_{260\ nm}$ / $OD_{280\ nm}$ quotient. A ratio of 1.8 – 2 indicates a pure DNA, a pure RNA should have a quotient higher than 2.

Nucleic acids concentrations were calculated according to the rule, that one unit of $OD_{260\ nm}$ equals 50 µg/ml of aqueous suspension of dsDNA, or 40 µg/ml of aqueous suspension of RNA, or 33 µg/ml of aqueous suspension of ssDNA or oligonucleotide, respectively.

Additional analysis of quality and quantity of isolated nucleic acids was performed by horizontal agarose gel electrophoresis and ethidium bromide staining, along with molecular mass markers (see below).

2.3 Protein concentration measurement

Protein concentration measurement was performed according to an established method of Bradford 1976, using bovine serum albumine (BSA) as a standard.

2.4 Gel electrophoresis of macromolecules

During electrophoresis, nucleic acids or protein molecules are separated according to their migration velocities in an electrical field, which is applied to the gel matrix. The migration velocity is dependant on the strength of the electric field, the netto charge on

the molecule, its size and conformation, as well as the ionic strength, the pore size and the temperature of the gel matrix.

2.4.1 Agarose gel electrophoresis of nucleic acids

The size, quantity and quality (shearing) of nucleic acids (isolated plasmids, endonuclease restricted fragments, PCR products, genomic DNA, total RNA) were determined by electrophoresis in agarose gels of agarose concentration appropriate to nucleic acid fragments (Sambrook et al. 1989), containing 30 µg/l of ethidium bromide. Agarose was solved in TAE buffer. Samples to be analysed were mixed with 0.1 volume of 10 × loading buffer, loaded on and separated by gel electrophoresis in TAE buffer with 10 V/cm in horizontal gel chambers. Results were visualized under UV light and recorded with a video camera system (Herolab, Wiesloch). The size and quantity of nucleic acid bands were estimated by comparison with co-migrating molecular weight standards.

10 × DNA loading buffer

Component	Final concentration
bromophenol blue	0,25% (w/v)
xylene cyanol	0,25% (w/v)
Tris-HCl, pH 7.6	50 mM
glycerol	40% (v/v)

50 × TAE buffer

Component	Final concentration
Tris–acetate, pH 7.5	40 mM
Na_2EDTA, pH 8.0	1 mM
glacial acetic acid	20 mM

2.4.2 Electrophoresis in denaturating sodium dodecyl sulphate polyacrylamide gels

The experiments were carried out using Laemmli SDS-PAGE reducing system (Laemmli 1970, Sambrook et al. 1989). In this method, protein samples are denaturated in presence of both, SDS and reducing agent, enabling denaturation of tertiary and quaternary structures, thus maximizing protein linearization. Stacking gel, separating gel (8 – 15% by volumes, depending on application; sample separation by 20 V/cm) and running buffer contains SDS to keep migrating proteins uniformly charged. Molecular mass of proteins separated was estimated by co-migrating mass standard. Proteins were visualized by staining for 15 min in Coomassie staining solution and subsequent destaining with Coomassie destaining solution for 1 h.

SDS gels

Component	Stacking gel	Separating gel
acrylamid/bisacrylamid (37.5:1)	5% (v/v)	8 – 15% (v/v)
Tris-HCl, pH 6.8	135 mM	-
Tris-HCl, pH 8.8	-	375 mM
SDS	1% (w/v)	0.5% (w/v)
APS	0.1% (w/v)	0.1% (w/v)
TEMED	0.01% (v/v)	0.01% (v/v)

Sample buffer, 2 ×

Component	Final concentration
Tris-HCl, pH 6.8	125 mM
glycerol	20% (v/v)
DTT	200 mM
bromophenol blue	0.2% (v/v)
SDS	4% (v/v)

SDS-PAGE running buffer, 10 ×

Component	Final concentration
Tris	3.03% (w/v)
glycine	14.41% (w/v)
SDS	1% (w/v)

Coomassie staining solution

Component	Final concentration
Coomassie Brilliant Blue G-250	0.2% (w/v)
acetic acid	10% (v/v)
methanol	30% (v/v)

Coomassie destaining solution

Component	Final concentration
acetic acid	10% (v/v)
methanol	30% (v/v)

2.5 Protein immunoblot

By employing the protein-specific detection antibodies, the protein immunoblot (Western blot) allows to determine the molecular weight of a sample protein and to measure the relative amounts of the protein present in different samples. Western blotting was used to analyse cloned tagged plant cardiolipin synthase. Membrane proteins isolated from *E. coli* or *S. cerevisiae* cells over-expressing plant CLS were separated by SDS-PAGE electrophoresis and then transferred (Semi-Dry Blotter, Carl Roth GmbH, Karlsruhe) to a sheet of pre-soaked PVDF membrane (Roche Diagnostics, Penzberg). The blot was incubated with milk proteins suspension (5% dry milk in TTBS Buffer) to block any remaining sticky places on the membrane. Anti-His antibody suspension, which is able to bind to 6 × His-tagged proteins (Penta-His, Qiagen; 1:2,000 dilution in TTBS buffer), was then applied (shaken overnight, 4 °C). Goat anti-mouse horse radish peroxidase (HRP) conjugate, (Dianova GmbH, Hamburg; 1:2,000 dilution in TTBS buffer) was used as a secondary antibody and the chemiluminescent western

blotting substrate (Lumi-Light^{PLUS} Roche Applied Science, Mannheim) was used for detection.

TTBS buffer

Component	Final concentration
Tris-HCl, pH 7.5	20 mM
NaCl	500 mM
Tween 20	0.05% (v/v)

2.6 Cloning and expression in *Escherichia coli*

2.6.1 Cultivation of *Escherichia coli*

E. coli strains were cultured according to standard protocols (Sambrook et al. 1989). Wild-type and transformed strains of *E. coli* were grown in Luria-Bertani (LB) medium, Terrific Broth (TB) medium or on agar-fixed LB plates at 37 °C supplemented with appropriate antibiotics for selection purposes.

The absorbance of growing bacterial cultures was measured by a spectrophotometer. After comparing the absorbance of grown cultures with that of sterile medium at wavelength of 600 nm, the optical density (OD) can be measured. One unit of $OD_{600\ nm}$ of an *E. coli* culture approximates 10^9 cells (Sambrook et al. 1989).

To prepare glycerol stocks of grown cultures, 500 µl of fresh culture was mixed with 500 µl of sterile glycerol solution (25 mM Tris-HCl, pH 7.5; 70% (v/v) glycerol) and stored at - 80 °C.

LB medium, pH 7.0 (NaOH)

Component	Final concentration
tryptone	1% (w/v)
yeast extract	0.5% (w/v)
NaCl	0.5% (w/v)
For plates: agar	1.5% (w/v)

TB Medium

Component	Final concentration
tryptone	1.2% (w/v)
yeast extract	2.4% (w/v)
glycerol	0.4% (w/v)
KH_2PO_4	17 mM
K_2HPO_4	72 mM

Antibiotic	Solvent used	Final concentration
carbenicillin	water	50 mg/l
chloramphenicol	70% (v/v)ethanol	30 mg/l
kanamycin	water	25 – 50 mg/l
streptomycin	water	100 mg/l
spectinomycin	water	50 – 100 mg/l
rifampicin	70% (v/v)ethanol	20 mg/l

2.6.2 Preparation of chemically competent cells

E. coli competent cells were prepared according to the rubidium chloride transformation protocol (New England Biolabs). This procedure improves the transformation with most strain backgrounds, when compared with the $CaCl_2$ procedure. A single colony coming from an *E. coli* strain streaked on an agar-LB plate and grown at 37 °C was shaken overnight in 5 ml of LB medium. 2 ml of this pre-culture was inoculated into 200 ml of pre-warmed LB medium and grown at 37 °C until the culture reached a logarithmic growth phase (OD_{600} of 0.4 - 0.6). The culture was cooled on ice and all following steps of the procedure were carried out at 4 °C. After pelletting the cells (10,000 × *g*, 10 min, 4 °C), the supernatant was removed, the remaining pellet was washed with 50 ml of ice-cold TfB I buffer and incubated 10 min on ice. The pellet resulting from second centrifugation (3,200 × *g*, 10 min, 4 °C) was resuspended in 10 ml of TfB I buffer and centrifuged as before. The resulting cell pellet was gently suspended in 4 ml of TfB II buffer, aliquoted to pre-chilled tubes, and instantly snap-frozen in liquid nitrogen. Aliquots of competent cells were stored at - 80 °C.

TfB I buffer,

pH 5.8 (Acetate)

Component	Final concentration
KOAc	30 mM
$MnCl_2$	50 mM
RbCl	100 mM
$CaCl_2$	10 mM
glycerol	15% (v/v)

TfB II buffer,

pH 6.8 (NaOH)

Component	Final concentration
RbCl	10 mM
$CaCl_2$	75 mM
MOPS	10 mM
glycerol	15% (v/v)

2.6.3 Transformation of *Escherichia coli*

Competent cells obtained according to the protocol described above were used for transformation. An aliquot of thawed competent cells upon thawing was incubated for 30 min on ice in presence of DNA (100 – 200 ng) or ligation product (maximum 20 µl). After a 60 s heat shock at 42 °C, 0.4 ml of SOC medium was added and cells recovered at 37 °C for one hour. 50 – 200 µl of transformed cell suspension was spread on LB plates supplemented with appropriate antibiotics and incubated overnight at 37 °C.

SOC medium

Component	Final concentration
tryptone	2% (w/v)
yeast extract	0.5% (w/v)
NaCl	0,05% (w/v)
KCl	25 mM
$MgCl_2$	10 mM
glucose	20 mM

2.6.4 Isolation of plasmid DNA from *Escherichia coli*

The method applied for small scale isolation of plasmid DNA is a modified rapid boiling method published previously (Riggs and Mclachlan 1986). A single colony was inoculated into 5 ml of LB containing appropriate antibiotics and shaken overnight at 37 °C. The cell pellet harvested by centrifugation of the culture (20,000 × *g*, 1 min, 20 °C) was suspended in 200 µl of BF buffer containing 1 mg/ml lysozyme. After 5 min digestion at 20 °C, the cell suspension was incubated at 95 °C for 1 min and quickly cooled down on ice for 5 min. The cell debris, denatured proteins and chromosomal DNA were pelletted by centrifugation (20,000 × *g*, 30 min, 20 °C). By adding isopropanol to 60% (v/v) and ammonium acetate to 600 mM to the resulting supernatant, plasmid DNA was precipitated for 30 min at 20 °C and sedimented by centrifugation (20,000 × *g*, 30 min, 20 °C). After washing the plasmid DNA pellet with 500 µl of 70% (v/v) ethanol, it was dried and, finally, suspended in 50 µl of 10 mM Tris-HCl, pH 8.0 with 50 µg/ml RNase A.

To obtain high quality and quantity plasmid DNA, Invisorb Spin Plasmid mini Kit (Invitek, Berlin) was employed. The isolation of plasmid DNA from *E. coli* cultures was conducted according to the instructions of the manufacturer.

For restriction analysis of plasmid DNA, 300 ng of DNA suspension was digested with appropriate restriction endonuclease according to the instructions of the enzyme manufacturer, and analysed by horizontal agarose gel.

BF buffer

Component	Final concentration
sucrose	8% (w/v)
Triton X-100	0.5% (w/v)
EDTA, pH 8.0	50 mM
Tris-HCl, pH 8.0	10 mM

2.6.5 Cloning and functional expression of cardiolipin synthase in *Escherichia coli*

The cDNA of genomic sequence AF118223.2 (Müller and Frentzen 2001) was obtained by RT-PCR (One-Step RT-PCR Kit, Qiagen) on *A. thaliana* ecotype Columbia RNA, using primers CLS_F1 and CLS_Rv (for details, see 2.9; primers sequences are listed in primers table, Appendix, 5.1). The sequence encoding the putative mitochondrial transit peptide was removed and 5'- or 3'-terminal His-tag sequences were inserted by amplifying full-length construct with *Pfu* polymerase (Promega) and respectively modified primers: CLS_F1-28, CLS_RvHis, CLS_F1-NHis (Appendix, 5.1). Amplification of the same sequence missing the putative signal sequence and Exon 8 of the predicted open reading frame was achieved by employing primers CLS_F1-28 in combination with CLS7_Rv or CLS7_RvHis (for addition of 3'-terminal His-tag). The correctness of each construct was confirmed by sequencing. The modified cDNA sequences were introduced to pBAD-TOPO plasmid (pBAD-TOPO Expression Kit, Invitrogen), and over-expressed in *E. coli* TOP10 strain according to the instructions of the manufacturer. This strain was cultivated at 37 °C and exponentially growing cultures were induced with 0.2% (w/v) L-arabinose and 1 mM isopropyl-β-d-thiogalactopyranoside. Cells were harvested 3 h after induction (3,200 × *g*, 10 min, 20 °C), unless otherwise stated.

2.6.6 Preparation of subcellular fractions from *Escherichia coli*

The induced *E. coli* cells harvested after 3 h of induction were washed with 50 mM Tris-HCl, pH 8.0, and suspended in isolation buffer (0.1 g fresh weight cells per ml of buffer). The cells were sonicated 2 times for 3 min at 50% power on ice (sonicator model UW70, Bandelin electronic, Berlin). The supernatant obtained by 10 min

centrifugation at 5,000 × *g*, 4 °C was used for ultracentrifugation (110,000 × *g* 35 min, 4 °C). The pelletted membranes were resuspended in the isolation buffer containing 50% glycerol (v/v) and stored at - 80 °C.

Isolation buffer

Component	Final concentration
Tris-HCl, pH 8.0	50 mM
DTT	5 mM
Pefabloc SC	200 µM

2.7 Cloning and expression in *Saccharomyces cerevisiae*

2.7.1 Cultivation of *Saccharomyces cerevisiae*

To cultivate *S. cerevisiae*, two media were used: YPD as full medium, whereas complete dropout medium (CMdum, Ausubel et al. 1995) was employed as minimal medium for the growth of yeast cells transformed with pYES vector constructs carrying uracil selection. For growth, both media were supplemented with 2% (w/v) glucose as a carbon source. To induce a galactose dependent promoter of the pYES vector, complete minimal dropout medium containing glucose was exchanged for medium containing 2% (w/v) galactose.

In brief, a 20 ml of selective medium inoculated with a single colony was incubated under constant shaking (100 rpm) for 48 h at 30 °C. The cells were introduced into a main culture of 200 ml and incubated for 48 hours at 30 °C under constant shaking (see above). The cells were pelletted, resuspended in 200 ml medium containing 2% (w/v) galactose and cultivated under constant shaking (see above) for 24 h at 30 °C. The induced cells were harvested by centrifugation (4,000 × *g*, 10 min, 4 °C), washed twice with water, and used for the preparation of subcellular fractions, as described below.

YPD medium

Component	Final concentration
pepton	2% (w/v)
yeast extract	1% (w/v)
NaOH	5 mM
glucose	2% (w/v)
For plates: agar	1.5% (w/v)

YNB medium

Component	Final concentration
YNB	1.7% (w/v)
$(NH_4)_2SO_4$	44.6 mM

DP dum mix, pH 1.1 (HCl)

Component	Final concentration
amino acids mix	0.9% (w/v)

Amino acids mix

Component	Final amount
adenine (hemisulfate)	2.5 g
L-arginine	1.2 g
L-aspartate	6.0 g
L-glutamate (Na salt)	6.0 g
L-lysine (HCl)	1.8 g
L-methionine	1.2 g
L-phenylalanine	3.0 g
L-serine	22.5 g
L-threonine	12.0 g
L-tyrosine	1.8 g
L-valine	9.0 g

CMdum minimal medium

Component	Final concentration
YNB medium	10% (v/v)
DP dum Mix	5% (v/v)
glucose or galactose	2% (w/v)

2.7.2 Transformation of *Saccharomyces cerevisiae*

A modified lithium acetate method (Ausubel et al. 1995) was applied to prepare competent *S. cerevisiae* cells. A single colony of the *S. cerevisiae* D2000 strain was inoculated into 10 ml of YPD medium and grown overnight under constant shaking at 30 °C. 2 ml of this pre-culture was then inoculated into 200 ml YPD medium (main culture), and grown under constant shaking at 30 °C until $OD_{600\,nm}$ reached 0.5 (6 – 8 h). After pelletting the cells (4,000 × *g*, 5 min, 20 °C), they were washed with 10 ml sterile water, then washed with 10 ml LiSORB buffer and finally, the cell pellet was resuspended in 100 µl LiSORB buffer.

For transformation, a bait assay was used. In short, 10 µl fragmented carrier-DNA (Yeastmaker Carrier DNA, BD Biosciences Clontech, Heidelberg) per transformation was pre-warmed for 5 min at 95 °C, mixed with 200 µl LiSORB buffer and cooled on ice to room temperature.

In a sterile reaction tube, 2 – 3 µg plasmid DNA were mixed with 25 µl carrier-DNA suspension and 25 µl competent cells and incubated for 30 min at room temperature. After adding 450 µl LiPEG buffer, the mixture was incubated one more time at room temperature for 30 min. Finally, 50 µl DMSO was added and the whole mixture was heat-shocked for 10 min at 42 °C. The cells were harvested by centrifugation (4,200 × *g*, 2 min, 20 °C). The resulting cell pellet was resuspended in water, plated on selection medium, and ultivated for 2 - 3 days at 25 °C.LiSORB buffer

Component	Final concentration
LiAc, pH 7.5	100 mM
sorbitol	1 M
Tris-HCl, pH 7.5	10 mM
Na_2EDTA, pH 8.0	0.1 mM

LiPEG buffer

Component	Final concentration
LiAc, pH 7.5	100 mM
PEG	40% (v/v)
Tris-HCl, pH 7.5	10 mM
Na_2EDTA, pH 8.0	0.1 mM

2.7.3 Cloning and functional expression of cardiolipin synthase gene in *Saccharomyces cerevisiae*

To clone the whole CLS ORF with and without a 6 × His-tag sequence at its 3'-terminus into the pYES vector (Invitrogen, Karlsruhe), yielding the pYCLS, or pYCLS-his constructs, respectively. The restriction sites were added by PCR and the correctness of the constructs was verified by sequencing. The expression of the cloned ORF was performed according to the procedure described above.

