The Importance of Cardiolipin Synthase for Mitochondrial Ultrastructure, Respiratory Function, Plant Development, and Stress Responses in Arabidopsis

Bernard Pineau, a,b,1 Mickaël Bourge, b Jessica Marion, c Caroline Mauve, a Francoise Gilard, a Lilly Maneta-Peyret, d Patrick Moreau, d Béatrice Satiat-Jeunemaître, b,c Spencer C. Brown, b,c Rosine De Paepe, a and Antoine Danon a,e,2

a Institut de Biologie des Plantes, Saclay Plant Science, Université de Paris-Sud XI, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8618, 91405 Orsay cedex, France
b Imagif, Centre National de la Recherche Scientifique, Institut Fédératif de Recherche 87/Fédération de Recherche du CNRS 3115, 91198 Gif-sur-Yvette cedex, France
c Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, Unité Propre de Recherche 2355, 91198 Gif-sur-Yvette cedex, France
d Laboratoire de Biogenèse Membranaire, Centre National de la Recherche Scientifique, Université Bordeaux Segalen, Unité Mixte de Recherche 5200, 33076 Bordeaux, France
e Institut de Biologie Physico-Chimique, Laboratoire de Biologie Moléculaire et Cellulaire des Eucaryotes, Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, FRE3354, 75005 Paris, France

ORCID ID: 0000-0001-6311-6076 (A.D.).

Cardiolipin (CL) is the signature phospholipid of the mitochondrial inner membrane. In animals and yeast (Saccharomyces cerevisiae), CL depletion affects the stability of respiratory supercomplexes and is thus crucial to the energy metabolism of obligate aerobes. In eukaryotes, the last step of CL synthesis is catalyzed by CARDIOLIPIN SYNTHASE (CLS), encoded by a single-copy gene. Here, we characterize a cls mutant in Arabidopsis thaliana, which is devoid of CL. In contrast to yeast cls, where development is little affected, Arabidopsis cls seedlings are slow developing under short-day conditions in vitro and die if they are transferred to long-day (LD) conditions. However, when transferred to soil under LD conditions under low light, cls plants can reach the flowering stage, but they are not fertile. The cls mitochondria display abnormal ultrastructure and reduced content of respiratory complex I/complex III supercomplexes. The marked accumulation of tricarboxylic acid cycle derivatives and amino acids demonstrates mitochondrial dysfunction. Mitochondrial and chloroplastic antioxidant transcripts are overexpressed in cls leaves, and cls protoplasts are more sensitive to programmed cell death effectors, UV light, and heat shock. Our results show that CLS is crucial for correct mitochondrial function and development in Arabidopsis under both optimal and stress conditions.

INTRODUCTION

Mitochondrial internal membranes have a specific lipid composition involved in both specific protein–protein and protein–lipid interactions that are essential for mitochondrial function (Schlame et al., 2000). Cardiolipins (CLs) are dimeric anionic phospholipids localized in bacterial membranes and in mitochondrial internal membranes of eukaryotes (Bligny and Douce, 1980). They are composed of four acyl chains, carry two phosphate groups, and can adopt different structures. In mitochondria, CLs are involved in membrane curvature and cristae formation (Mileykovskaya and Dowhan, 2009). In all eukaryotes examined so far, the last step of CL synthesis is catalyzed by cardiolipin synthase (CLS) from phosphatidyl-4cytidine monophosphate (CMP) and phosphatidylglycerol (PG; Tamai and Greenberg, 1990; Schlame and Hostetler, 1997). The CLS gene is present in single copy in yeast (Saccharomyces cerevisiae; Chang et al., 1998), Arabidopsis thaliana (Katayama et al., 2004), and mammals (Nowicki et al., 2005). In yeast, cls1 disrupted (crd1) mutants are viable, although they display reduced oxidative phosphorylation activities and mitochondrial membrane potential (Jiang et al., 1997; Zhong et al., 2004). CLs have been shown to bind with high affinity to a large portion of the proteins of the inner mitochondrial membrane, such as complexes I, III, IV, and V, the carrier family (ADP–ATP carrier, phosphate carrier, uncoupling protein), and two peripheral membrane proteins (cytochrome c and creatine kinase) in yeast and mammals (Sharpley et al., 2006). CL was shown to be required for the formation and stabilization of respiratory chain supercomplexes, which have been proposed to be essential for proper functioning of the mitochondrial electron transport chain in the human respirasome and for supramolecular organization of ATP synthase (Acehan et al., 2011). CLs are thus crucial for physiological ATP production. Moreover, it was shown that CLs are implicated in the mitochondrial transport machinery in yeast (Jiang et al., 2000) by interacting with translocases of the outer mitochondrial membranes (Gebert et al., 2009, 2011). In human tissues, decreased CL content, alterations in its acyl chain composition, and/or peroxidation have been...
results in plant development and function both under normal and stress conditions. Plant mitochondria maintain a constant \( \text{inherently associated with mitochondrial dysfunction in different pathological conditions, such as cardioskeletal myopathy Barth syndrome (Vreken et al., 2000; Gu et al., 2002; Scherer and Schmitz, 2011). Peroxidized CLs have also been reported to play a crucial role in the animal apoptotic process (Ott et al., 2007).}