2.7.4 Preparation of subcellular fractions from *Saccharomyces cerevisiae*

The preparation of subcellular fractions from galactose induced *S. cerevisiae* D2000 carrying either pYES (negative control) or a pYCLS construct was performed according to a modified method published previously (Zinser and Daum 1995). The method bases on the enzymatic lysis of the yeast cell wall by zymolyase under light hyperosmotic conditions and disintegration of the spheroplasts in a Dounce homogenizer.

The harvested cell pellet was suspended in 100 mM Tris-HCl, pH 9.4 (5 ml/g wet cell weight) and 10 mM DTT. The cell suspension was shaken for 30 min at 30 °C. After sedimenting the cells (4,000 × *g*, 10 min, 20 °C), they were resuspended in zymolyase buffer (5.7 ml/g wet cell weight) and digested for 1 h at 30 °C under light shaking with zymolyase (5 mg/g wet cell weight).

The obtained spheroplasts were sedimented at 4,000 × *g* for 10 min at 4 °C and gently washed with ice-cold zymolyase buffer, and washed twice with ice-cold homogenization buffer (1 ml/g wet cell weight) containing 1 mM PMSF (phenylmethylsulfonfluorid, Boehringer, Mannheim; dissolved in DMSO). Finally, the spheroplasts were resuspended in same buffer, homogenized in a Dounce homogenizer

by 15 strokes, and centrifuged (4,000 x *g*, 10 min, 4 °C). The supernatant was stored on ice, while the pellet was subjected to two more cycles of suspending, homogenizing and sedimenting. The combined supernatant fractions were further separated by differential centrifugation (10,000 × *g*, 20 min, 4 °C). The mitochondria present in the 10,000 × *g* pellet were resuspended in homogenization buffer and separated from spheroplast debris (3,000 × *g*, 10 min, 4 °C). Mitochondria present in the supernatant were sedimented at 10,000 × *g* for 10 min at 4 °C, washed twice with homogenization buffer and, finally, resuspended in 10 mM Tris-H_2SO_4, pH 7.4 containing 50% (v/v) glycerol and stored at -80 °C for further analyses.

The microsomes present in the 10,000 x *g* supernatant fraction were densified by ultracentrifugation (120,000 × *g*, 1 h, 4 °C) and the pellet was suspended in a small volume of 10 mM Tris-H_2SO_4 pH 7.4 containing 50% (v/v) glycerol. The microsomal suspension was stored at -80 °C.

Zymolyase buffer

Component	Final concentration
sorbitol	1.2 M
H_2KPO_4, pH 7.4	20 mM

Homogenization buffer

Component	Final concentration
mannitol	0.6 M
Tris-H_2SO_4, pH 7.4	10 mM

2.8 Analyses and manipulations of *Arabidopsis thaliana*

2.8.1 Cultivation of *Arabidopsis thaliana*

A. thaliana plants were grown under sterile conditions on MS-agar medium (liquid and solid) or as callus cultures (on plates and in cell suspension cultures) and on earth. The seeds of T-DNA insertional mutants of *A. thaliana* were obtained from NASC (Alonso et al. 2003).

Wild-type and mutants *A. thaliana* seeds were surface sterilized by shaking for 5 min in 70% ethanol. After washing the seeds 5 times with sterile water, they were plated on MS medium plates. The plates were incubated overnight at 4 °C in darkness to stratify the seeds, and subsequently at 22 °C with 8 h excitation, 16 h darkness. Seedlings grown under these conditions for 10 – 14 days were transferred to earth pots. For ^{33}P labelling experiments or to obtain material for callus culture, sterile grown seedlings were transferred to 1 ml plant liquid medium in 50 ml conical flasks, and grown under short day conditions and constant shaking (80 rpm). For flowering and producing next generation, plants were grown under long day conditions (16 h excitation, 8 h darkness).

MS medium plates

Component	Final concentration
Murashige & Skoog medium (incl. Gamborg B5 vitamins)	0.44% (w/v)
sucrose	5% (w/v)
agar	1.5% (w/v)

Plant Liquid Medium

Component	Final concentration
Murashige & Skoog medium (incl. Gamborg B5 vitamins)	0.44% (w/v)
sucrose	3% (w/v)
6-benzylaminopurine (BAP)	0.0045% (w/v)
α-naphtalene acetic acid (NAA)	0.04% (w/v)

2.8.2 Analysis of T-DNA insertional mutants

For precise location of T-DNA insertion, seeds of T-DNA transformed lines for *At4g04870* obtained from NASC collections were grown in earth under short day conditions, genomic DNA was isolated and T-DNA flanking plant DNA was recovered by PCR and sequenced. A list of primers for amplification of the flanking regions of T-DNA transformed lines is presented in 5.1. Details regarding recovery of flanking DNA are provided in 2.9.

2.8.3 Isolation of plant genomic DNA

The genomic DNA of *A. thaliana* was isolated by phenol/chloroform/isoalmyalcohol (25:24:1, by volumes) extraction. 100 – 300 mg of liquid nitrogen frozen leaf material was grinded and homogenized in 500 µl of DNA extraction buffer under constant shaking (800 rpm) for 10 min at 25 °C. Genomic DNA extracted by adding the same volume of phenol/chloroform/isoamylalcohol. The phases were separated by centrifugation (20,000 x *g*, 10 min, 20 °C), the water phase was transferred to a new vessel and re-extracted in the same manner. The resulting supernatant was mixed with 2 volumes ethanol and 0.1 volumes 3 M Na-acetate, pH 5.2 and incubated at – 20 °C for 20 min. The precipitated genomic DNA was pelletted (20,000 x *g*, 10 min, 20 °C), suspended in water containing 50 µg/ml RNase A, and stored at – 20 °C.

DNA extraction buffer

Component	Final concentration
Tris-HCl, pH 8.5	100 mM
NaCl	100 mM
EDTA, pH 8.0	10 mM
SDS	0.2% (v/v)

2.8.4 Isolation of plant RNA

A. thaliana RNA isolation was carried out using a modified TRIZOL-method (Chomczynski 1993). The method is similar to phenol/chloroform extraction (2.8.3), but using water-equilibrated phenol pH 5.5 a separation of nucleic acids was achieved. A leaf fragment of 10 mg was frozen in liquid nitrogen directly upon harvesting, homogenized using pestle (1 min, pre-cooled metal holder), and suspended in 1 ml TRIZOL by short mixing. After 10 min incubation at room temperature, 100 µl bromchloropropane was added to improve separation of the phases. The suspension was thouroughly mixed for 10 s and incubated for 10 min at room temperature. Phases were separated by centrifugation (20,000 × *g*, 4 °C, 10 min). The aqueous upper phase was transferred to a new vessel and RNA was precipitated by adding 1 volume of isopropanol and incubating at room temperature for 15 min. The precipitated RNA was sedimented (20,000 × *g*, 10 min, 4 °C), washed with 70% (v/v) ethanol, centrifuged (20,000 × *g*, 5 min, 4 °C), and, after complete removing traces of supernatant, air-dried

shortly. Isolated RNA was suspended in 30 µl water and stored at - 80 °C. Quality and quantity of isolated RNA were investigated as described in 2.2.

TRIZOL

Component	Final concentration
guanidinium thiocyanate	0.8 M
ammonium thiocyanate	0.4 M
NaAc, pH 5.0	0.1 M
glycerol	5% (w/v)
phenol (water equillibrated)	38% (v/v)

2.8.5 Transformation of Arabidopsis thaliana

Creation of Cre/lox constructs

Cre/lox is a method, allowing to remove a sequence of interest flanked by recombination sites from the bacteriophage P1 Cre/lox recombination system (Dale and Ow 1991). The full ORF of *A. thaliana At4g04870* gene in the pB6actin1 vector, carrying additionally a Cre recombinase ORF under the control of a heat-shock gene promoter, was introduced into *A. thaliana* mutant plants by means of *A. tumefaciens* floral dip transformation to obtain transgenic plants in which the *CLS* gene can be excised by heat treatment. Vectors for creating the final construct were kindly provided by S. Kushnir (Gent, Belgium).

Dip transformation of *Arabidopsis thaliana* plants

The method for floral dip transformation of *A. thaliana* was applied, as described by Clough and Bent 1998. Seedlings of mutant plants were transferred from MS plates into pots and grown under long day conditions (see 2.8). When most plants had produced the first inflorescence, its termial part was clipped to increase the flower nubmer and plants were cultivated for a further week. The *A. tumefaciens* strain carrying respective construct was grown in 250 ml YEP medium containing respective antibiotics for at least 48 h at 26 °C, until $OD_{600\ nm}$ reached 2. Cell pellet harvested by centrifugation (4,000 × *g*, 10 min, 20 °C) was suspended in transformation medium to an $OD_{600\ nm}$ of 0.8. Aerial parts of blossoming *A. thaliana* plants were submerged in bacteria

suspension 3 times for 3 minutes. The plants were sprayed with bacteria suspension, returned to a 16 °C dark chamber, covered with a plastic dome and incubated for 24 h. Finally, the plants were grown under long day conditions (see 2.8) and seeds were harvested from dried brown siliques.

Transformation medium

Component	Final concentration
sucrose	3% (w/v)
Silwet L-77	0.02% (v/v)

2.8.6 Analysis and manipulations of *Agrobacterium tumefaciens*

Cultivation of *Agrobacterium tumefaciens*

Wild-type and transformed *A. tumefaciens* C58C1 ATHV strains were grown at 28 °C in YEP Medium (An et al. 1988) supplemented with Rifampicin (20 mg/l) and, when necessary, with appropriate antibiotics (Walkerpeach and Velten 1994).

YEP medium, pH 7.2

Component	Final concentration
pepton	1% (w/v)
yeast extract	1% (w/v)
NaCl	0.5% (w/v)
For plates: agar	1.5% (w/v)

Transformation of *Agrobacterium tumefaciens*

A. tumefaciens cells were transformed according to a modified method published (An et al. 1988). 50 ml YEP medium containing rifampicin was inoculated with 5 ml overnight pre-culture of *A. tumefaciens* and incubated until an $OD_{600\ nm}$ of 0.5 was reached under constant shaking (250 rpm) at 28 °C. After pelleting the cells (3,000 × *g*, 5 min, 4 °C), they were resuspended in 1 ml ice-cold 20 mM $CaCl_2$ and aliquoted into pre-chilled tubes at 100 µl each. The competent cells were snap frozen in liquid nitrogen with 1 µg

plasmid DNA and thawed in a water bath of 37 °C for 5 min. The regeneration of transformed cells was conducted for 4 h in 1 ml of YEP Medium. 200 µl of transformed cell suspension was plated on agar-fixed YEP, containing respective antibiotics, and grown 2 – 3 days at 28 °C.

Isolation of plasmid DNA from *Agrobacterium tumefaciens*

The modified alkaline lysis method (Birnboim and Doly 1979) was applied to isolate plasmid DNA from *A. tumefaciens*. A fresh overnight culture of 5 ml coming from a single colony was sedimented (20,000 × *g*, 1 min, 20 °C) and the cell pellet was thouroughly resuspended in 400 µl lysis buffer I. 100 µl of lysis buffer I with lysozyme (20 mg/l) was added and the cell suspension was shaken in a water bath of 37 °C for 30 min. After adding 500 µl of lysis buffer II and incubating for 10 min at 37 °C, 500 µl of lysis buffer III was added; the suspension was mixed thoroughly and incubated on ice for 5 min. The cell debris, denatured proteins and chromosomal DNA were pelletted by centrifugation (20,000 × *g*, 10 min, 4 °C) and the resulting supernatant was mixed with an equal volume of and incubated on ice for 5 min. The plasmid DNA pellet obtained by centrifugation (20,000 × *g*, 20 min, 4 °C) was suspended in 300 µl water and subjected to phenol/chloroform/isoamylalcohol (25:24:1, per vol.) extraction. Equal volume (300 µl) of phenol/chloroform/isoamylalcohol mixture was added to the plasmid DNA suspension and mixed thouroughly. Phase separation was achieved by centrifugation (20,000 × *g*, 5 min, 20 °C). The inorganic upper phase was transferred to a new vessel and plasmid DNA was precipitated by adding 0.1 volume of 3 M NaAc, pH 5.2 and 2 volumes of ethanol and incubating for 10 min on ice. The precipitated DNA was sedimented (20,000 × *g*, 20 min, 4 °C), washed with 500 µl 70 % ethanol, air-dried, suspended in 50 µl water containing 50 µg/ml RNase A. 10 µl of such isolated plasmid DNA was analysed by endonuclease restrictions.

Lysis buffer I

Component	Final concentration
glucose	50 mM
EDTA, pH 8.0	10 mM
Tris-HCl, pH 8.0	25 mM

Lysis buffer II

Component	Final concentration
SDS	1% (w/v)
NaOH	0.2 M

Lysis buffer III

Component	Final concentration
KAc	3 M
formic acid	1.8 M

2.8.7 Establishing *Arabidopsis thaliana* callus cultures

The roots of sterile grown *A. thaliana* seedlings (2.8) were cut into small pieces and laid out on B5 medium plates. The plates were incubated 3 – 4 weeks in darkness at 24 °C. The resulting callus was transferred to 10 ml of liquid B5 medium supplemented with ampicilin (20 mg/l) and cultured at 24 °C in darkness under constant shaking (80 rpm). The medium was exchanged every week. After 3 – 4 weeks the callus suspension cultures were scaled-up to 100 ml in 500 ml conical flasks.

B5 medium, pH 5.8 (KOH)

Component	Final concentration
Gamborg B5 medium	0.316% (w/v)
sucrose	3% (w/v)
kinetin (N6-furfuryladenine)	0.005% (w/v)
2,4-D (dichlorphenoxyacetic acid)	0.05% (w/v)
For plates: agar	1% (w/v)

2.8.8 *In vivo* labelling of *Arabidopsis thaliana* phospholipids with ^{33}Phosphate

To obtain information on lipid patterns in T-DNA insertional mutants, *A. thaliana* plants and callus suspension cultures of the mutants and the wild type were *in vivo* labelled with [^{33}P]-phosphoric acid (Hartmann Analytic, Braunschweig, specific radioactivity 20 mCi/ml). For callus suspension cultures, 0.3 g callus was transferred to 10 ml of fresh medium and labelled with 100 µCi [^{33}P]-phosphoric acid for 6 h, 24 h and 44 h by shaking at 80 rpm at 25 °C in darkness. 4 sterile-grown plants with 8 – 10 developed leaves were transferred into 6 ml plant liquid medium diluted 1 : 2 with water (see 2.8), and incubated under constant shaking at 80 rpm for 15 h at 25 °C in the presence of 50 µCi of [^{33}P]-phosphoric acid. Plant and callus lipids were isolated as follows. After transferring plant material to 25 ml conical glass tube and removing the rests of the medium (3,200 × *g*, 5 min, 20 °C), it was washed twice with 3 ml of salt solution (0,45% NaCl (w/v), 20 mM EDTA) and then boiled for 10 min in 3 ml of the same salt solution to inactivate lipases. Lipids were extracted from the plant material by adding 3 ml chloroform/methanol (1:2, v/v) and mixing for 30 min at room temperature. After addition of 3 ml chloroform and further 30 min incubationand mixing, 1.5 ml salt solution was added and phase separation was achieved by centrifugation (5,000 x *g*, 2 min, 20 °C). The organic phase containing the lipids was filtered through glass fibre. The remaining water phase was re-extracted with 3 ml chloroform/methanol 2:1 (v/v). The filtered organic phases were combined, evaporated and lipids dissolved in 500 µl chloroform/methanol were analysed. The radioactivity in 2 µl of each isolated organic fraction was counted (in 10 ml of OptiSafe scintillation cocktail, PerkinElmer, Wiesbaden; liquid scintillation counter LS 5000 TD, Beckmann, Krefeld). Equal amounts of labelled lipids originating either from wild type or from mutant plant material were applied to thin layer chromatography (TLC) plates (Silica gel 60, 0.25 mm, Merck, Darmstadt) and separated in chloroform/methanol/25% NH_3 (60:30:3, per vol.) together with 50 µg lipid standards (Sigma, Taufkirchen): phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), cardiolipin (CL). After developing the plates, the labelled lipids were visualized (Bioimager FLA3000, Raytest, Straubing) and phospholipids were identified by co-migrating lipid standards after spraying with phosphatidate reagent (Dittmer and Lester 1964). Subsequently, the silica gel from each band was transferred to a scintillation vial to determine the labelling of the seperated lipids.

Phosphatidate reagent

Component	Final concentration
Mo	1 mM
MoO_3	15 mM
sulphuric acid	12.5 M

2.8.9 Measurement of respiration rates of suspension cultures

Oxygen consumption measurement followed the previously published method (Vanlerberghe and McIntosh 1992). The experiments were performed in a model DW1 oxygen electrode (Hansatech, Reutlingen). 0.3 g of callus was transferred to 10 ml of fresh callus medium and after 24 h shaking in darkness at 26 °C, the samples of 20 µg, 30 µg and 40 µg were suspended in 1 ml of fresh medium. Steady rates of respiratory O_2 consumption could be determined after about 5 min under these conditions. The cytochrome pathway capacity was taken to be the portion of the O_2 consumption inhibited by 1 mM KCN in the presence of 2 mM SHAM and the alternative pathway capacity was taken to be the portion of the O_2 consumption inhibited by 2 mM SHAM in the presence of 1 mM KCN. Residual respiration (in the presence of KCN and SHAM) was subtracted from all measures of the total respiration.

2.8.10 Preparation of mitochondria from the *A. thaliana* cell suspension cultures

The mitochondria isolation procedure followed closely this established by Werhahn et al. 2001. The plant material gathered (approximately 50 g of kallus grown in the cell suspension cultures) were filtered through two layers of muslin and suspended in 200 ml of ice-cold grinding buffer. The cells were disrupted by homogenizing for three periods of 15 s using a Waring blender. Mitochondria were enriched by a four-step centrifugation: centrifugation at 3,500 x *g*, 10 min at 4 °C, two centrifugations at 3,000 x *g* for 5 min at 4 °C (organelles in the supernatant) and one centrifugation at 17,000 x *g* for 10 min at 4 °C (organelles in the pellet). The mitochondrial fraction was resuspended in washing buffer and layered on top of three-step Percoll gradients (six gradients of 30 ml each containing 10 ml of 18% (v/v), 10 ml of 23% [v/v], and 10 ml of 40% [v/v] Percoll in Percoll buffer). After centrifugation at 50,000 *g* for 45 min at

4 °C the mitochondria were isolated from the 23%/40% interphase. To remove the Percoll, the purified mitochondria were centrifuged twice in the 30 ml resuspension buffer at 12,000 *g*. for 10 min at 4 °C. Finaly, the isolated mitochondria were resuspended in small volume of resuspension buffer and stored at – 20 °C in the presence of 50% (v/v) glycerol. Such isolated of mitochondria were active for several weeks.

Grinding buffer

Component	Final concentration
MOPS/KOH, pH 7.4	15 mM
Sucrose	450 mM
EGTA	1.5 mM
BSA	0.2% (w/v)
PVP-40	0.6% (w/v)
DTT	10 mM
PMSF	0.2 mM

Washing buffer

Component	Final concentration
MOPS/KOH, pH 7.4	10 mM
Sucrose	300 mM
EGTA	1 mM
PMSF	0.2 mM

Percoll buffer

Component	Final concentration
MOPS/KOH, pH 7.4	10 mM
Sucrose	300 mM

Resuspension buffer

Component	Final concentration
Tricine/KOH, pH 7.2	10 mM
Mannitol	400 mM
EGTA	1 mM
PMSF	0.2 mM

2.9 PCR methods

PCR (polymerase chain reaction) is a method for specific amplification and modification of DNA sequences (Mullis and Faloona 1987). The target DNA sequence is amplified by multiple repetition of denaturation, oligonucleotides (primers, see 5.1) annealing, and primer by DNA polymerase. Target sequence modifications (e.g. 6 × His-tag, restriction endonuclease recognition sites, point mutations) were introduced by constructing primers of an appropriate sequence. Reaction conditions (template, primer and Mg^{2+}buffer concentrations, annealing temperature, extension duration and number of cycles) were optimized for individual experiments. For high fidelity amplification, *Pfu* DNA polymerase (Promega, Mannheim) was employed, according to the instructions of the manufacturer. Unlike the *Taq* DNA Polymerase, the *Pfu* DNA polymerase exhibits 3'→5' exonuclease (proof-reading) activity, which enables the polymerase to correct nucleotide incorporation errors. The error rate of the *Taq* DNA polymerase is 8 fold higher in comparison with *Pfu* DNA polymerase, however the advantages of the enzyme include the 2 fold quicker DNA synthesis (approximately 1,000 bp/min) and terminal transferase activity, which adds the 3' A-overhangs desired in certain techniques.

The PCR-reaction mixture of 25 µl contained template DNA, 2 µM primers, 1 – 2 units DNA polymerase, 1 × PCR buffer, 2 – 10 mM $MgCl_2$ and 0.8 mM dNTP mixture.

PCR thermoprofile

Step	Temperature	Time
Template denaturation	95 °C	3 min
35 - 40 cycles of:		
Denaturation	95 °C	45 sec
Primer annealing	Depending on primer combination	1 min
Extension	72 °C	[a]
Final extension	72 °C	15 min

[a] Dependent on expected product size and polymerase used

PCR products were analysed by agarose gel separation and visualization (2.4).

10 × *Taq* polymerase PCR Buffer

Component	Final concentration
Tris-HCl, pH 8.5	10 mM
KCl	500 mM
$MgCl_2$	22.5 mM
Tween 20	0.5% (v/v)

10 × *Pfu* polymerase PCR Buffer

Component	Final concentration
Tris-HCl, pH 8.8	200 mM
KCl	100 mM
$(NH_4)_2SO_4$	100 mM
$MgSO_4$	20 mM
Triton X-100	1% (v/v)
BSA, nuclease-free	1 mg/ml

Performed PCR experiments:

Experiment	Primers combination	Annealing temperature used	Number of cycles	Enzyme used	Extension time	Expected lenthg of the product[bp]
Cloning of cDNA of CLS	CLS_F1 CLS_Rv	52 °C	40	*Pfu*	150 s	1050
Truncation of ORF of CLS	CLS_F1-28 CLS_Rv	52 °C	40	*Pfu*	120 s	940
3’ His-tag sequence addition	CLS_F1 CLS_RvHis	45 °C	40	*Pfu*	120 s	1070
3’ His-tag sequence addition and ORF truncation	CLS_F1-28 CLS_RvHis	45 °C	40	*Pfu*	120 s	960
5’ His-tag sequence addition and ORF truncation	CLS_F1NHis CLS_Rv	45 °C	40	*Pfu*	120 s	960
Yeast construct analysis	CLS_F1 CYC1terminator	52 °C	40	*Pfu*	120 s	960
Analysis of Salk_026633, *cls-1* (wt)	022633_LP3 022633_RP3	59 °C	45	*Taq*	120 s	940
Analysis of Salk_026633, *cls-1* (ins. mutant)	022633_LP3 LBa1_TDNA	59 °C	45	*Taq*	120 s	700
Analysis of Salk_049840, *cls-2* (wt)	049840_LP 049840_RP	59 °C	45	*Taq*	120 s	940
Analysis of Salk_049840, *cls-2* (ins. mutant)	049840_RP LBa1_TDNA	59 °C	45	*Taq*	120 s	640
Analysis of Sail_1231C07, *cls-3* (wt)	SAIL_1231_LP SAIL_1231_RP	59 °C	45	*Taq*	120 s	1030
Analysis of Sail_1231C07, *cls-3* (ins. mutant)	SAIL_1231_RP LB2_SAIL	59 °C	45	*Taq*	120 s	300
Analysis of Salk_022655, *cls-4* (wt)	026655_LP 026655_RP	59 °C	45	*Taq*	120 s	920

Analysis of Salk_022655, *cls-4* (ins. mutant)	026655_RP LBa1_TDNA	59 °C	45	*Taq*	120 s	600
Cloning of ORF of CLS into plant binary vector	Actin3pB6 CLS_RvHis	56 °C	45	*Taq*	120 s	1500
Cloning of Cre into plant binary vector	RBpB6 CMrev	48 °C	45	*Taq*	150 s	3500

2.9.1 Reverse transcription - 1st strand synthesis

The first strand cDNA synthesis was preceded by DNase I treatment of isolated RNA, in order to remove traces of DNA from the preparation. The DNase I inactivation (15 min, 75 °C) was followed by addition of sequence degenerated primers d(9)N to final concentration of 2.5 µM, and their annealing for 10 min at 25 °C. Finally, $RT^{(+)}$ (containing Moloney Murine Leukemia Virus reverse transcriptase (Promega); M-MLV RT) and $RT^{(-)}$ (without the enzyme) assays were set up, both in presence of 1 mM dNTP, and carried out for 60 min at 37 °C. The reaction was terminated by heat inactivation of M-MLV RT (10 min, 70 °C), and 1st strand cDNA was applied to further analyses.

To clone the ORF of plant CLS, OneStep RT-PCR Kit (Qiagen GmbH) and gene specific primers (CLS_F1, CLS_Rv; see the table above and 5.1) were used with isolated plant RNA in a reaction consisting of 25 µl.

The cDNA of *At4g04870* from *A. thaliana* carrying a point mutation G199D was obtained by F. Müller and used for subsequent cloning into pBAD and expression studies, alongside with non-mutated open reading framed cloned in this study. Respective primers used for amplification of both sequences were the same, i.e. CLS_F1 / CLS_Rv or CLS_F1-28 / CLS_Rv. Amplification of the same sequence missing the putative signal sequence and Exon 8 of the predicted open reading frame was achieved by employing primers CLS_F1-28 in combination with CLS7_Rv or CLS7_RvHis (for addition of 3'-terminal His-tag) (see the table above and Appendix, 5.1).

OneStep RT-PCR composition

Component	Final concentration
5 × OneStep RT-PCR buffer	1 x
dNTP mix	1.6 mM
CLS_F1	0.8 µM
CLS_Rv	0.8 µM
OneStep enzyme mix	N/A
5 × Q-solution	1 ×
Template RNA	2 µg

2.9.2 Quantitative real-time PCR

The real-time PCR procedure based on fluorescence-kinetic PCR enables quantification of the PCR product in "real-time" (Giulietti et al. 2001). This sensitive and accurate technique measures PCR product accumulation during the exponential phase of amplification.

The real-time PCR experiments were performed with SYBRGreen I, which has an undetectable fluorescence when it is in free form, but once bound to the dsDNA it starts to emit fluorescence. Using this method, the information of PCR product length is not directly available; following agarose gel electrophoresis and detection are necessary. However, since SYBRGreen I intercalates any dsDNA present in reaction, by analysis of melting curves one obtains information with regard to primer dimers or background level.

All real-time PCR assays were conducted using qPCR Core Kit for SYBRGreen I (Eurogentec, Seraign, Belgium), according to the protocol of the manufacturer. The PCR thermoprofile applied was the default one of real-time PCR appliance ABIPrism 7000 Sequence Detection System (Applied Biosystems, Darmstadt). The sequences detected and sequences of the primers are described in 5.1.

Conditions of real time PCR

Experiment	Primers combination	Annealing temperature used	Number of cycles	Enzyme used	Extension time	Expected lenthg of the product[bp]
Real time: Actin	Actin2_Fw Actin2_Rv	60 °C	40	Hotstart Gold Taq	60 s	110
Real time: CLS	RT-CLS F2 RT-CLS Rv1	60 °C	40	Hotstart Gold Taq	60 s	130

2.10 Enzymic assays

2.10.1 Assay for CLS

Cardiolipin synthase catalyses a transfer of phosphatidic moiety onto phosphatidyl-glycerol (PG), so that cardiolipin is formed.

CDP-dioleoyl-[U-^{14}C]diacylglycerol (CDP-DAG) was synthesized from *sn*[U-^{14}C]glycerol 3-phosphate (150 mCi/mmol, Amersham Biosciences Europe, Freiburg) as described previously (Frentzen and Griebau 1994). Briefly, three consecutive enzymic reactions were applied. In the first step, 1-oleoyl[U-^{14}C]glycerol 3-phosphate was obtained from [U-^{14}C]glycerol 3-phosphate and by action of oleoyl-CoA and purified glycerolphosphate acyltransferase form pea chloroplasts (Bertrams and Heinz 1981). Subsequently, labelled 1-oleoyl[U-^{14}C]glycerol 3-phosphate was condensed with oleoyl-CoA in the reaction performed by acylglycerolphosphate acyltransferase, over-expressed by *E. coli* JC201/p*PLSC* strain (Coleman 1992). Finally, CDP-dioleoyl-[U-^{14}C]glycerol was obtained in a reaction catalyzed by memrane fractions of *E. coli* harboring pCD100 (Tamai and Greenberg 1990) and was subsequently purified by TLC. [U-14C]glycerol 3-phosphate was converted to the final product with a yield of 50-60%.

CLS activity was assayed in a standard volume of 50 µL at 30 °C for 30 min (unless otherwise indicated). Then the reactions were stopped and lipids were extracted with 240 µL chloroform/methanol (1:1, v/v). After phase separation (2,000 × *g*, 5 min, 20 °C), the organic layer was analysed by TLC on boric acid activated silica gel plates in chloroform/methanol/water/25% NH_3 (70:30:3:2, by volumes). Plates were visualized in Bioimager FLA3000 (Raytest), and labelled lipids were quantified by scintillation counting.

In reconstitution assays, different concentrations of phospholipid suspensions were added to the assay. Phospholipids were suspended in 20 mM PIPES, pH 7.4, but PI was suspended in 10 mM Tris-HCl, pH 7.4; 1 mM EGTA.

CLS standard assay composition

Component	Final concentration
BisTrisPropane-HCl, pH 8.8	50 mM
$MnCl_2$	20 mM
PG, dioleoyl	600 µM
CDP-dioleoyl-[U-^{14}C]glycerol	2 µM
membrane fraction protein	0.5 – 20 µg

2.10.2 Assay for PCS

Condensation of CDP-[*methyl*-^{14}C]choline (59 mCi/mmol; Amersham Biosciences Europe, München) with 1,2-diacylglycerol to phosphatidylcholine by yeast subcellular fractions was performed as described by Kuchler et al. 1986. The 100 µl reaction mixtures were incubated 30 min at 30 °C. Assays were stopped and reaction products extracted in 1 ml chloroform/methanol (1:1, v/v) with 0.5 ml of 1 M KCL, 0,1 M H_3PO_4. The labelling rates of the extracted PC was quantified scintillation counting.

PCS assay composition

Component	Final concentration
MOPS, pH 7.4	50 mM
DTT	0.5 mM
$MgCl_2$	30 mM
CDP-[*methyl*-^{14}C]choline	85 µM
Yeast subcellular fraction protein	2 – 50 µg

2.10.3 Assays for PGPS

Phosphatidylglycerolphosphate synthase activity was measured by determining the condensation rates of *sn*-[U-^{14}C]-glycerol-3-phosphate (150 mCi/mmol, Amersham Biosciences Europe, München) with CDP-diacylglycerol, dioleoyl (Sigma, Taufkirchen) into the lipophilic product phosphatidylglycerolphosphate (PGP) and phosphatidylglycerol (PG), as described before (Müller and Frentzen 2001). The radioactive substrate, glycerol 3-phosphate was in form of mixture of *sn*-[U-^{14}C] glycerol 3-phosphate with unlabeled glycerol 3-phosphate (specific radioactivity: 11 dpm/pmol). After incubation (30 min, 30 °C) the assay was stopped and organic products were extracted with 2.4 ml chloroform/methanol (1:1, v/v) and 1 ml of 1 M KCl, 0.1 M H_3PO_4. The phases were separated, and the incorporation rates of glycerol 3-phosphate into lipophilic products were quantified by scintillation counting.

PGPS assay composition

Component	Final concentration
BisTrisPropane, pH 7.0	50 mM
Triton X-100	0.06% (v/v)
CDP-diacylglycerol, dioleoyl	50 µM
$MnCl_2$	0.1 mM
sn-[U-^{14}C] glycerol 3-phosphate	0.5 mM
membrane fraction protein	2 – 50 µg

2.11 Partial purification of CLS

2.11.1 Solubilization of CLS over-expressed in *Escherichia coli* membranes

Aiming at CLS purification, protein solubilization from over-expressing *E. coli* cells was performed. A protocol for solubilizaiton with Triton X-100 was developed, and a modified method of CLS solubilization using Zwittergent 3-14 published previously (Schlame and Hostetler 1991) were applied. Triton X-100 (Merck) and Zwittergent 3-14 (Anatrace Inc., Maumee, USA) were applied in the following manner.

For solubilization of transmembrane proteins with Triton X-100, membrane protein fraction isolated from over-expressing *E. coli* was incubated with 1% (v/v) Triton X-100 at detergent/protein ratio of 6.3:1. After 30 min stirring on ice, the mixture

was subjected to ultracentrifugation (120,000 × *g*, 30 min, 4 °C). The supernatant containing the solubilized proteins was transferred to a new vessel and the pellet was washed with 20 mM MOPS, pH 7.4 and suspended in the same buffer, in volume equal to the volume of used membrane fractions. CLS activities in both, solubilized supernatant and pellet were analysed as described above. The fractions were stored at - 80 °C.

Solubilization assay with Zwittergent 3-14 was performed at detergent/protein ratio of 2.1:1, at a final detergent concentration of 0.25% (w/v). The solubilization conditions and handling of solubilized fractions were similar to the Triton X-100 assay.

Composition of solubilization assay with Triton X-100

Component	Final concentration
Tricine-HCl, pH 8.0	25 mM
EDTA, pH 8.0	5 mM
EGTA, pH 8.0	5 mM
glycerol	25% (v/v)
Triton X-100	1% (v/v)
membrane fraction protein	1 mg

Composition of solubilization assay with Zwittergent 3-14

Component	Final concentration
Tricine-HCl, pH 8.0	25 mM
EDTA, pH 8.0	5 mM
EGTA, pH 8.0	5 mM
glycerol	25% (v/v)
Zwittergent 3-14	0.25% (w/v)
membrane fraction protein	1 mg

Anion-exchange column elution of solubilized plant cardiolipin synthase

The CLS solubilized from *E. coli* membranes with Zwittergent 3-14 was purified by anion exchange column chromatography (UNOsphere Q, BioRad, München). Solubilized supernatant fractions were diluted 1:1.5 (by volumes) with 25 mM Tricine-HCl, pH 8.0, 25% (v/v) glycerol and applied on 0.5 ml UNOsphere resin, pre-equilibrated with 30 ml equilibration buffer. The column was washed two times with 1 ml wash buffer I, followed by two washes with 1 ml wash buffer II of increased ionic strength. Finally, 3 washes with 0.3 ml with elution buffer were used. The fractions were analysed by CLS assays, SDS-PAGE and Western-blot.