Much less information is available on CL function in plants, as the characteristics of plant cls mutants and of CL-lacking plants are unclear. Although stable mitochondrial supercomplexes containing complex I (CI) and dimeric complex III have been described in several species (Eubel et al., 2003, 2004) and a stable respirasome-like complex has been described in spinach (Spinacia oleracea) green leaf mitochondria (Krause and Durner, 2004), their association with CL in the plant kingdom is unclear. Here, we characterize three mutants disrupted in the Arabidopsis CLS single-copy gene, named cls mutants. In vitro, germinating seedlings of cls mutants are able to develop under low-illumination conditions only, and all three have a slow-growing phenotype. When transferred to soil, they can eventually reach the flowering stage, but they are not fertile. We show that the cls mutant is fully devoid of CL, confirming that CLS activity is essential for CL biosynthesis in plants as in other eukaryotes. The cls mitochondria have an abnormal ultrastructure and reduced content in respiratory CI and supercomplex I/III (CI/CIII), showing alteration in mitochondrial electron transport. Amino acid accumulation occurs in the mutants, which is indicative of extensive metabolic perturbations. Antioxidant systems and programmed cell death are also exacerbated in cls, indicating a general perturbation of stress responses. Our results show that CL is necessary for correct mitochondrial function and development in Arabidopsis, especially under stress conditions.

RESULTS

Morphological Characterization of the cls Phenotype in Arabidopsis

A single gene encoding CLS, named CLS, was identified in the ecotype Columbia (Col-0) nuclear genome (At4g04870; Katayama et al., 2004). The SALK_49835, SALK_4984, and SALK_2263 lines from the Salk Institute (Alonso et al., 2003) were identified as containing independent T-DNA insertions within this gene. We confirmed the localization of the predicted T-DNA insertions in the CLS gene by PCR using different primer combinations (Figures 1A to 1C). Homozygous cls mutants were easily recognized when these lines were germinated in vitro on gelose medium under continuous light and very low intensity (30 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). Indeed, a few days after germination, approximately one-quarter of the seedlings of the three lines exhibited a strong blond phenotype (Figures 2A to 2C). No CLS mRNA could be detected in these seedlings (Figure 1D), which were therefore named cls1 (SALK_49835), cls2 (SALK_4984), and cls3 (SALK_2263). The mutants were able to develop under higher light intensity, \(-80 \mu \text{mol m}^{-2} \text{s}^{-1} \), but under short-day (SD) conditions only, and they displayed a pale green phenotype (Figures 2D and 2E). When transferred to long-day (LD) conditions, most cls plantlets started to bleach and die (Figure 2F; see Supplemental Figure 1 online). Taken together, these observations indicate a higher susceptibility of cls seedlings to light quantity. When transferred to soil in LD conditions under low light (<50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)), only a few cls plantlets were able to survive and initiate the transition from vegetative to reproductive growth. cls rosette plants developed inflorescence stems in synchrony with the wild type;
however, the size of leaves and inflorescence stems was severely reduced (Figure 2G; see Supplemental Figure 1 online). Moreover, cls adult plants produced inordinately small siliques (arrowed in insets of Figure 2G) containing only a few and irregularly-sized seeds that were not able to germinate. Similar results were obtained with all three SALK lines and provide evidence that the drastic phenotypes we observed come from the \textit{CLS} mutation.

In further analyses, we used the SALK_49835 line as a source of cls mutant plants, referred to as \textit{cls1}.

**Absence of CL and Membrane Lipidic Changes in cls1**

The cls1 mutant was expected to be devoid of CLs. Therefore, we quantified CL content in Col-0 and cls1 by high performance thin layer chromatography (HPTLC; see Methods). While the concentration of CL in Col-0 reached 34 ± 12 ng mg⁻¹ fresh weight, no trace of CL could be detected in cls1 seedling lipids (Figure 3A). In addition, we examined the content of some of the main membrane lipids. The content of total polar lipids was similar between Col-0 and cls1, but phosphatidylethanolamine (PE) + PG increased by ~19% in the mutant and phosphatidylcholine by 40%, while phosphatidylinositol (PI) and phosphatidylserine (PS) decreased by 30% (Figure 3B). As HPTLC analyses were first specifically designed to detect the very-low-abundance CL, we could not in this case discriminate phosphatidylinositol and phosphatidylserine, on the one hand, and PE and PG, on the other hand (Figure 3B).

**The cls Mutation Affects Mitochondrial Structure and Function**

Disruption of the \textit{CLS} gene was expected to have marked consequences on mitochondrial populations in plants as in the yeast \textit{crd1} mutant. Col-0 and cls1 tissues were stained with the Nernst potential-sensitive markers MitoTracker Red and 3,3′-dihexyloxycarbocyanine iodide [DiOC6(5)].

These two probes were used in parallel not only to explore complementary spectral domains but also to appreciate nonspecific...
binding (to lipids and waxes), which tends to be higher with DiOC6(5), even though it is a potential-sensitive dye, than with MitoTracker Red (Bourdon et al., 2012). Using confocal microscopy, we clearly identified active mitochondria in Col-0 leaf mesophyll (Figure 4A) and epidermal (Figure 4B) cells. Both markers gave comparable observations (the former fluoroscences red and the latter green), the organelles were punctate (1 µm or less), sometimes fusiform, often mobile. Aggregation of mitochondria was occasionally observed in mesophyll cells (Figure 4A). In cls1, labeling similar to Col-0 was observed in mesophyll (Figure 4D) and epidermal (Figure 4E) cells; however, very small mitochondria appeared, while mitochondrial aggregates were more frequent in the mesophyll, and fivefold larger globular structures (considering their axes) were observed in labeled DiOC6(5) epidermal cells (Figure 4E). Using 10-N-nonyl acridine orange (NAO), which was initially described to specifically bind CLs in mitochondrial membranes (Petit et al., 1992), similar classes of structures appeared in Col-0 as with the other dyes (Figure 4C). Unexpectedly, mobile NAO* mitochondria were also observed in cls1 epidermal cells and stomata, which also contain large globular mitochondria. Furthermore, in many cells of both genotypes (Col-0 and cls1), NAO strongly labeled other membranes.

Similar observations were done in protoplasts isolated from young in vitro-grown seedlings (Figures 4G to 4N). In Col-0 protoplasts, MitoTracker Red revealed typical small punctate mitochondria with high contrast (Figures 4G and 4H), while labeling of both small punctate mitochondria and of large organelles (arrows) was observed in cls1 protoplasts (Figures 4I and 4J). Similarly, NAO labeled both punctate and large organelles in Col-0 protoplasts (Figures 4K and 4L) and in cls1 protoplasts (Figures 4M and 4N). Moreover, in addition to mitochondria, the stain labeled a whole range of structures, such as cytoplasmic membranes and the chloroplast periphery in both genotypes, showing that the dye is not specific for CL in our experimental conditions. This was confirmed using flow cytometry (Figure 4C), gating on light scatter, and exclusion of propidium iodide in order to target only intact protoplasts. In contradiction with any putative binding of NAO to CLs, cls1 protoplasts were even more fluorescent than Col-0 protoplasts after 5 min of staining with 100 nM NAO. The capacity to label with NAO decreased if the protoplasts had first been stored, but the same relationship was observed (Figure 4C for protoplasts stored 2 or 4 h). If the geometric mean of NAO fluorescence after 2 h of incubation is set at 100 (arbitrary units) for intact wild-type protoplasts, the cls1 population had a mean of 142 arbitrary units.

In summary, all fluorescent markers used, including NAO, outlined the effects of the cls mutation on the size and three-dimensional organization of the mitochondrial pool. The unspecificity of NAO for CL binding is possibly due to the low concentration used (100 nM). Similar unspecificity has already been reported in the yeast cdt1 mutant (Gohil et al., 2005a; Mileykovskaya and Dowhan, 2009).

The cls Mutation Affects the Mitochondrial Ultrastructure

The lack of CL was previously shown to affect mitochondrial shape and ultrastructure in the yeast cdt1 mutant and in human Barth cells (Acehan et al., 2007; Mileykovskaya and Dowhan, 2009). The ultrastructure of Col-0 and cls1 leaf cells of plantlets maintained in SD conditions was examined by transmission electron microscopy. Col-0 mitochondria showed the typical features of functional mitochondria in plant cells (Frey and Mannella, 2000), consisting of ovoid organelles, ranging in size from 0.5 to 1.2 µm (longer axis; Figures 5A and 5B). The osmium/potassium ferrocyanide impregnation outlined the numerous cristae. The mitochondrial population of cls1 leaf cells appeared far more heterogeneous in size and aspect (Figures 5C to 5H). While a few cls1 mitochondria (roughly 2%) had size and shape similar to those of Col-0 mitochondria (Figure 5C), most (roughly 70%) were oblong or asymmetrical and exhibited fewer cristae, reminiscent of young, undifferentiated mitochondria (Figures 5E to 5G). In addition, giant mitochondria (roughly 28%) were observed, with a 2- to 6-µm-long axis (Figures 5D and 5H). Giant mitochondria could correspond to the large fluorescent structures shown in Figure 4. Neither the shape, size, and thylakoid organization of cls1 chloroplasts (Figure 5H) nor the endomembrane system (endoplasmic reticulum, Golgi, vacuoles) appeared to be affected in the mutant leaf cells.

These results confirmed that the cls mutation induces drastic changes in mitochondrial morphogenesis and ultrastructure.

Importance of CLs for the Assembly of Respiratory Complexes of the Inner Mitochondrial Membrane

CLs have been shown to interact with different complexes of the inner membrane in mammals and yeast (McCayle et al., 1999; Zhong et al., 2004; Sharpley et al., 2006) and to stabilize respiratory chain supercomplexes (Pfeiffer et al., 2003; Gebert et al., 2009). We compared the accumulation of respiratory CI and CI/CI11 in Col-0 and the cls1 mutant by blue native electrophoresis (Figure 6). In dodecyl maltoside–solubilized proteins of Col-0 and cls1 leaf membrane extracts (Figure 6A), both in-gel NADH/nitroblue tetrazolium (NBT) staining, which reveals NADH dehydrogenase activity, and immune detection using an antibody directed against the NAD9 subunit of the CI peripheral arm (Klodmann et al., 2010) showed that CI accumulation was lower in cls1 than in Col-0. In contrast, the accumulation of complex IV, revealed by the anti-COXII antibody, was unaffected (Figure 6A).

Moreover, in proteins solubilized by digitonin, which maintains the association of supercomplexes (Fineau et al., 2008), NAD9 was essentially incorporated into CI/CI11 in Col-0, whereas such supercomplexes were hardly detectable in cls1 (Figure 6B). The level of CI/CI11 in cls1 mitochondrial membranes also appeared low after NADH/NBT staining (Figure 6B). The lack of accumulation of CI and of CI/CI11 indicates an alteration of the mitochondrial electron transport and possibly of respirasome function (Krause and Durner, 2004) in the cls1 mutant.