Equilibration Buffer

Component	Final concentration
Tricine-HCl, pH 8.0	25 mM
glycerol	20% (v/v)
Zwittergent 3-14	0.1% (w/v)

Wash Buffer I

Component	Final concentration
Tricine-HCl, pH 8.0	25 mM
glycerol	20% (v/v)
Zwittergent 3-14	0.1% (w/v)
EDTA, pH 8.0	5 mM
EGTA, pH 8.0	5 mM

Wash Buffer II

Component	Final concentration
Tricine-HCl, pH 8.0	25 mM
glycerol	20% (v/v)
Zwittergent 3-14	0.1% (w/v)
KCl	200 mM
EDTA, pH 8.0	5 mM

EGTA, pH 8.0	5 mM

Elution Buffer

Component	Final concentration
Tricine-HCl, pH 8.0	25 mM
glycerol	20% (v/v)
Zwittergent 3-14	0.1% (w/v)
KCl	1 M
EDTA, pH 8.0	5 mM
EGTA, pH 8.0	5 mM

2.11.2 Ni-NTA column elution of solubilized plant cardiolipin synthase

The CLS solubilized from *E. coli* membranes with Zwittergent 3-14 was diluted 1:1.5 with dilution buffer, and incubated (150 rpm, 1 h, 4 °C) with 0.5 ml Ni-NTA agarose (Qiagen) resin, prewashed with 15 ml activation buffer. The column was washed four times with 4 ml wash buffer, followed by four washes with 0.5 ml elution buffer of higher imidazol concentration. Eluted fractions were analysed by CLS assays, SDS-PAGE and Western-blot.

Activation Buffer

Component	Final concentration
NaH2PO4, pH 8.0 (NaOH)	50 mM
NaCl	300 mM
Imidazol	10 mM
Zwittergent 3-14	0.1% (w/v)
Glycerol	20% (v/v)

Dilution Buffer

Component	Final concentration
NaH2PO4, pH 8.0 (NaOH)	50 mM
NaCl	300 mM
Imidazol	10 mM
Glycerol	20% (v/v)

Wash Buffer

Component	Final concentration
NaH2PO4, pH 8.0 (NaOH)	50 mM
NaCl	300 mM
Imidazol	20 mM
Zwittergent 3-14	
Glycerol	20% (v/v)

Elution Buffer

Component	Final concentration
NaH2PO4, pH 8.0 (NaOH)	50 mM
NaCl	300 mM
Imidazol	250 mM
Zwittergent 3-14	
Glycerol	20% (v/v)

3 Results and Discussion

In order to characterize the plant cardiolipin synthase (CLS) and to investigate the functional role of its product cardiolipin in plant mitochondria, the *Arabidopsis thaliana* cardiolipin synthase gene was cloned. The properties of the encoded enzyme were determined by heterologous over–expression in microorganisms. Additionally, molecular and biochemical analyses of *A. thaliana* mutants carrying a T - DNA insertion in the *CLS* gene were performed.

3.1 Identification of a putative *A. thaliana CLS* gene

Database searches using the sequences of the *CLS* gene of yeast (Jiang et al. 1997, Tuller et al. 1998, Chang et al. 1998) and the two *PGS* genes of *A. thaliana* encoding PGP synthase (Müller and Frentzen 2001) resulted in the identification of the *A. thaliana* gene *At4g04870*. The gene is located on the (-) genomic DNA strand of chromosome 4 and comprises 8 exons of different lengths. It contains an open reading frame of 1026 bp that encodes a polypeptide of 341 amino acids with a calculated isoelectric point of 10.52 and a calculated mass of 38.04 kDa. As highlighted in Figure 3-1, the encoded protein contains the CDP-alcohol binding motif $DX_2DGX_2ARX_8GX_3DX_3D$ typical for the class I CDP-alcohol phosphatidyltransferases. Hence, it belongs to the same protein class as PGS from bacteria and plants, as well as CLS from other eukaryotes (Houtkooper et al. 2006, Jiang, Rizavi et al. 1997, Tuller, Hrastnik et al. 1998, Chang, Heacock et al. 1998). According to TargetP prediction[1)], the protein is located in mitochondria, and possesses a cleavable transit peptide of 28 amino acids at its N-terminus, (Figure 3-1) while MitoProtII[2)] predicts a mitochondrial transit peptide of 85 amno acids. In addition, the protein is predicted to represent a type II membrane protein with a membrane spanning domain in its C-terminal region, although the location of the transmembrane domain differs, depending on the programme used for the prediction[3)].

[1])http://www.cbs.dtu.dk/services/TargetP/; Emmanuelsson et al. 2000 ; Nielsen et al. 1997

[2]) Ver.1.04a, http://ihg.gsf.de/ihg/mitoprot.html; Claros et al. 1996

[3]) SOSUIsignal: http://www.proteome.bio.tuat.ac.jp/sosuisignal/sosuisignal_submit.html;
TMHMM: http://www.cbs.dtu.dk/services/TMHMM/;



Figure 3-1: Alignment of the amino acid sequences of eukaryotic phosphatidyltransferases
The sequences of yeast CLS (Accession NP_010139), human CLS (DQ386730) and *A. thaliana* enzymes (PGS1: BAB68508; PGS2: AAL15250; CLS: AAL24147) were aligned with Clustal_X, and the alignment was viewed with GeneDoc. The cleavable transit peptide of the plant CLS predicted by TargetP is underlined. CDP - alcohol phosphatidyl transferase domains are highlighted with a grey background; amino acids constituting DGAR motif are indicated. Identical amino acids are marked with asterisks.

As depicted in Figure 3-2, the protein encoded by the *At4g04870* shows higher sequence similarity to the CLS of yeast and human than to the bacterial or plant PGP synthases (PGS), but the highest one to two rice proteins of yet unknown functions (Figure 3-1).

The homology searches of the *A. thaliana* genomic databases[4)] show no further hits for the putative CDP-alcohol transferases. Based on this fact and on its amino acid sequence similarity, it is likely that *At4g04870* does not encode a further mitochondrial PGS as annotated in the databases (Beisson et al. 2003), but the CLS of *A. thaliana.*

[4)] http://www.ncbi.nlm.nih.gov/blast/; Zhang et al. 2000

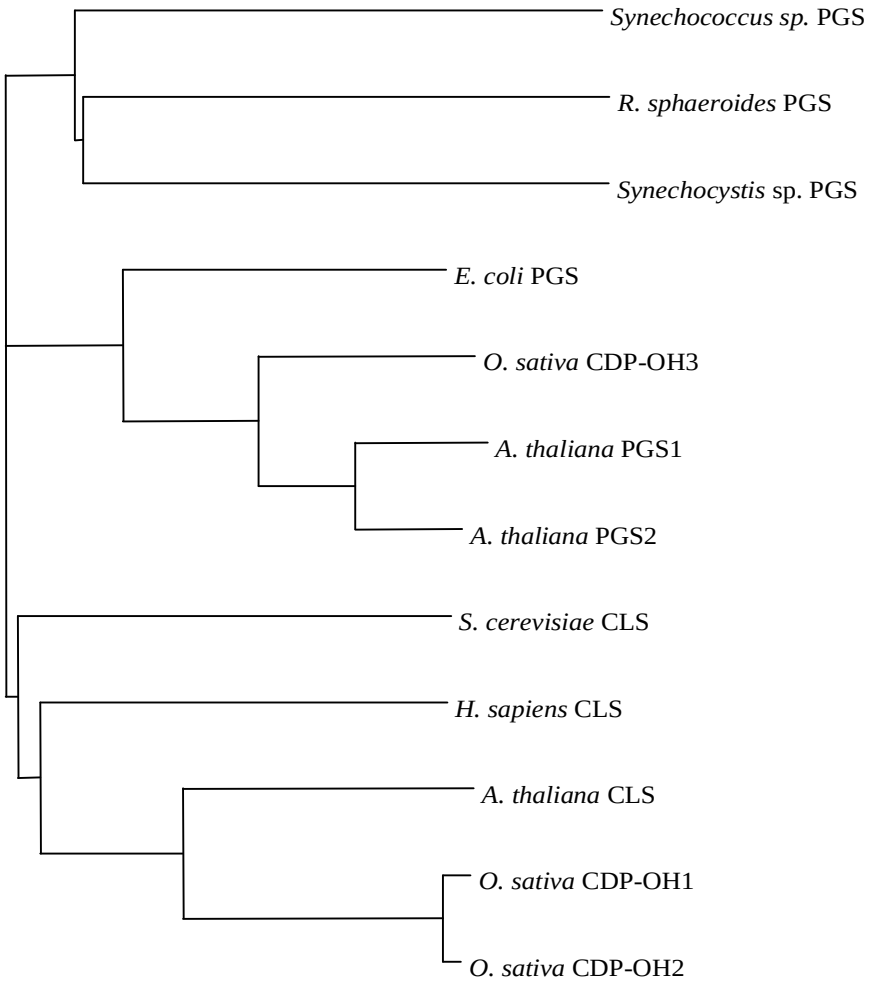


Figure 3-2: Homology tree of phosphatidyltransferases
The amino acid sequences of depicted proteins were aligned with Clustal_X; the dendrogram was viewed by TreeView. (Accession numbers: *E. coli* PGS: P06978; *R. spaeroides* PGS: U29587; *.S cerevisiae* CLS: NP_010139; *H. sapiens* CLS: DQ386730; *A. thaliana* CLS: AAL24147; *A. thaliana* PGS1: BAB68508; *A. thaliana* PGS2: AAL15250; *Synechococcus* sp. PGS: AAN46178; *Synechocystis* sp. PGS: BAA18467; *O. sativa* CDP-OH phosphatidyltransferase 1: NP_915744 *O. sativa* CDP-OH phosphatidyltransferase 2: BAD81959; *O. sativa* CDP-OH phosphatidyltransferase 3 XP_493849).

3.2 Cloning and expression of plant cardiolipin synthase

To functionally characterize the translation product of the *At4g04870* gene, its open reading frame was amplified by RT-PCR using RNA isolated from the *A. thaliana* ecotype Columbia as template and the respective CLS_F1, CLS_Rv primers (Appendix 5.1). The amplified open reading frame lacking the sequence of the putative transit peptide of 28 amino acids was cloned in such a way into the pBAD vector that either His-tag sequence was fused to its 5'- and 3'-end, respectively, or not. The different constructs were termed pCLS_N-His, pCLS_C-His, pCLS. Additionally, a pCLS_G199D construct with the plant *CLS* open reading frame lacking the putative transit peptide of 28 amino acids with the His-tag sequence fused to its 3'-sequence and

carrying a nucleotide substitution resulting in an amino acid change from a conserved glycine to aspartatate position 199 was procured. The truncated open reading frame of the *At4g04870* missing the last exon of the gene coding for the C-terminal 30 amino acids with the His-tag sequence was cloned in the pBAD and the resulting construct was termed pCLSΔ8. All five constructs were over-expressed in *E. coli* and the subcellular fractions of the transgenic bacterial cells were analysed by Western blots and enzymic assays.

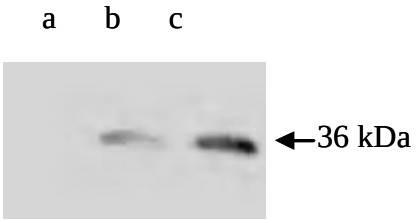


Figure 3-3: Western blot analysis of subcellular fractions from *E. coli* cells expressing the pCLS_C-His construct
Soluble protein fraction (a; 10 µg protein) and the membrane protein fractions (b and c; 2 µg and 5 µg respectively) were isolated from the *E. coli* cells 3 h after induction, separated by SDS-PAGE, transferred on the PVDF membrane and detected with Penta-His antibodies (Qiagen).

The analysis of soluble and membrane fractions of the transgenic bacterial cells harbouring the pCLS_C-His construct or the empty vector as a control revealed that the recombinant *A. thaliana* protein of 36 kDa accumulated in significant levels in the membranes of the bacterial cells (Figure 3-3). It was undetectable, however, in the respective soluble protein fractions or in the control fractions (data not shown). The observed accumulation of the CLS protein in the *E. coli* membranes is consistent with its amino acid sequence, according to which it contains at least one membrane spanning domain in its C-terminal region.

To optimize the level of the recombinant plant protein in the bacterial membranes, the transgenic *E. coli* cells were cultivated in two different media and harvested at different times after induction. As shown in Figure 3-4, higher protein levels were obtained from *E.coli* cells cultivated in LB medium, than in TB medium. For both media, the optimal expression time was found to be 3 h (lanes A3 and B3). A further prolongation of the time after induction did not improve expression levels, but led to a degradation of the His-tagged protein. Hence, transgenic *E. coli* cells were routinely cultivated in the LB medium and harvested 3 h after induction.

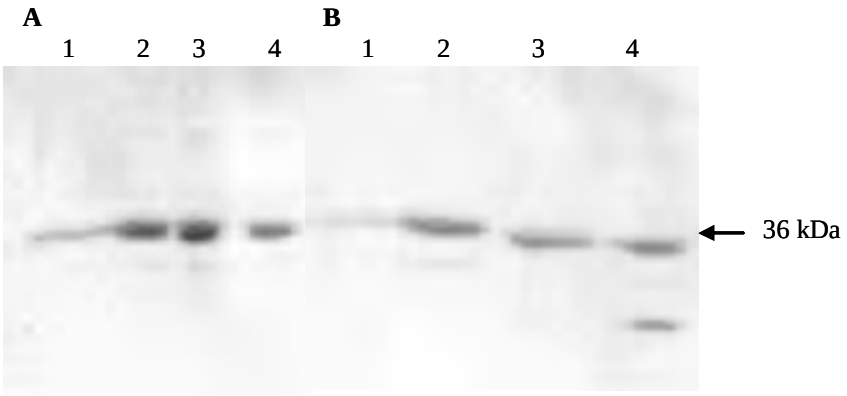


Figure 3-4: Time course of expression of the recombinant plant CLS in *E. coli* cells cultivated in different media
E. coli cells harbouring the pCLS_C-His construct were cultivated in either LB media (A) or TB media (B) and harvested 1, 2, 3 or 4 h (lanes 1 – 4) after induction. 5 µg protein of the membrane fractions isolated from transgenic bacterial cells were analysed by Western blot.

In the next step, the isolated membrane fractions of the transgenic *E coli* cells were used for enzymic assays with labelled CDP-DAG and unlabelled PG or glycerol-3-phosphate as the substrate. The analyses of the reaction products formed by the membrane fractions from labelled CDP-DAG and unlabelled PG revealed that fractions bearing an *A. thaliana* protein produced appreciably higher levels of CL than the control fractions (Figure 3-5).

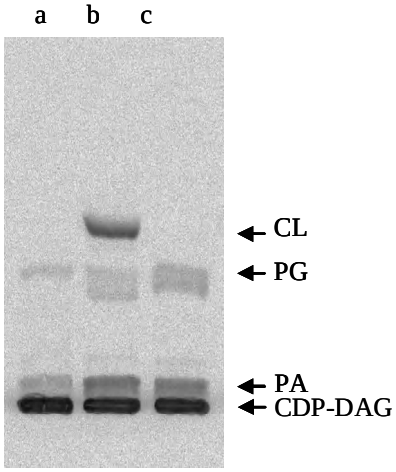


Figure 3-5: Functional expression of the *CLS* cDNA from *A. thaliana* in *E. coli*
TLC analysis of the reaction products formed from the labelled CDP-DAG and unlabelled PG (lanes b and c) or unlabelled glycerol-3-phosphate (lane a) by membrane fractions of *E. coli* harbouring either the empty vector (lane c) or the pCLS_C-His construct (lanes a and b). The enzymic assays were conducted under standard conditions. The labelled products were visualized by Bioimager (Raytest). CL: cardiolipin; PG: phosphatidylglycerol; PA: phosphatidic acid; CDP-DAG: cytidine-diphosphate diacylglycerol.

In contrast to the CL synthesis, the expression of the *A. thaliana* sequence did not increase the labelling rates of PGP or PG within the membranes, no matter whether PG was substituted by glycerol-3-phophate in the enzyme assay (Figure 3-5 lane a) or assay conditions optimized for plant PGP synthases were used (Müller and Frentzen 2001). Thus, these data suggest that the At4g04870 gene of *A. thaliana* does not encode a PGS but a CLS, which are in line with the data recently published (Katayama et al. 2004).
The addition of a His-tag at the N- or C-terminus of the protein was found to have no obvious effect on the CLS activity. The N-His tagged CLS protein acculmulated in the bacterial membranes in levels very similar to those of the C-his tagged protein depicted in Figure 3-4. At the same time, the specific CLS activities in the membrane fractions isolated from the *E. coli* strains carrying pCLS-28, pCLS_C-His or pCLS_N-His constructs display nearly identical specific activities (Figure 3-6).

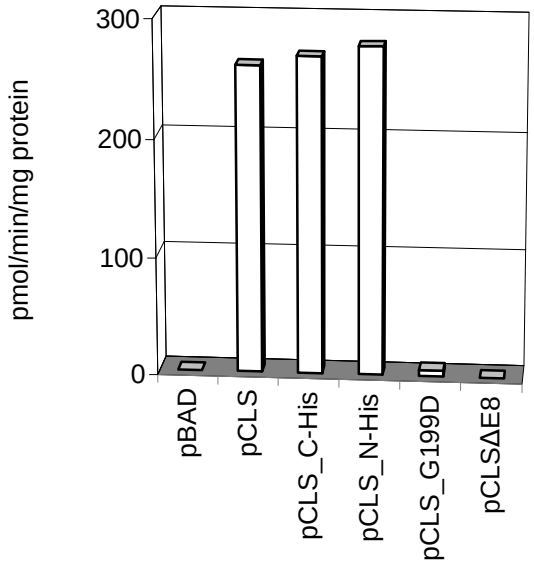


Figure 3-6: CLS activites displayed by induced *E. coli* carrying respective constructs
The bacterial membranes isolated from strains harbouring respective constructs were assayed for the CLS activities under standard conditions.