Changes in Antioxidant Gene Expression in cls

Alteration of mitochondrial electron transport in cls1 was expected to increase the probability of the formation of reactive oxygen species (ROS) by respiratory complexes, essentially CI and CI1 (Moller, 2001). To test this hypothesis, transcript levels of several genes previously reported to reflect cellular ROS levels were quantified (Figure 7). First, transcript levels of AOX1 and NDB2, genes encoding alternative oxidase and external NAD
Figure 4. Confocal Microscopy of Col-0 and cls1 Cells and Flow Cytometry of NAO Intensity in Protoplasts.

(A) to (F) Leaves. In Col-0 mesophyll (A) and epidermal (B) cells, the Nernst potential-sensitive dyes MitoTracker Red (red) and DiOC6(5) (green) revealed punctate (arrowhead) or fusiform mitochondria ~1 µm in length; chloroplasts are coded blue. Occasional clusters of mitochondria were also observed (arrows). In cls1 mesophyll (D) and epidermal (E) cells, both very small punctate mitochondria (arrowhead) and large reticulate mitochondria (arrows) were labeled with MitoTracker Red, and immobile globular structures were observed with DiOC6(5). The putatively potential-insensitive mitochondrial dye NAO (green) revealed a similar range of mitochondrial forms in Col-0 mesophyll (IC; chloroplasts coded red); in cls1, punctate mitochondria were observed in epidermal cells (IF; arrowhead), and large globular mitochondria (IF; arrows) were present in stomata. The lipidic osteolemma was also labeled by NAO. Bars = 5 µm.

(G) to (N) Protoplasts isolated from young in vitro–grown seedlings. In Col-0 protoplasts, MitoTracker Red revealed typical small punctate mitochondria with high contrast [IG] and [IH]. Both small punctate mitochondria and large organelles (arrows) were labeled in cls1 protoplasts [I] and [J]. Chloroplasts are coded in blue in (G) and (I), whereas (H) and (J) are single-color micrographs. NAO (green) labeled tiny punctate mitochondria and larger organelles in Col-0 protoplasts [IK] and [IL]; in cls1 protoplasts [IM] and [IN], both punctate mitochondria and a whole range of structures, such as cytoplasmic membranes and the chloroplast periphery (arrow in IN], were labeled by NAO. Bars = 5 µm.

(O) Flow cytometry of Col-0 and cls1 protoplasts to quantify NAO uptake. Histograms indicate the relative intensity of NAO charge in protoplasts after 5 min of staining with 100 nM NAO and counterstaining with PI. The capacity to label with NAO was measured after storing the protoplasts at room temperature 2 h (left histogram) and 4 h (right histogram). The geometric mean of NAO fluorescence was determined for Col-0 (shaded histogram) and cls1 protoplasts (unshaded histogram) using analytical cytometry to exclude PI-permeant protoplasts. For each histogram, the geometric mean was obtained and reexpressed in arbitrary units (au), assigning 100 to the mean of the wild type at 2 h. Unstained protoplasts were <3 arbitrary units.

**CLS Mutation Affects Tricarboxylic Acid–Derived Metabolites and Amino Acid Levels**

Mitochondria play a crucial role in the plant nitrogen/carbon balance (Fernie et al., 2004), and CI mutants have been shown to accumulate tricarboxylic acid (TCA) cycle–derived metabolites (Dutilleul et al., 2005; Meyer et al., 2009). Similarly, the balance of primary metabolites, examined by gas chromatography–time of flight–mass spectrometry (GC-TOF-MS; Figure 8), was markedly altered in cls1 as compared with Col-0 plants. In addition to the accumulation of soluble sugars in cls1 plants, most TCA cycle intermediates were more abundant in the mutant than in Col-0. Fumarate, the most abundant acid in Arabidopsis, synthesized in part by a cytoplasmic fumarase (Pracharoenwattana et al., 2010), was increased only marginally. In parallel to TCA cycle derivatives, most amino acids were also more abundant in cls1 than in Col-0 plants, except Glu and Gin, which were not significantly different, and (P)H dehydrogenase, respectively, two mitochondrial respiratory enzymes whose expression is induced under several stress conditions (Yoshida and Noguchi, 2009), are markedly increased in cls1 seedlings. Moreover, as overexpression of genes encoding extramitochondrial antioxidant enzymes has been reported previously in mitochondrial CI mutants (Dutilleul et al., 2003b; Meyer et al., 2009), several markers of cellular oxidative stress were tested, such as Catalase1 (CAT1) to CAT3 for the peroxisome catalase (Queval et al., 2007; Du et al., 2008) and Ascorbate peroxidase1 (APX1) and APX2 for the cytosolic ascorbate peroxidase (Koussevitzky et al., 2008). Whereas no significant transcript changes were detected for the major CAT2 and APX1 genes, CAT1, CAT3, and APX2 transcript levels were markedly increased in cls1. Among the genes previously reported to be implicated in the chloroplastic response to ROS (op den Camp et al., 2003; Kim et al., 2012), we tested distinctive markers that are selectively induced either by superoxide/hydrogen peroxide, FERRITIN1 (FER1), or by singlet oxygen, BONZAI-ASSOCIATED PROTEIN (BAP1). In cls1, transcript levels of BAP1 but not of FER1 were strongly increased, suggesting singlet oxygen but not superoxide/hydrogen peroxide overproduction in cls1 plastids. Taken together, these results suggest that in cls1, oxidative stress occurs, probably from both mitochondria (superoxide/hydrogen peroxide) and plastids (singlet oxygen).
Asp, which was decreased. In particular, levels of photorespiratory Gly and Ser were markedly increased in the mutant, as was the level of glycolate but not of glycerate, the end product of the photorespiratory cycle. The glycolate:glycerate, Gly:Ser, and Ser:glycerate ratios were higher in cls1 than in Col-0 leaves, suggesting impairment of the photorespiratory cycle and marked limitation at the level of glycolate oxidase and Gly decarboxylase activities (Douce et al., 2001). In contrast, integration of glycerate into the Calvin cycle in the chloroplast appeared not to be limited.

**Increased Sensitivity of cls Protoplasts to Programmed Cell Death Effectors**

CLs have been reported to mediate programmed cell death (PCD) in animals (Schug and Gottlieb, 2009). The impact of the lack of CL on the initiation of PCD in plants was tested using UV-C light and heat shock, two treatments known to induce a mitochondria-dependent PCD in Arabidopsis (Danon et al., 2004; Vacca et al., 2006; Gao et al., 2008). The quantification of cell death reactions was achieved using protoplasts and Evans Blue dye, which selectively stains dead cells (Wright et al., 2000; Danon et al., 2005). Protoplasts from Col-0 and cls1 seedlings were isolated, and the percentage of dead cells was calculated 2, 4, and 6 h after treatment. The frequency of dead cells increased in cls1 even after 2 h of treatment with UV-C light (Figure 9A) or heat shock (Figure 9B), whereas there was no significant increase in Col-0. For later time points, dead cells increased both in Col-0 and cls1, but more rapidly and more intensively in the mutant. In the absence of treatments, the number of dead Col-0 and cls1 cells remained almost constant throughout the experiment (Figure 9). Collectively, these results suggest that, in response to different stimuli, the cls1 mutant develops an exaggerated and accelerated PCD.

**DISCUSSION**

We have identified knockout mutants for the CLS gene in Arabidopsis, which has allowed us to evaluate the importance of CLs for mitochondrial ultrastructure and function as well as for development and adjustment to light conditions and response to...
PCD treatments. The cls1 mutants did not display a detectable amount of CL (Figure 3A), which was expected since CLS is the last step of CL synthesis in plants as in other eukaryotes and the CLS gene is in single copy. CL deficiency induces changes in the lipid homeostasis of whole plant cells, phosphatidylcholine, PE, and PG being in higher amounts in the cls1 mutant than in Col-0. Interestingly, PG is the direct precursor of CL, while PE and CL have been reported to have overlapping functions and to cooperate in maintaining mitochondrial morphology (Gohil et al., 2005b; Joshi et al., 2012). CLs are key molecules for plant mitochondrial structure and function. Indeed, cls1 plants present some of the characteristics of CL-deficient cells described in yeast and mammals, where the lack of CL results in an aberrant morphology of mitochondrial cristae (Mileykovskaya and Dowhan, 2009; Gonzalez et al., 2013) and the instability of respiratory chain supercomplexes (see Introduction). In the cls1 mutant, most mitochondria have abnormal shape with few cristae (Figure 5), and the low abundance of CI/CII of the respiratory chain (Figure 6) strongly suggests that mitochondrial electron transport is affected. Moreover, giant mitochondria are observed in cls1 cells (Figures 4 and 5), as has been observed in other CL-deficient cells (Gonzalez et al., 2013), suggesting impairment of the plant mitochondrial fusion/fission machinery (Westermann, 2010). Such a function of CL might be supported by several nonexclusive mechanisms, including either intrinsic properties (Schlame, 2008; Furt and Moreau, 2009) and/or properties related to its importance in mitochondrial ATP production and membrane protein import (Herlan et al., 2004; Joshi et al., 2009; Acehan et al., 2011; Gebert et al., 2011). Increased levels of TCA-derived organic acids (Figure 8) suggest the blocking of glycolysis and the TCA cycle following impaired respiration and mitochondrial protein import. Whatever the mechanism(s) involved, these results demonstrate strong alterations of mitochondrial biogenesis and activity in cls1 mutant plants. However, the presence of at least partially functional mitochondria, in particular giant mitochondria, is indicated by the accumulation of membrane potential–sensitive dyes and NAO (Figure 4), showing that this dye is definitely not specific for CL, as demonstrated previously in yeast (Gohil et al., 2005a; Rodriguez et al., 2008; Mileykovskaya and Dowhan, 2009).

While mitochondrial activity is sufficient to allow the development of the cls1 mutant under low light, although at a very low rate, the mutant is not able to develop and then bleaches under higher illumination conditions (either higher light intensity or LD conditions). An analysis of several markers of oxidative stress (Figure 7) suggests that light sensitivity can result, in last in part, from ROS overproduction.

First, restricted mitochondrial electron transport is expected to generate more superoxide/H2O2 (Møller, 2001), and the accumulation of mitochondrial and extramitochondrial antioxidant transcripts was previously observed using inhibitors of the respiratory chain (Vidal et al., 2007; Umbach et al., 2012). Accordingly, transcripts of AOX1 and NDB2, which are responsive to H2O2 (Wagner, 1995), accumulate in cls1 (Figure 7), as reported previously in Nicotiana sylvestris (Sabar et al., 2000; Dutilleul et al., 2003b; Vidal et al., 2007), maize (Zea mays; Karpova et al., 2002), and Arabidopsis CI mutants (Meyer et al., 2009). The accumulation of peroxisomal CAT1 and CAT3 mRNAs, which, unlike CAT2, are not related to photorespiratory activity (Du et al., 2008; Mhamdi et al., 2012), might also respond to H2O2 accumulation and favor accumulation of the cls1 mutant to mitochondrial impairment.