A substitution of glycine 199, one of the two highly conserved glycine groups within the CDP-alcohol binding motif (Figure 3-1), by an aspartate group encoded by the pCLS_G199D construct gave a CLS protein possessing low but detectable enzymatic activity, reaching about 5% of the wild-type activity (Figure 3-6). Moreover, a deletion of 30 amino acids of the C-terminal region of the protein of the pCLSΔE8 construct resulted in a complete loss of the CLS activity (Figure 3-9), although the protein was detected in the bacterial membranes in levels very similar to those of shown in Figure

3-4. On the other hand, recently published results (Katayama, Sakurai et al. 2004) demonstrated CLS activity with the *A. thaliana* protein lacking not 28 but 100 amino acids at the N-terinus of the preprotein. Hence, the conserved CDP-alcohol phosphotransferase domain of the central and C-terminal region, unlike the N-terminal one (Figure 3-1) appears to be indispensable for the CLS activity.

3.3 CLS expression studies in *Saccharomyces cerevisiae*

The identity of the *A. thaliana* gene was further supported by expressing its open reading frame in the *S. cerevisiae* mutant strain D2000 deficient in CL synthesis (Tuller, Hrastnik et al. 1998).

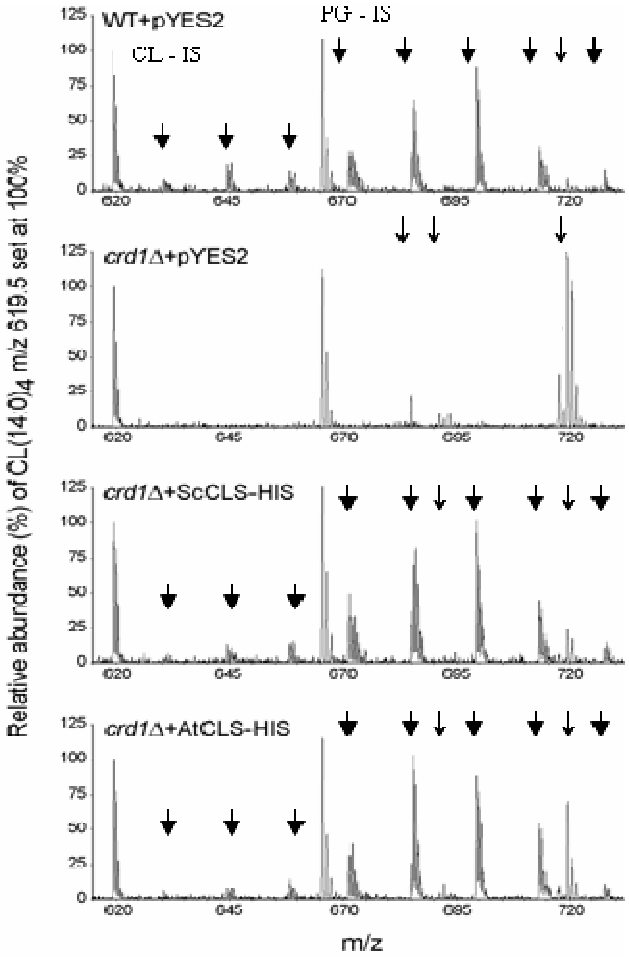


Figure 3-7: Complementation analysis by CL profile analysis
CL (closed arrows) and PG (opened arrows) profiles of *S. cerevisiae* wild type (WT) and the D2000 strains (*crd1*Δ) harbouring the empty vector pYES2 (WT+pYES2, *crd1*Δ+pYES2), yeast CLS gene construct (*crd1*Δ+ScCLS-His) or a plant CLS gene construct (*crd1*Δ+AtCLS-His) are shown in the region from *m/z* 615 to *m/z* 720. Peaks at *m/z* 619.5 (CL i.s.) and *m/z* ´665.5 (PG i.s.) represent the internal standards of CL and PG, respectively. The abundance of the CL internal standards was set at 100% (PG internal standard is not fully depicted).

To investigate whether the CL levels were restored in the D2000 strain expressing the plant CLS, CL and PG were analysed by HPLC-MS in the mutant cells transformed with either the *A. thaliana* or the yeast CLS cDNA or the empty vector. Comparison of the PG and CL spectra with the respective wild type control (Figure 3-7) shows that CL is present in the transformants expressing not only the yeast but also the plant open reading frame. On the other hand, the *cls* deletion mutant transformed with the empty vector almost completely lacks CL but contains significant levels of P. To compensate for the lack of CL, the D2000 strain is known to produce high levels of PG (Chang, Heacock et al. 1998).

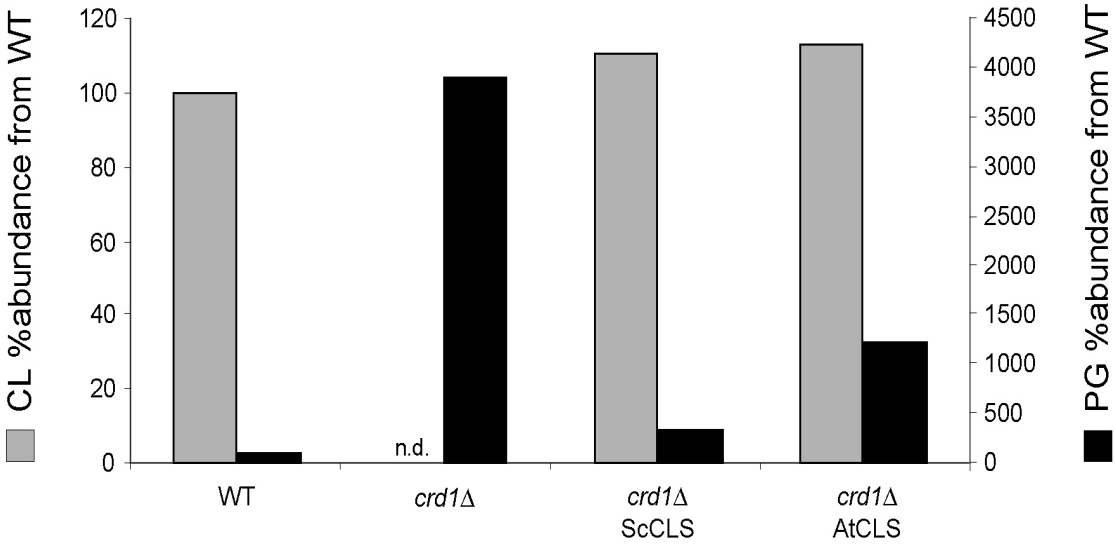


Figure 3-8: Estimation of CL and PG levels
CL and PG levels of wild type and the D2000 mutant strain carrying the empty vector pYES (WT+pYES2, *crd1*Δ+pYES2), yeast CLS gene construct (*crd1*Δ+ScCLS) or plant CLS gene construct (*crd1*Δ+AtCLS) were determined by HPLC / MS analysis. WT levels were set at 100% for both CL and PG. PG levels are highly elevated (almost 40 times) in the D2000 strain, whereas CL is undetectable (n.d.).

An estimation of the CL and PG levels in the different transformants is depicted in Figure 3-8. The D2000 strain contained almost 40 times more PG than the wild-type strain. Expression of the *A. thaliana* CLS caused the formation of CL and reversed the accumulation of PG to nearly the same extent as expression the yeast CLS.

The lipid analysis data were clearly supported by *in vitro* enzyme assays using the mitochondrial and microsomal membrane fractions of the transgenic yeast cells as enzyme source. While in the membrane fractions of the cells carrying the empty vector the CLS activity was not detectable, the membrane fractions of yeast cells expressing the *A. thaliana CLS* gene clearly displayed the CLS activity which was distinctly higher in the crude mitochondrial fractions than in the microsomal fractions (Figure 3-9).

As shown in Figure 3-9, the activity pattern of the plant CLS expressed in the yeast strain D2000 was similar to that of the yeast PGS located in the inner mitochondrial membrane, but different to that of the yeast phosphatidylcholine synthase (PCS) located in the endoplasmic reticulum.

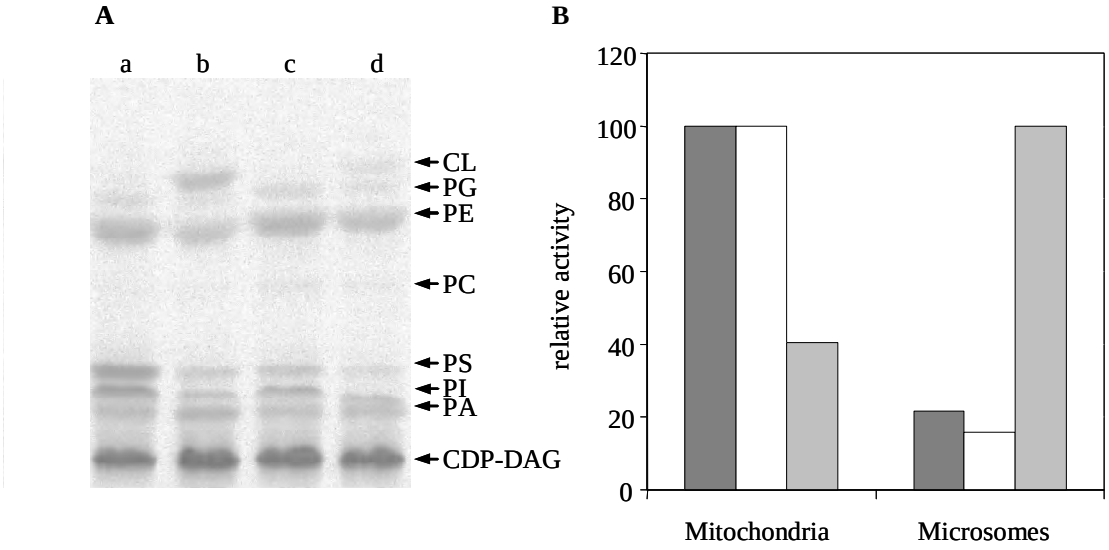


Figure 3-9: Functional expression of *A. thaliana CLS* cDNA in *Saccharomyces cerevisiae*
A: TLC analysis of the lipophilic reaction from labelled CDP-DAG and unlabelled PG the mitochondrial (a,b) or microsomal (c, d) fractions of the yeast *cdr1* deletion strain carrying empty vector (a, c) or *A. thaliana* CLS gene (b, d). Lanes a – d: 10 µg of the fraction protein; CL: cardiolipin; PG: phosphatidylglycerol; PE: phosphatidylethanolamine; PC: Phosphatidylcholine; PS: phosphatidylserine; PI: phosphatidylinositol, PA: phosphatidic acid; CDP-DAG: cytidyldiphophate-diacylglycerol (start).
B: Relative activities of CLS (dark grey bars) as well as endogenous PGP synthase (white bars) and PC synthase (light grey bars) in mitochondrial and microsomal fractions of the yeast *crd1* deletion strain harbouring an open reading frame of the *A. thaliana* CLS are given (100% CLS activity corresponds to 72 pmol/min/mg protein).

In summary, the functional expression studies in *E. coli* and yeast provide strong evidence that the locus *At4g04870* represents the *CLS* gene of *A. thaliana* and suggest that the preprotein expressed in yeast can be imported into the mitochondria and processed to a catalytically active protein.

3.4 Characterization of the *A. thaliana* cardiolipin synthase

To determine the properties of the plant CLS, the recombinant protein from membranes isolated form *E. coli* strain carrying pCLS_C-His construct was used as the enzyme source.

The *A. thaliana* CLS required divalent cations for its activity. The highest activities were measured in the presence of 10 mM $MnCl_2$ (Figure 3-10, A), while $MgCl_2$ or $CoCl_2$ stimulated the activity only slightly (Figure 3-10, B). While the dependance on divalent cations appears to be characteristic of the mitochondrial CLS of different organisms, the enzymes differ in their cation specificities. The yeast CLS shows preference for Mg^{2+} (Tamai and Greenberg 1990), the enzyme isolated from rat liver favors Co^{2+} (Schlame 1993), and plant CLS requires Mn^{2+} (Frentzen and Griebau 1994).

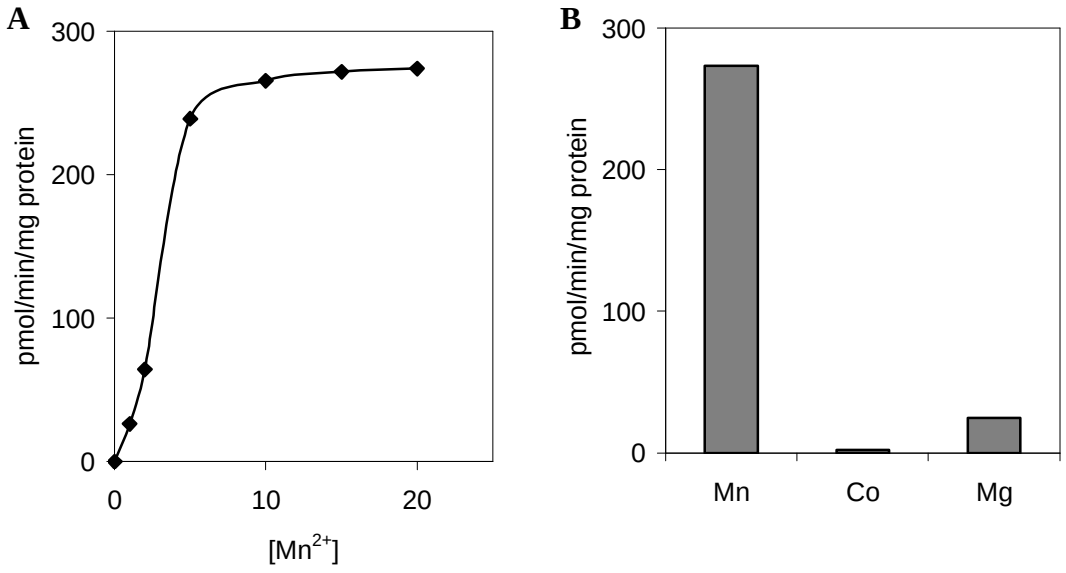


Figure 3-10: Dependance of the *A. thaliana* CLS activation on dialent cations.
The pCLS_C-His construct was over-expressed in *E. coli*, and 0.5 µg protein of isolated membrane fractions were assayed for CLS activity under otherwise standard conditions. A: CLS activity as a function of the $MnCl_2$ concentration**.** B: CLS activity in the presence of 20 mM of the given divalent cations.

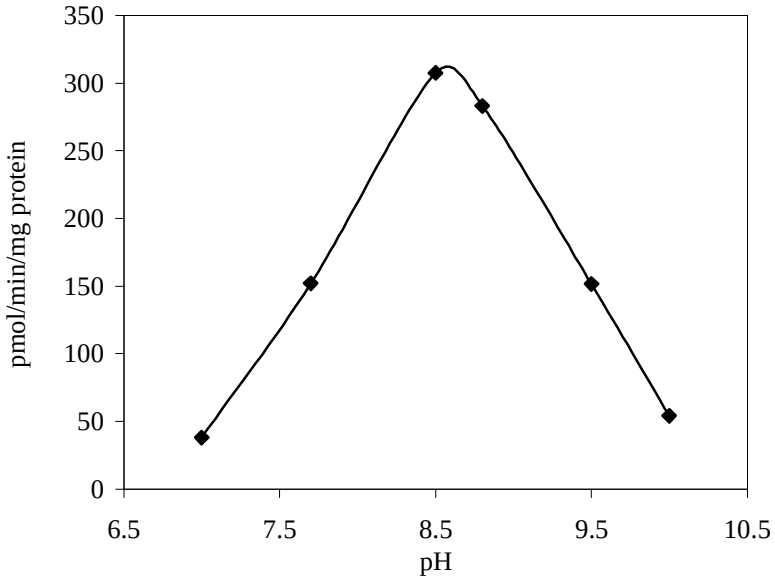


Figure 3-11: The pH optimum of the *A. thaliana* CLS
The pCLS_C-His construct was over-expressed in *E. coli* for 3 h and the isolated membranes were assayed for CLS activity. Standard assay conditions were applied (see the description of the Figure 3-6 for details), with the exception of the Mn^{2+} concentration (see text). The 100% of the specific activity corresponds to 154 pmol / min*mg.

In accordance with the CLS of yeast, mammals and mung bean, the *A. thaliana* enzyme displayed a basic pH optimum of about 8.5, as presented in Figure 3-11 (Tamai and Greenberg 1990, Schlame and Hostetler 1991, Frentzen and Griebau 1994).

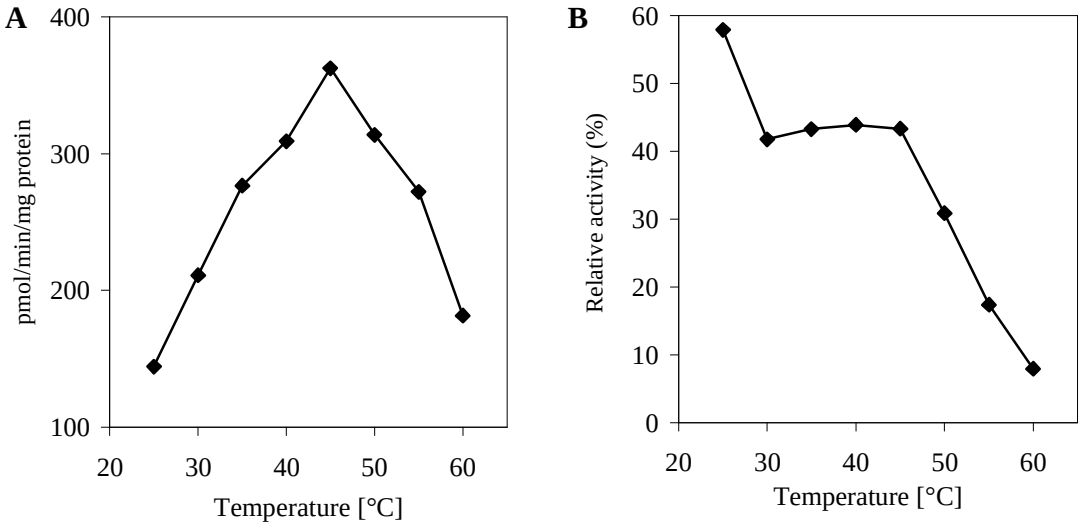


Figure 3-12: Influence of temperature on plant CLS activity (A) and stability (B)
The membrane fraction isolated from the *E. coli* cells carrying pCLS_C-His construct were assayed for 30 min at the given temperatures under standard conditions (A), or incubated at indicated temperatures for 20 min, stored on ice for 30 min, and assayed for 30 min at 30 °C under standard conditions.

The *A. thaliana* CLS showed highest activities at 45 °C (Figure 3-12, A) and thus, it resembled the respective enzyme of yeast (Tamai and Greenberg 1990). On the other hand, the plant enzyme was significantly less heat resistant than the yeast CLS (Tamai and Greenberg 1990). Incubation at 25 °C for 20 min led to more than 40% loss in activity when compared with the membranes stored on ice (Figure 3-12, B). Whether this low heat stability is a typical property of the plant protein or is caused by the recombinant protein expressed in a heterologous environment awaits clarification.

Unlike the PGS of *A. thaliana* requiring 15 mM Triton X-100 for activity (Müller and Frentzen 2001), the CLS activity of the same origin was severly inhibited by detergents (Figure 3-13). Even low concentrations of about 0.2 mM Triton X-100 or Zwittergent 3-14 gave a 50% inhibition, while concentrations higher than 0.6 mM resulted in almost complete loss of activity (Figure 3-13).

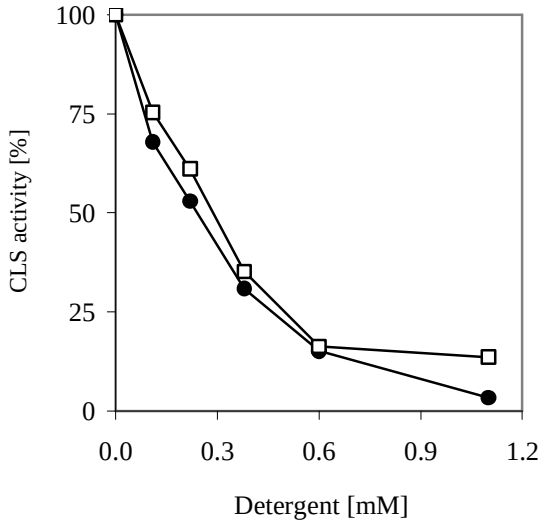


Figure 3-13: Inhibition of the activity of the *A. thaliana* CLS by detergents
Enzyme assays were conducted with membrane fraction of *E. coli* cells expressing the pCLS_C-His construct in the presence of the given detergent concentrations: Zwittergent 3-14 (●) and Triton X-100 (□). 0.5 µg protein was applied per assay under standard conditions (see the description of Figure 3-6).

With regard to its detergent sensitivity, the *A. thaliana* CLS resembles the respective enzyme from rat liver (Schlame and Hostetler 1991) but differs from the yeast CLS, the activity of which was stimulated by Triton X-100 up to a concentration of 3 mM (Tamai and Greenberg 1990).

The *A. thaliana* CLS showed typical saturation pattern when its activity was determined as a function of the substrate concentrations (Figure 3-14). With the dioleoyl species of both substrates the enzyme displayed maximal activities at 600 µM PG and 2.5 µM CDP-DAG and apparent K_m values of about 150 µM and 1.4 µM, respectively, were determined. Distinctly lower K_m values for CDP-DAG than for PG were also reported for the CLS from mung bean seedlings (Frentzen and Griebau 1994) as well as the CLS from yeast and mammals (Schlame and Hostetler 1991, Tamai and Greenberg 1990).
Under optimal conditions, the CL synthesis rates were found to be constant for at least 1 h and up to a protein amount of at least 5 µg. Specific CLS activities of about 320 ± 50 pmol/min/mg protein were determined in the bacterial membrane fractions, which were about 5-fold higher than those of isolated mitochondrial fractions from the *A. thaliana* cell suspension cultures (see below).

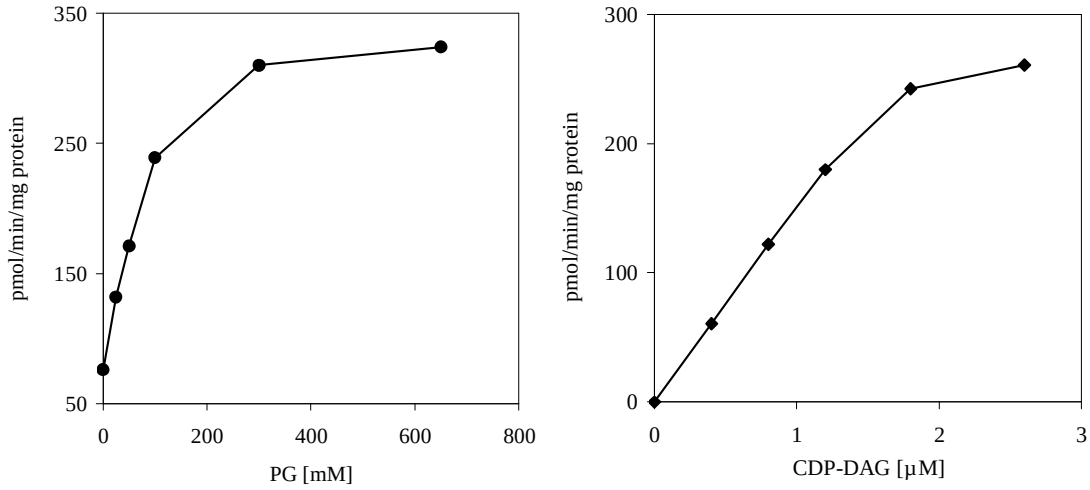


Figure 3-14: Substrates dependency of the *A. thaliana* CLS activity
Formation rates of CL by the CLS expressed in *E. coli* as a function of the CDP-dioleoyl[U-^{14}C]glycerol (A) and dioleoyl-PG (B) concentration. 0.5µg of membrane protein isolated form the *E. coli* carrying pCLS-C-His construct were assayed under otherwise standard conditions.

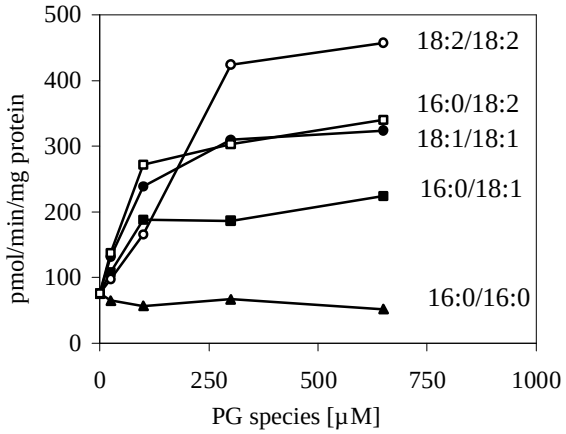


Figure 3-15: PG species specificity of the *A. thaliana* CLS
Formation rates of CL by the CLS expressed in *E. coli* are given as a function of the concentration of five different PG species (●, dioleoyl; ○, diolinoleoyl; ■, 1-palmitoyl-2-oleoyl species; □, 1-palmitoyl-2-linoleoyl; ▲ dipalmitoyl). 0.5 µg of protein of isolated membrane fraction from the *E. coli* carrying pCLS-C-His induced for 3 h were assayed under standard conditions.

As depicted in Figure 3-15, the *A. thaliana* CLS displayed highest activity with the dilinoleoyl than with the dioleoyl species of PG, while the 1-palmitoyl-2-oleoyl species was processed at the same rate as the dioleoyl species of PG did. Consequently, the *A. thaliana* CLS activity increased with the unsaturation level of the PG species. In that way, the plant CLS differs from the respective human enzyme (Houtkooper, Akbari et al. 2006). Heterologously expressed plant CLS was less active with the dioleoyl species of PG, progressively less active with the 1-palmitoyl-2-oleoylPG, and inactive with the dipalmitoyl-PG. The PG species specificity of the *A. thaliana* CLS is in line with the fatty acid composition of CL from plants that contain almost exclusively unsaturated C_{18} acyl groups, unlike the fatty acid pattern of PG from plant mitochondria, predominantly consisting of palmitic acid (Bligny and Douce 1980). According to the determined relatively high activities of the *A. thaliana* CLS with mixed PG species carrying a saturated and an unsaturated acyl group (Figure 3-15). It is unlikely however, that the enzyme can exclude such PG species from CL biosynthesis, especially if it is provided with high levels of these species. The inability of the *A. thaliana* CLS to discriminate against mixed substrate species became even more obvious with regard to its second substrate CDP-DAG.

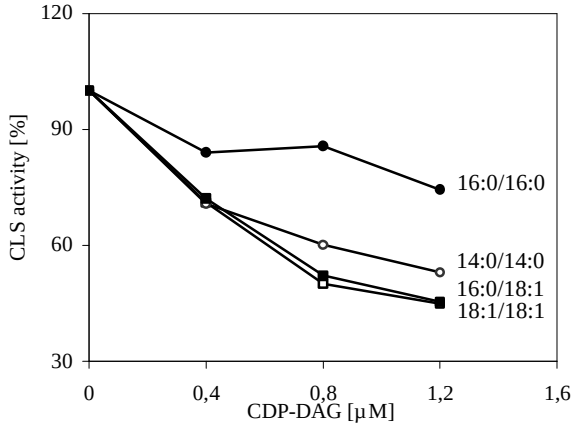


Figure 3-16: CDP-DAG species selectivity of the *A. thaliana* CLS activity
Effect of increasing concentrations of unlabeled CDP-dipalmitoylglycerol (o), CDP-dioleoylglycerol (•), CDP-1-almitoyl-2-oleoylglycerol (■), or CDP-dimirystoylglycerol (□) on the incorporation rates of 1.2 µM CDP-dioleoyl[U-^{14}C]glycerol into CL. Per assay, 0.5 µg of membrane protein isolated from E. coli carrying the pCLS_C-His construct was applied.

CDP-DAG species selectivity of the *A. thaliana* CLS was determined by competition experiments. The formation rates of CL from PG and labelled CDP-dioleoylglycerol was measured in the presence of increasing concentrations of different unlabelled CDP-DAG species. As shown in Figure 3-16, the CDP-dipalmitoylglycerol hardly competed with CDP-dioleolyglycerol as substrate. The dimirystoyl species and those derived from egg-lecithin, predominanty containing the 1-dipalmitoyl-2-dioleoyl species displayed clear competition pattern very similar to that of CDP-dioleoylglycerol.

Hence, the data which are very similar to these reported for the mung bean and human CLS clearly show that the incorporation of the physiologically irrrelevant substrate species cannot be prevented by the properties of the enzyme.

In summary, the *A. thaliana* CLS displays properties very similar to those of the respective enzymes from mung bean and cucumber (Frentzen and Griebau 1994) as well as fromother eukaryotes (Jiang et al. 1997, Tuller et al. 1998, Chang et al. 1998, Houtkooper et al. 2006). An alkaline pH optimum, the absolute requirement for divalent cations and distinctly lower K_m values for CDP-DAG than for PG appear to be typical properties of the mitochondrial CLS from various eukaryotes. However, the enzymes from various organisms differ with regard to their cation and substrate species specificities (Schlame 1993, Schlame et al. 2000, Houtkooper, Akbari et al. 2006). Although the plant CLS possesses more pronounced substrate species specificities than the respective enzymes of mammals, it cannot prevent the incorporation of mixed

species into CL. Consequently, the observed properties of the *A. thaliana* CLS suggest that in plants as in mammals (Xu et al. 2003, Ma et al. 1999) remodelling mechanisms following CL synthesis, such as deacylation/reacylation or transacylation, are required to establish the typical fatty acid pattern of CL.

3.5 Solubilization and reconstitution of the cardiolipin synthase activity

In order to solubilize and purify the CLS protein of *A. thaliana* over-expressed in *E. coli*, the bacterial membranes were extracted with Zwittergent 3-14 or Triton X-100 under various conditions. Best results were obtained with Zwittergent 3-14 at a final concentration of 6.9 mM (approximated to 0.25% (w/v)) and a detergent/protein ratio of about 1.5 (Figure 3-17).

According to the CLS activities remaining in the unsolubilized sediment fractions resuspended in detergent-free buffer, more than 90% of CLS was extracted from the membranes. This, however, was not reflected by the CLS activities of the solubilized fractions. These fractions showed less than 40% of the CLS activity because the detergent concentrations of 0.22 mM in the assay caused a severe inhibition (Figure 3-13). The addition of salts improved the solubilization rate by a few percent only (Figure 3-17). Therefore, salt was omitted during membrane extraction when the solubilized protein fractions obtained by high speed centrifugation should be separated by an anion exchange column.

Attempts to purify the solubilized fractions on Ni^{2+} chelate affinity resin were not successful since the recombinant CLS protein with neither C-teminal nor N-terminal His-tag bound to the affinity resin. On the other hand, the solubilized CLS protein bound to an anion exchange column and CLS activity was detected in the protein fractions eluted with 1 M salt from the column. The recovery of enzymatic activity, however, was very low (less than 2%) even when the inhibition caused by the detergent present in the assays (0.1 mM; Figure 3-13) was considered.

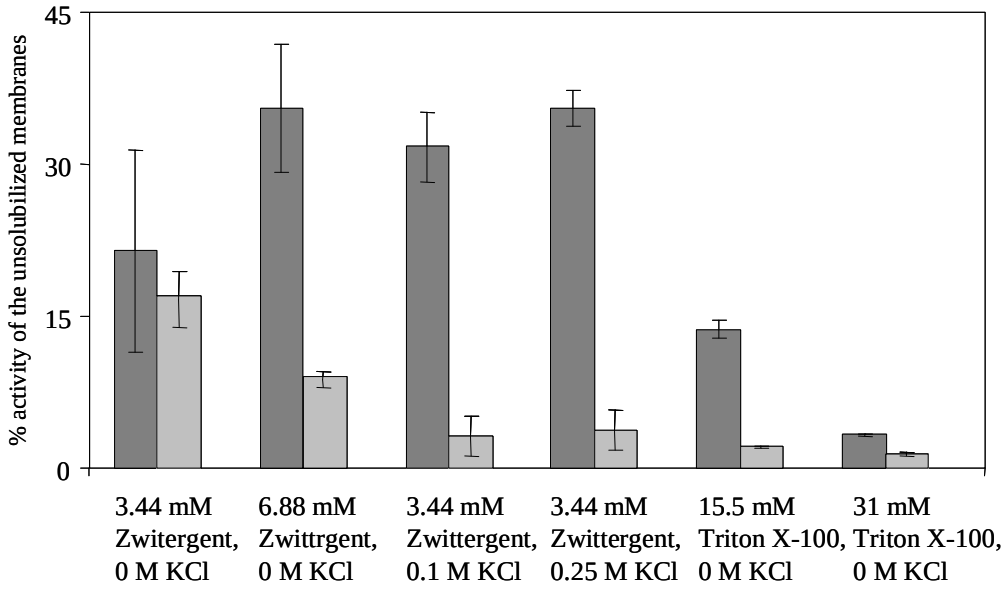


Figure 3-17: The optimization of plant CLS solubilization
The membranes isolated from *E. coli* harboring the pCLS_C-His construct were extracted with the given detergent and salt concentration for 30 min at 4 °C. After 40 min high.speed centrifugation, CLS activity was determined in the supernatants (dark grey) and the pellet fractions (light grey) resuspended in the detergent-free 20 mM MOPS, pH 7.4. The given values were not corrected by inhibition caused by the detergent concentration in the reaction mixture.

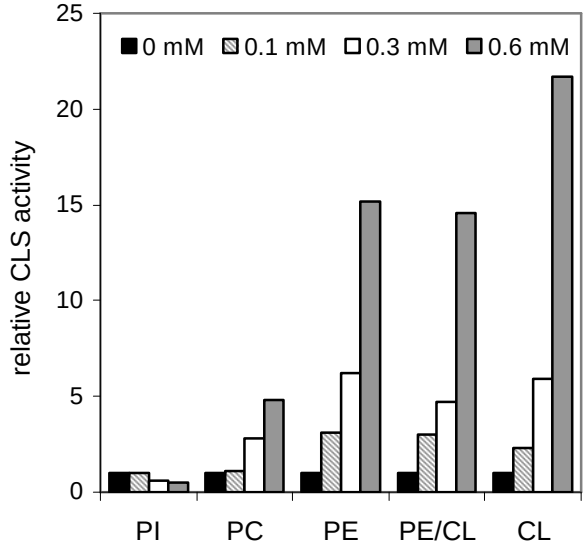


Figure 3-18: Reactivation of the solubilized *A. thaliana* CLS by phospholipids
Enzyme assays were carried out with the 1 M KCl Mono Q eluate in the presence of 0.22 mM Zwittergent 3-14 and the given phospholipid concentrations (PE/CL, 2/1 mixture of the two phospholipids). CLS activity is given as stimulation rate relative to the activity without phospholipids.