Second, the impairment of the photorespiratory pathway originating from the absence of CL in cls mitochondria might also have an impact on plastid functioning, as a deficiency in photorespiration has been shown to interrupt the Calvin cycle and to affect the stability of the photosystem II (Takahashi et al., 2007). In cls plants, a photosynthetic impairment might generate singlet oxygen and result in photooxidative stress, as reported elsewhere (Apel and Hirt, 2004; Krieger-Liszkay, 2005). In cls1 seedlings, we found that mRNAs of the singlet oxygen–responsive gene BAP1 (op den Camp et al., 2003; Kim et al., 2012) strongly accumulate, which is consistent with the production of singlet oxygen in plastids, as would be expected from a lack of stability in photosystem II. Another possible but not exclusive explanation

![Figure 7. Changes in Antioxidant Gene Expression Levels in the cls1 Mutant.](image-url)

The accumulation of transcripts related to oxidative stress in mitochondria (AOX1a and NDB2), plastids (BAP1 and FER1), cytosols (APX1 and APX2), or catalases (CAT1, CAT2, and CAT3) was quantified by real-time PCR in Col-0 and cls1 seedlings. Results shown are mean relative mRNA levels ± se from at least three independent samples. Asterisks indicate significant differences between cls1 and Col-0 values.
Figure 8. Leaf Metabolic Content Is Altered in the cls1 Mutant.

TCA-derived metabolites, amino acids, and total sugars are from GC-TOF-MS analyses. Results shown are mean metabolite contents according to fresh weight (arbitrary units) ± se from three independent samples. Asterisks indicate significant differences between cls1 and Col-0 values. TCA-fumarate indicates all TCA-derived organic acids except fumarate, by far the most abundant organic acid in Arabidopsis.
for singlet oxygen production in cls1 plastids comes from the fact that mitochondria are able to dissipate redox equivalents from the chloroplast to protect them from photoinhibition (Woodson and Chory, 2008); therefore, the altered ultrastructure and respiratory function in cls1 could also explain the overproduction of singlet oxygen in plastids. The expression of \textit{FER1}, a plastidic superoxide/H\textsubscript{2}O\textsubscript{2}-responsive gene (op den Camp et al., 2003; Kim et al., 2012), was not affected in the mutant, suggesting that only singlet oxygen, but not superoxide/H\textsubscript{2}O\textsubscript{2}, was produced in cls1 plastids (Figure 7). Mitochondria were reported to have an impact on proper chloroplast functioning in a \textit{N. sylvestris} cytoplasmic male sterility II mutant lacking Cl, which has low photosynthesis levels (Sabar et al., 2000; Dutilleul et al., 2003a) and impaired adjustment to higher growth irradiance (Priault et al., 2006).

Taken together, these results show that various putative sources of ROS overproduction are present in cls1 following CL deficiency, which would explain why cls1 seedlings are highly susceptible to high-illumination conditions. This is not the first time that the crucial role of CL has been described, not only in the functioning of mitochondria but also in its relationship with other cell compartments. In yeast, a new mitochondria–vacuole signaling pathway was identified in a \textit{crt1} mutant lacking CL (Chen et al., 2008).

Mitochondria play a key role in most types of PCD signaling identified so far. In animals, CLs have been shown to be important regulators of PCD mainly because they anchor cytochrome c, which is released from mitochondria into the cytosol during apoptosis (Garrido et al., 2006; Vacca et al., 2006). Peroxidation of CLs has been shown to be involved in the regulation of cytochrome c release (Kagan et al., 2009). However, the impact of the lack of CLs on the onset of PCD is still controversial, as it has been described as either enhancing (Choi et al., 2007; Kagan et al., 2009) or inhibiting (Huang et al., 2008; Schug and Gottlieb, 2009), depending on the system used. Mitochondria are considered to be key players in the execution of PCD in plants as well, and the release of cytochrome c during PCD has been described in several situations (Reape et al., 2008). The cytoplasmic male sterility II Cl mutant has been shown to display enhanced resistance to \textit{Tobacco mosaic virus} in an incompatible interaction (Dutilleul et al., 2003b). We took advantage of the cls1 mutant to test the impact of the lack of CLs on the onset of mitochondria-dependent PCD induced by UV-C light (Danon et al., 2004; Gao et al., 2008) and heat shock (Vacca et al., 2006, 2007). While untreated protoplasts from Col-0 and cls1 exhibited the same death frequency, for both PCD treatments, cls1 protoplasts were much more sensitive than the wild type (Figure 9).

This could suggest that, as described for animals, the lack of CL would facilitate the release of cytochrome c into the cytosol, exacerbating the onset of PCD (Choi et al., 2007; Kagan et al., 2009). It is also possible that ROS accumulation in the mutant, indicated by the upregulation of ROS-responsive marker genes, might have an impact on the onset of PCD in cls1 cells under stress conditions. Indeed, mitochondrial ROS appear to be involved in the onset of PCD in both animals and plants (Fleury et al., 2002; Rhoads et al., 2006), and more recently, the importance of chloroplastic ROS has also been demonstrated in \textit{Arabidopsis} PCD (Kim et al., 2012).