The partial reconstitution of the CLS activity was achieved by the addition of phospholipids. As shown in Figure 3-18, CL effected a distinctly higher reactivation than phosphatidylethanolamine (PE) or a mixture of PE and CL. On the other hand, PC,

the major phospholipid of mitochondrial membranes, stimulated the CLS activity only slightly and phosphatidylinositol (PI) even inhibited the activity.

Data similar to those depicted in Figure 3-18 were obtained with partially purified CLS fractions from mung bean mitochondria (Frentzen, unpublished). These data provide strong evidence that the CLS of plants require a defined phospholipid environment, particularly the presence of CL, to acquire the catalytically active conformation. A phospholipid requirement has also been reported for the CLS from rat liver, but unlike the plant enzyme, the mammalian enzyme was found to require interaction with both PE and CL for full enzymic activity (Schlame and Hostetler 1991).

3.6 Analyses of the *A. thaliana* insertional mutants

3.6.1 Selection of the T-DNA insertional mutants of *A. thaliana*

To study the functional role of CL in plant mitochondria, seeds of the T_3 generation of the *A. thaliana* plants carrying T-DNA insertion in the *CLS* gene obtained from ABRC[5)] and NASC[6)] were cultivated and analysed. The position of the T-DNA insertion within the *CLS* gene was verified and mutant plants homozygous with respect to the T-DNA insertion were identified by PCR with gene and T-DNA specific primers.

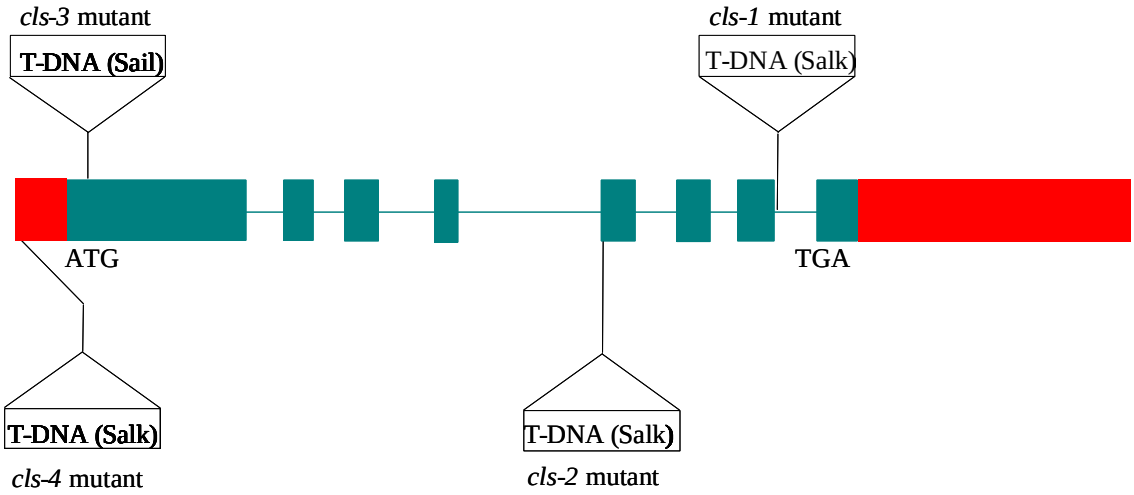


Figure 3-19: Mutants carrying a T-DNA insertion at dfferent positions of the CLS gene
Source collection of the T-DNA insert is given in parentheses. The indicated respective insertion sites were confirmed by sequencing the amplified flanking regions. Coding regions are depicted in blue (exons- thick lines, introns- thin lines), non-coding regions in red (scaled correspondingly). Start and stop codons are marked.

As shown in Figure 3-19, the mutant lines carry a T-DNA insertions at different positions of the *CLS* gene, namely intron 7 (*cls-1* mutant), exon 5 (*cls-2* mutant), exon 1 (*cls-3* mutant) and in the promoter region (*cls-4* mutant). Within the T_3 generation, *cls-1* and *cls-4* mutant plants homozygous with respect to the T-DNA insertion were readily identified by the PCR screens (Figure 3-20; Table 3-1). These homozygous plants were propagated after selfting and used for further analyses as described below.

[5]) http://signal.salk.edu/;

[6]) http://www.arabidopsis.org/

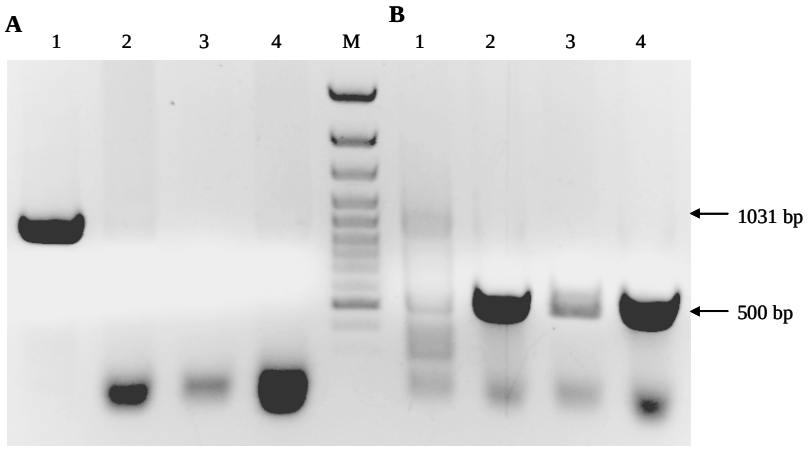


Figure 3-20: PCR screening of the *cls-4* mutant plants
Plant DNA extracted from wild-type (1) and mutant plants (2-4) was analyzed by PCR. Samples were amplified with wild-type *CLS*-specific (026655_LP and 026655_RP) and (B) *CLS* and T-DNA insert-specific (026655_RP and LBa1_TDNA) primers, respectively. Expected product lengths were 920 bp (A) and 600 bp (B). M: 100 bp PLUS marker.

Table 3-1: Phenotypes of the different insertional mutants of *A. thaliana*
Genomic DNA isolated form the leaves of the T_3 and T_4 plants were analysed by PCR using the given *CLS*-aspecific and T-DNA insert-specific primers.

Mutant analysed	Generation	Number of homozygous plants	Number of heterozygous plants	Number of wild-type plants
cls-1[a)]	T_3	6	8	1
cls-2[b)]	T_3	0	1	9
	T_4	0	107	58
cls-3[c)]	T_3	0	25	0
	T_4	0	80	8
cls-4[d)]	T_3	8	8	0

PCR analyses were performed using the following primer combinations: a) 022633_LP3 and 022633_RP3 (wild type); 022633_LP3 LBa1_TDNA (insertion); b) 049840_LP and 049840_RP (wild type); 049840_RP and LBa1_TDNA (insertion); c) SAIL_1231_LP and SAIL_1231_RP (wild type); SAIL_1231_RP and LB2_SAIL (insertion); d) 026655_LP and 026655_RP (wild type); 026655_RP and LBa1_TDNA (insertion).

On the other hand, the *cls-2* and *cls-3* mutant plants showed wild-type or heterozygous phenotype only. Seeds of the selfted heterozygous plants were therefore germinated and plants of the resulting T_4 generations were analysed. The attempts to select the transgenic plants by germinating the seeds on kanamycin-containing plates were not successful, suggesting that the *NPTII* gene present in the T-DNA insert was silenced in both, *cls-2* and *cls-3* mutants, as reported for other T-DNA insertional mutants[7)]. As

[7)] http:\\signal.salk.edu\tdna_FAQs.html

shown in Table 3-1, PCR-based screen of the T_4 plants resulted in the identification of heterozygous and wild-type plants only.

Consequently, these data suggest that *cls-2* and *cls-3*, unlike *cls-1* and *cls-4*, represent loss-of-function mutations and that a loss-of-function in the *CLS* gene is lethal for *A. thaliana*.

3.6.2 Analyses of the homozygous insertional mutants of *A. thaliana*

To investigate whether the expression level of the plant *CLS* gene changed upon the T-DNA insertion, transcript levels were estimated in the wild-type plants and homozygous *cls-1* and *cls*-4 mutants (Giulietti et al. 2001). The *CLS*-specific primers used for the transcript quantification (see 2.8.2 and 5.1) aligned to the exon 7 (RT-CLS F2) and the exon 8 (RT-CLS Rv1), thus the PCR product overlapped the insertion position of the *cls-1* mutant. The results obtained were calibrated according to the abundance of the *Actin 2* transcript used as internal reference (*At3g18780*; Bari et al. 2004) in the respective cDNA preparations.

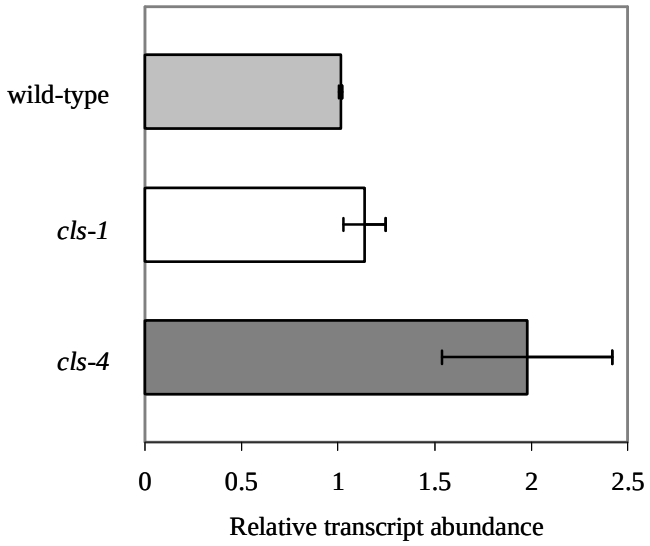


Figure 3-21: The abundance of the plant *CLS* and *Actin2* transcripts
Presented are the results of the real-time quantification of the plant CLS transcripts level as the ratio to the *Actin 2* (At3g18780; internal standard) abundance. The transcripts were quantified three times in duplicates in the cDNA prepared on the total RNA from the fresh plant leaf material by means of real-time-PCR. The sequences detected and sequences of the primers used are described in 2.8 and 5.1.

As presented in Figure 3-21, none of the mutants contained significantly lower *CLS* transcript levels than the wild type, rather the transcript levels of the mutants were very similar or even higher than that of the wild type. These data suggest that neither the T-DNA insertion in the promoter region nor in the intron 7 (Figure 3-19) significantly inhibits the *CLS* transcript synthesis and maturation processes.

To determine whether the T-DNA insertions caused alterations in the CLS activity and CL level in the mitochondrial membranes, cell suspension cultures of the two mutants and wild-type plants were developed. Such cultures have been shown to be the optimal starting material for the isolation and analysis of mitochondria from *A. thaliana* (Werhahn et al. 2001). A comparison of the CLS and PGS activities determined in the mitochondrial fractions isolated from the wild type and the two mutants revealed differences between the *cls-1* and *cls-4* mutants (Figure 3-22). In the mitochondria of the *cls-4* mutant, the CLS displayed two-fold higher activity than the wild type. These data are in line with these of the transcript levels depicted in Figure 3-21. The mitochondria of the *cls-4* mutant, however, showed as well higher PGS activities than the wild-type fractions. Hence, the increase in the CLS activity of the *cls-4* mutant cannot merely be attributed to the T-DNA insertion within the *CLS* gene, but might be caused to certain extend by the overall vitality, as its growth rate was 1.5-fold higher than that of the wild type.

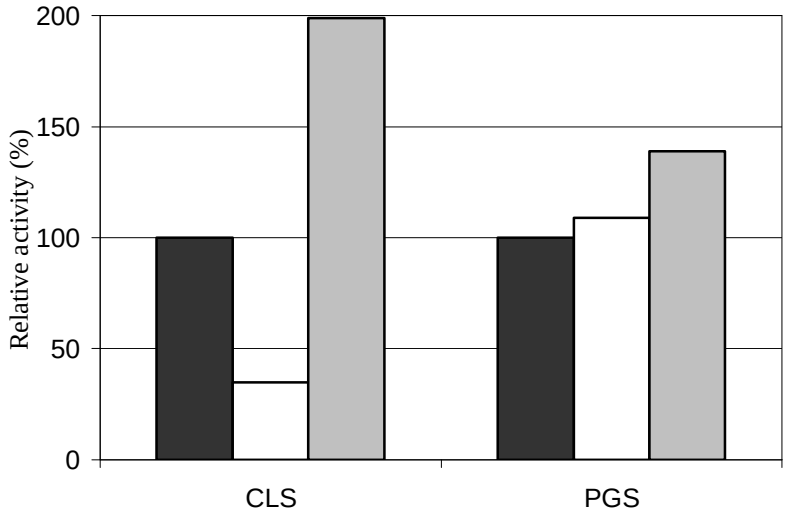


Figure 3-22: Activities of the CLS and PGS in mitochondria
The mitochondria isolated from the cell suspension cultures of the wild type (dark grey bars), the *cls-1* (white) and *cls-4* (light grey bars) mutants of *A. thaliana* were investigated on the CLS and PGS (internal control) activities. To assess the CLS activity, 1 – 10 µg of protein was applied to standard assay. For the PGS activity assays, 2 – 40 µg mitochondrial protein was assayed under standard conditions. 100% CLS activity corresponds to 64 pmol/min/mg and 100% of the PGS activity corresponds to 243 pmol/min/mg).

Unlike the *cls-4* mutant, the cell suspension culture of the *cls-1* mutant grew slightly slower than the wild type and their organelles showed PGS activities very similar to these of the wild type. At the same time, the CLS activity amounted to 35% of the wild type activity only (Figure 3-22). The results of the enzymic assays with mitochondrial fractions of the *cls-1* mutant indicate that the T-DNA insertion partially inhibits *CLS* gene expression, although this was not reflected in the *CLS* transcript level of the mutant (Figure 3-21).

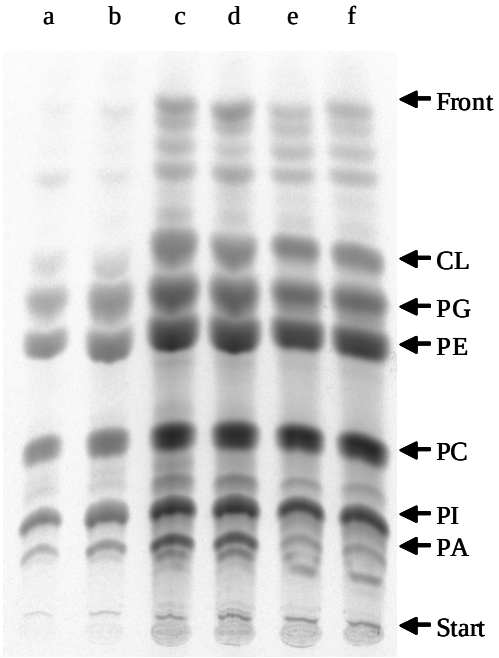


Figure 3-23: ^{33}P phospholipid labelling pattern of wild type and *cls-1* mutant
The TLC analysis was performed on the lipids isolated from the 0.3 g cell suspension culture of wild type (a, c, e) and *cls-1* (b, d ,f) incubated with 50 µCi ^{33}P for 6 h (a, b), 24 h (c, d) or 44 h (e, f) in 10 ml medium. The same labelling experiments were performed with the *cls-4* mutant and gave the same results. Lanes a, b: 30.000 dpm loaded; lanes c – f: 250.000 dpm loaded.

In spite of the differences in the CLS activities determined in the isolated mitochondrial fractions of the *cls* mutants by the *in vitro* enzymic assays (Figure 3-22), *in vivo* phosphate labelling experiments with the cell suspension cultures revealed no significant differences between the two mutants and the wild type. As shown in Figure 3-23, CL was labelled to the same extends in both, the *cls-1* mutant and the wild type. Moreover, these results were not altered when the labelling experiments where conducted with seedlings instead of the cell suspension, or when the *cls-4* mutant substituted the *cls-1* mutant (Table 3-2). Thus, the labelling experiments with the *cls* mutants provide no evidence for an altered membrane lipid composition of their

mitochondria, even in case of the organelles of the *cls-1* mutant possessing CLS activities lower than the wild type.

Table 3-2: Labelling pattern of phospholipids from the cell suspension cultures of wild type and the *cls-1* and *cls-4* mutants
Labelling is expressed as the % of the total phospholipid labelling.

Phospholipid	wild type 1	*cls-1*	wild type 2	*cls-4*
PA	7.33	6.83	2.35	2.15
PI	15.70	16.75	13.25	18.80
PE	29.01	31.24	36.55	34.45
PC	29.66	29.24	29.05	31.30
PG	10.47	8.81	10.15	8.50
CL	2.95	2.30	1.92	1.57

This was further supported by measuring the respiration rates of the cell suspension cultures from the wild type and the two *cls* mutants with an oxygen electrode (Vanlerberghe and McIntosh 1992). These determinations revealed no differences between the wild type and the mutant cultures (data not shown).

In summary, the analyses of the homozyous c*ls-1* and *cls-4* mutants from the *A. thaliana* showed that the T-DNA insertion in the *CLS* gene, either in intron 7 or in the promoter region, respectively, causes at most alterations in the CLS activity. They also provide evidence that such alterations in the CLS activities are not sufficient to affect the membrane lipid composition of the mitochondria and, thus, mitochondria structure and function.

3.6.3 Analyses of the heterozygous insertional mutants

Unlike the *cls-1* and *cls-4* mutant plants, the screenings of the *cls-2* and *cls-3* mutant plants resulted in identification of plants heterozygous with respect to the T-DNA insertions in the exons of plant *CLS* gene, but not homozygous ones, suggesting that *cls-2* and *cls-3* are loss-of-function mutations and that these mutations are embryo lethal (Table 3-1). Analysis of the developing siliques of the heterozygous *cls-2* and *cls-3* mutant plants, however, provided no evidence for an embryo-lethal phenotype. This was further supported by the germination rates of seeds from the selfted heterozygous mutant plants, which showed germination rates very similar to those of the wild type plants (Table 3-3).

Table 3-3: Comparison of seed germination rates
Germination rates were determined after the seeds of respective plants were plated on MS-agar medium, vernalized at 4 °C for 24 h in darkness, and grown under short day conditions for 4 days at 23 °C.

Plant	Number of seeds plated	Germination rate [%]
wild type	315	63
cls-1	358	70
cls-2	242	72
cls-3	400	75
cls-4	249	75

These data point that the mutations primarily affect pollen development or pollen germination and growth rates and, thus, cause male sterility. To provide direct evidence for this assumption, and to overcome the apparent inability of the *cls-2* and *cls-3* mutants to produce homozygous plants with respect to the T-DNA insertion in the *CLS* gene (Figure 3-19), the T_5 selfings of the two mutants were re-transformed with the ORF of the plant *CLS* placed between the lox sequences on a plant binary vector (2.7.5). As shown in Figure 3-24, the construct carried the Cre recombinase gene (Osborne 1995) under the control of the heat-shock promoter as well.

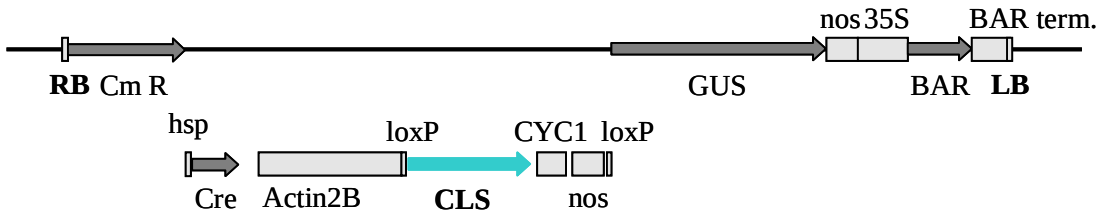


Figure 3-24: The T-DNA region of the pB6 CLS Cre construct integrated into plant genome
The region integrating into the plant genom stretched between the RB (right borader) and LB (left boarder) and additionally carried: Cre recombinase under heat shock promoter (hsp), Actin2B (promoter of *A. thaliana Actin2B* gene; U41998), including the 5'UTR and the first intron; loxP sites for site specific recombination; CLS CYC1 terminator region from the pYES2 CLS construct; nos terminator: nopaline transcription terminator sequence; GUS: coding region of *GUS* gene (transformation marker); 35S: 35S CaMV promoter, which drives the expression of BAR: BASTA resistance gene; BAR terminator: BAR transcriptional terminator sequence. Cm R: chloramphenicol resistance gene, for selection in *E. coli*.

Seeds of the *cls-2* and *cls-3* mutants re-transformed with the pB6 CLS Cre contruct were germinated and cultivated under short day conditions for 3 weeks. Subsequently, the tranfomants were selected by BASTA treatment. So far, BASTA resistant *cls-2* and *cls-3* mutant plants heterozygous with respect to the T-DNA insertion in the *CLS* gene have been selected. Analysis of the progeny of these transformants will show whether the introduced chimeric *CLS* coding sequence will complement the mutant phenotype,

so that mutant plants homozygous with respect to the T-DNA insertion in the native *CLS* gene can be isolated. In such plants CL deficiency can be induced by heat-shock treatment and, thus, the functional role of CL in plants can be assessed.

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5 Appendix

5.1 Primers

Name, Function	Sequence	Position, Comment
CLS_F1 [a)]	cacc**atg**gcg atttacagat ctctaag	Coding region of *At4g04870*, cDNA 140→162
CLS_F1-28 [a)]	cacc**atg**gga accgtttctc tgactcc	Coding region of *At4g04870*, cDNA 227→246
CLS_Rv [a)]	actatgatct cttaatcata gatatag	Coding region of *At4g04870*, cDNA 1165→1140(C)[f)]
CLS_RvHis [b)]	actaatggtg atggtgatgg tgtgatctct taatcatag	Coding region of *At4g04870*, cDNA 1162→1146(C)
CLS_F1NHis[b)]	**atg**caccatc accatcaccat ggcggaac	Coding region of *At4g04870*, cDNA 224→230
CLS7_Rv[a)]	actatgatct cttaatcata gatatag	Coding region of *At4g04870*, cDNA 1026→1001(C)
CLS7_RvHis[b)]	actataccac taccactacc actgatctct taatcataga tatag	Coding region of *At4g04870*, cDNA 1023→1001(C)
LBa1_TDNA[c)]	tggttcacgt agtgggccatc g	T-DNA left boarder
022633_LP3 [c)]	cccgactcag atatcagaaga aatgtac	Coding region of *At4g04870*; genomic DNA, 188709→188736
022633_RP3 [c)]	ggtggatgca tgggacatttg tgc	Coding region of *At4g04870*; genomic DNA, 189645→189622 (C)
049840_LP [c)]	aaaacagtga gagctgcgtat tcc	Coding region of *At4g04870*; genomic DNA, 188814→188837
04980_RP [c)]	ttgaagggcg gaattttctct tat	Coding region of *At4g04870*; genomic DNA, 189928→189905(C)
SAIL_1231_LP[c)]	tatacagttt ggtttcggttt ggatg	Promoter region of *At4g04870*; genomic DNA, 191763→191737(C)
SAIL_1231_RP[c)]	agagaggtcc attaagaggaa cgaac	Coding region of *At4g04870*; genomic DNA, 190828→190853
LB2_SAIL [c)]	gcttcctatt atatcttccc aaattaccaa taca	T-DNA left boarder
022655_LP[c)]	acaaagttga aatctttccg gattc	Promoter region of *At4g04870*; genomic DNA, 192698→192674(C)

022655_RP[c)]	gataataaac ttcttcatcc aaattatgcg	Promoter region of *At4g04870*; genomic DNA, 191781→191810
CYC1terminator[d)]	gcgtgaatgt aagcgtgac	pYES analysis
Actin3 pB6 [d)]	atttggatct gtgaacctcc act	Actin promoter of pB6
RBpB6 [d)]	ggataaacct tttcacgccct ttt	Right Boarder of pB6
CMfor [d)]	taagttggca gcatcacccga	CM^R cassette;
CMrev [d)]	acatacttcg ggtatacta	CM^R cassette;
RT-CLS F2 [e)]	actctagtcg ctggtgcaat acttc	Coding region of *At4g04870*, cDNA 998→1022
RT-CLS Rv1 [e)]	gtacaccata agctgcagtt gaagc	Coding region of *At4g04870*, cDNA 1121→1097 (C)
Actin2_Fw [e)]	ggtaacattg tgctcagtggt gg	*At3g18780* (Actin2) cDNA; 1222→1200(C)
Actin2_Rv [e)]	ggtgcaacca ccttaatcttc at	*At3g18780* (Actin2) cDNA; 1110→1132

a) used for cloning of CLS or clone analysis, b) His-Tag / restriction site modification, c). At4g04870 T-DNA insertional mutant analysis; numbers correspond to clone numbering of Salk Institute; d) used for creation of construct for yeast or plant transformation and CLS reconstitution; e) used for Real-Time PCR experiments; f) (C) denotes sequence on the strand complementary to ORF.

5.2 Chimeric construct maps

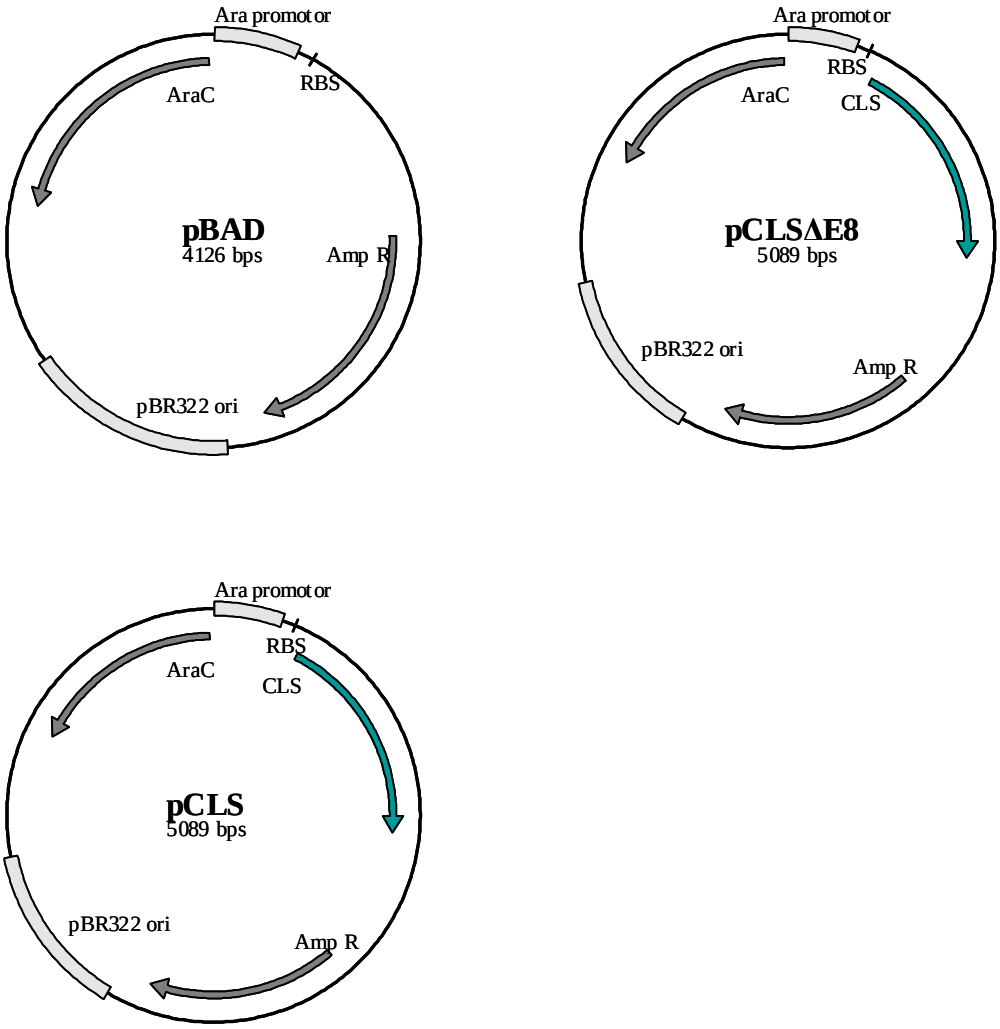


Figure 5-1: Chimeric constructs for expression studies in *E. coli*
The *CLS* constructs were cloned under the control of the Ara promoter that is positively and negatively regulated by the AraC protein, a transcriptional regulator that forms a complex with L-arabinose. Formation of such complex releases the DNA loop and allows the transcription to begin. RBS: ribosome binding site; Amp R: Ampicilin (Carbenicillin) resistance gene; pBR322 ori: pBR322 origin of replication; pBAD: empty vector; pCLSΔE8: pBAD carrying open reading frame of plant CLS missing 30 amino acids of the C-terminal region; pCLS: schematic representation of pCLS, pCLS_C-His, pCLS_N-His, and pCLS_G199D.

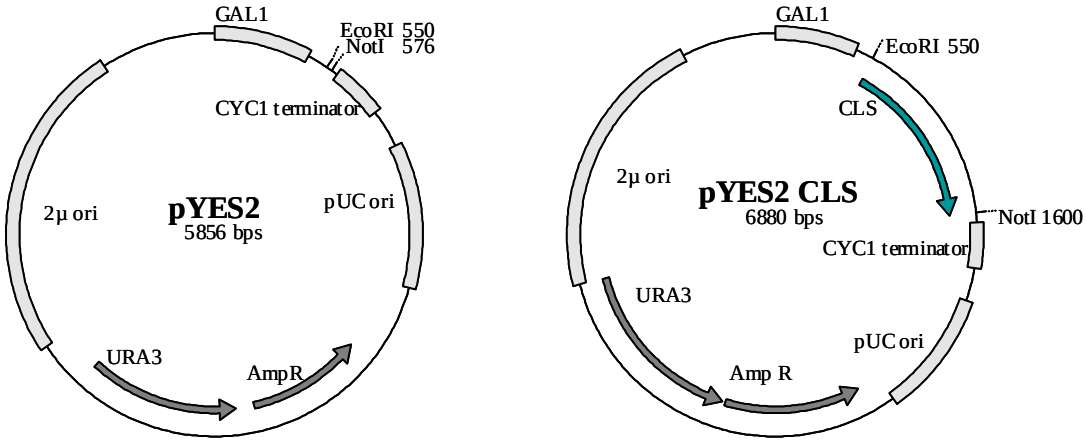


Figure 5-2: Chimeric construct maps for *S. cerevisiae* expression
To inducibly express the recombinant plant *CLS* sequence in yeast, it was introduced into pYES2 vector. Empty vector was used for control transformation. GAL1: promoter for high level inducible expression in yeast by galactose and repression by glucose; CYC1: transcriptional terminator; pUC ori: pUC origin of replication for bacterial amplification; Amp R: ampicilin (carbenicillin) resistance gene for selection in *E. coli*; URA3: *URA3* gene for selction of transformants in yeast host strains with *ura3* genotype; 2µ ori: 2 micron origin of replication.

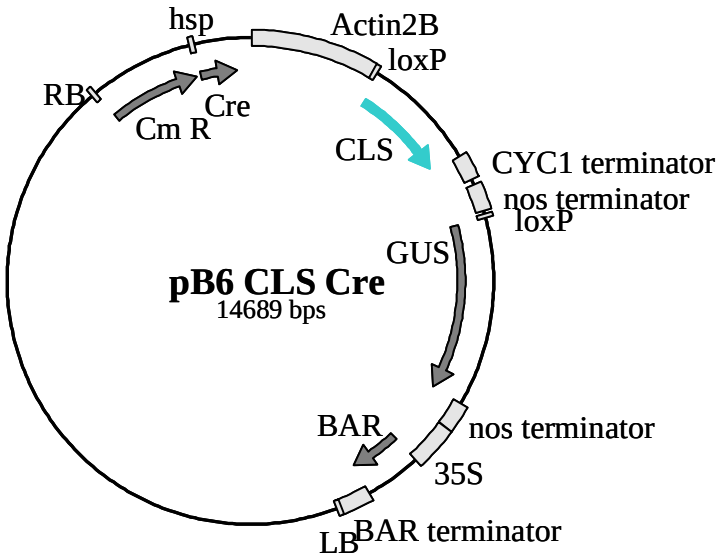


Figure 5-3: Plant transformation construct
The plant transformation construct developed was basing on the pPZP200 construct (Hajdukiewicz et al. 1994). Additionally, Cre recombinase (Cre) under control of heat-shock promoter (hsp) was introduced (kind gift of Dr. S. Kushnir). The T-DNA region of the construct integrated into plant genom stretched between RB (left borader) and RB (right boarder), and additionally carried: Actin2B (promoter of *A. thaliana Actin2B* gene (U41998; At3g18780). including the 5'UTR and the first intron; loxP sites for site.specific recombination; CLS CYC1 terminator region from the pYES2 CLS construct; nos terminator: nopaline terminator sequence; GUS: codig region of *GUS* gene (transformation marker); 35S:35S CaMV promoter, which drives the expression of BAR: Basta® resistance gene. BAR terminator: BAR transcriptional terminator sequence. Cm R: chloramphenicol resistance gene, for selection in *E. coli*.

5.3 Abbreviations

Abbreviation	Meaning
ABRC	Arabidopsis Biological Resource Center; http://www.biosci.ohio-state.edu/pcmb/Facilities/acrc/acrchome.htm
ADP	adenosine diphosphate
APS	ammonium persulfate
ATP	adenosine triphosphate
BSA	bovine serum albumine
BTHS	Barth syndrome
cDNA1	complementary DNA
CDP-DAG	cytidinediphosphate diacylglycerol
CL	cardiolipin
CLS	cardiolipin synthase
CoA	coenzyme-A
CTP	cytidine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotides, mixed
dpm	disintegration per minute
dsDNA	double-strand DNA
DTT	dithiothreitol
E.C.	The Enzyme Comission
EDTA	ethylenediamintetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
ER	endoplasmatic reticulum
g	gravitational constant
GroP	glycerol 3-phosphate
GPAT	glycerol 3-phosphate acyltransferase
HPLC-MS	high pressure liquid chromatography coupled with mass spectrometry
HRP	horseradish peroxidase
kDa	kilodalton
K_m	Michaelis-Menten constant
lacZ	β-galactosidase
LPAAT	lysophosphatidic acid acyltransferase
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LPG	lysophosphatidylglycerol
MLCL	monolysocardiolipin
M-MLV RT	Moloney Murine Leukemia Virus reverse transcriptase
MOPS	3-(N-morpholino)propanesulfonic acid

mRNA	messenger RNA
NASC	The European Arabidopsis Stock Centre; http://www.arabidopsis.info
OD	optical density
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PG	phosphatidylglycerol
PGP	phosphatidylglycerolphosphate
PGPas	phosphatidylglycerolphosphate phosphatase
PGS	phosphatidylglycerolphosphate synthase
PI	phosphatidylinositol
PMSF	phenylmethanesulfopnyl fluoride
PS	phosphatidylserine
PVDF	polyvinykidine fluoride
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
RT	room temperature
RT	reverse transcription
SDS	sodium dodecyle sulfate
TAZ	taffazine
T-DNA	transfer DNA
TEMED	tetramethylenediamine
TLC	thin layer chromatography

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