Taken together, our results show that CLs are essential for plant development, mitochondrial ultrastructure, and the assembly of Cl/CIII of the internal mitochondrial membrane. This diversity of CL roles explains the striking consequences for plant growth and morphology. The cls phenotype is much more accentuated in plants than in yeast, where development is slightly affected only under nonfermentable conditions. This might be attributable to the impaired functioning of mitochondria and altered metabolism leading to ROS production in mitochondria but also to photooxidative stress in plastids, which would explain why cls plants are only viable under a low-light regime. Even though the impact of CLs on the onset of PCD in \textit{Arabidopsis} is clear, an analysis of the peroxidation of CLs and the release of cytochrome c in cls would help us to understand further the mechanisms involved. The \textit{Arabidopsis} cls mutant, therefore, constitutes an important model for the study of the multiple roles of CL in mitochondrial biogenesis and function in obligate aerobes.
METHODS

Plant Material

Arabidopsis thaliana seeds of the SALK_49835, SALK_4984, and SALK_2263 lines carrying a T-DNA insertion in the Atmg04570 gene were obtained from the Nottingham Arabidopsis Stock Centre. Seeds were sterilized and sown under aseptic conditions on agar plates containing Gamborg B5 salt and vitamins (Duchefa) supplemented with 0.5% Suc. The plated seeds were kept at 4°C for 5 d and transferred to an illuminated, temperature-controlled growth chamber. Plants were grown at 20°C under an 8-h-day/16-h-night photoperiod (short days) of white light (Plants were grown at 20°C under an 8-h-day/16-h-night photoperiod (short days) of white light)

Lipid Extraction and Quantification

Lipids were extracted three times with 2 mL of chloroform:methanol (2:1, v/v) at room temperature for 1 h and then washed three times with 0.9% (w/v) NaCl. The upper phase was discarded, and the solvent was evaporated under at room temperature for 1 h and then washed three times with 0.9% (w/v) NaCl. The upper phase was discarded, and the solvent was evaporated under

RNA Isolation and RT-PCR Analysis

Total RNA was prepared using the Trizol reagent (Invitrogen) following the manufacturer’s instructions. RNA (1 µg) was treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed using random hexamers and the SuperScript III RNase H− reverse transcriptase (Invitrogen) following the manufacturer’s recommendations. One unit of the cDNA solution, 200 nM of each primer, and the LightCycler 480 SYBR Green I master mix (Roche Applied Science) were mixed in a final volume of 20 µL. Real-time PCR products were analyzed with the LightCycler 480 detection system. Melting curves were used to verify the specificity of amplification products. Relative mRNA abundance of the selected genes was calculated using the 2^(-Delta Delta C_T) method described by Mhamdi et al. (2010). Primers used for RT-PCR were designed by Mhamdi et al. (2010). The expression levels of AOX1α and NDB2 were determined using primers described by Ho et al. (2008), AOX1α, AOX2, CAT1, CAT2, and CAT3 mRNA levels were determined using primers described by Mhamdi et al. (2010). BAP1 and FER1 expression levels were determined using primers described by op den Camp et al. (2003). The expression levels of TIF4A were determined using TIF4A-5 (5′-AGTTCTGTGTATTCAACCTTC-3′) and TIF4A-3 (5′-CGACTCTTT- TCTCCCTCCTAC-3′) primers. Equivalent efficiencies between the different probes and our internal standard were observed during the PCRs. Primers used to detect the expression levels of CLS by RT-PCR were as follows: 

Preparation of Crude Leaf Membrane Extracts, Blue Native Electrophoresis, and In-Gel Activities

Preparation of crude leaf membrane extracts and blue native electrophoresis were performed as described previously (Pineau et al., 2008). After blue native electrophoresis, the NADH dehydrogenase activity of CI was revealed by incubation of the gel in the presence of 1 mM NBT and 0.2 mM NADH in 0.05 M 3-(N-Morpholino)propanesulfonic acid, 4-Morpholinepropanesulfonic acid, pH 7.6, after washing for 20 min in 0.1 M 3-(N-Morpholino)propanesulfonic acid, 4-Morpholinepropanesulfonic acid, pH 7.6. The cytochrome oxidase activity was revealed after washing the gel in 50 mM sodium phosphate, pH 7.4, by incubation in the same buffer but containing 0.22 M Suc, 0.5 mM bovine heart cytochrome c, 1.4 mM 3′,5′-diaminobenzidine, and 24 units mL⁻¹ catalase (Sigma-Aldrich).

Protein Gel Blot Analyses

Gels were electrophoresed onto nitrocellulose membranes for SDS-PAGE and polyvinylidene difluoride for blue native electrophoresis, and immunodetection studies (Pineau et al., 2008) were performed using wheat (Triticum aestivum) anti-NADH dehydrogenase subunit9 antibody at a 1:1000 dilution (a gift from J.M. Grienenberger, Institut de Biologie Physico-Chimique). Signals were visualized by chemiluminescence according to the manufacturer’s instructions (Roche Diagnostics).

Confocal Microscopy of Col-0 and cis1 Leaf Cells and Protoplasts with Mitochondrial Dyes

For imaging tissue, leaves were torn with tweezers in 5 µM dye solutions containing 0.1% (w/v) Tween 20, first using potential-sensitive mitochondrial markers (Fricker et al., 2001): DIOC6(3) (Kodak) and MitoTracker Red (Invitrogen). These were imaged with a confocal microscope (SP2 Leica) using a 40×, numerical aperture 1.25 oil objective with a 488-nm laser (8% power), measuring the emission at 505 to 540 nm, or for MitoTracker Red, excitation at 543 nm and emission at 570 to 630 nm. Chlorophyll was recorded simultaneously at 670 to 720 nm. Transmission images were taken in differential interference contrast mode. Likewise, leaves were torn in a 5 µM NAO solution containing 0.1% (w/v) Tween 20 prepared freshly from 2 mM NAO (ethanol stock of 10-nonyl acridine orange bromide [Invitrogen Molecular Probes]) and imaged with 488-nm excitation (as above). Protoplasts were imaged after 5 min of incubation in 100 nM solutions of these four dyes. The same material was also imaged on a wide-field microscope (DMIRE6000; Leica) using a 63×, numerical aperture 1.40 oil objective and the filter blocks fluorescein isothiocyanate or XF93 Red. Deconvolution was applied to the best Z-stacks.

Protoplast Preparation and Determination of Cell Death

Col-0 protoplasts were isolated from leaves of seedlings that were incubated for 15 h in the dark in the presence of the digestion medium: Gamborg B5 medium salt and vitamins (Duchefa), 0.5 M mannitol, 1.2% cellulose (Duchefa), and 0.8% macerozyme (Duchefa), pH 5.8. Protoplasts were then separated from cellular debris by filtration through a 100-µm mesh filter (Milan), followed by filtration on a 0.4 M Suc solution at 60g for 10 min. They were then collected and centrifuged at low speed (60g) for 5 min and washed. Finally, the protoplasts were resuspended in culture medium containing Murashige and Skoog medium (supplemented with 0.4 M Suc and 0.4 M mannitol). The frequency of dead cells was determined by light microscopy of protoplasts stained with Evans Blue (Sigma-Aldrich) at a final concentration of 0.04% (w/v). Protoplasts were exposed to 55°C for 10 min or to UV-C irradiation (254 nm) at a dose of 10 kJ m⁻² using UV Stratalinker 1800 (Stratagene).

Measurement of Intracellular NAO Fluorescence of Protoplasts by Flow Cytometry

Mesophyll protoplasts from Arabidopsis were suspended in B5-mannitol buffer on a 24-well plate (3 × 10⁷ mL⁻¹). For each point of kinetic
measurements, NAO was diluted at 10 μM in B5-mannitol buffer from 1 M stock solution in DMSO. Fifty microliters of protoplasts was taken from the plate and gently added to a tube with 250 μL of B5-mannitol. Then, 3 μL of 10 μM NAO (final concentration of 100 nM) was added, and the samples were incubated 5 min in darkness at room temperature. After the addition of 1 μg mL⁻¹ propidium iodide (PI) to check cell integrity, samples were analyzed by flow cytometry. Every kinetic point was prepared in duplicate to confirm measurements.

Acquisitions were made on an ELITE cytometer (Beckman-Coulter) using a 488-nm argon laser at 15 mW. Fluorescence emission of NAO was collected through a 525-nm band-pass filter, and fluorescence emission of PI was collected through a 610-nm band-pass filter. We checked for the presence of chlorophyll by autofluorescence through a 685-nm band-pass filter. Dead or leaky protoplasts were excluded from the analyzed population by gating out PI-positive objects.

Electron Microscopy
Small leaf fragments were processed as described by Hawes and Satiat-Jeunemaitre (2001), except that the osmium post fixation step was replaced by adding a mixture of 1% osmium and 1.5% potassium ferricyanide to increase the contrast of mitochondrial membranes (Aubert et al., 2011). Specimens were embedded in epoxy resin (for 80 nm) obtained with an ultramicrotome (EM UC6; Leica Microsystems) and polymerized for 16 h at 60°C. Ultrathin sections (80 nm) were collected on formvar carbon-coated copper grids, poststained with 2% uranyl acetate and lead citrate, and observed with a JEM-1400 transmission electron microscope (JEOL) operating at 120 kV. Images were acquired using a postcolumn high-resolution (11 megapixels) high-speed camera (AC1000 Orius; Gatan) and processed with Digital Micrograph (Gatan).

Metabolomic Analyses
GC-TOF-MS profiling was performed as described in detail (Noctor et al., 2007).

Statistical Methods
Significant differences at P < 0.05 were calculated using Student’s t test.

Accession Numbers
Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: CLS, At4g04870; AOX1a, At3g22370; NDB2, At4g05020; BAP1, At3g61190; FER1, At5g01600; APX1, At1g07890; APX2, At3g09640; CAT1, At1g02630; CAT2, At4g35090; CAT3, At1g20620.

Supplemental Data
The following material is available in the online version of this article.

Supplemental Figure 1. Phenotype of the cls2 Mutant.

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AUTHOR CONTRIBUTIONS
A.D. and R.D.P. supervised the study. S.C.B. and B.S.-J. were in charge of the cell biology strategy. A.D. ordered single mutant plants and performed their genetic characterization. B.P. performed blue native PAGE and immunodetection experiments. S.C.B., A.D., and M.B. performed cytometry, light microscopy, and fluorochrome experiments. J.M. performed the electron microscopy studies. P.M. and L.M.P. did lipid quantifications. Metabolomic quantifications were performed by C.M.

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**Supplemental Figure 1.** Phenotype of the cls2 mutant.

A. *In vitro* seedlings of the cls2 line (SALK4984) grown under LD at 80 μmol m⁻² s⁻¹. Bleaching homozygous cls2 plants are arrowed.

B. cls2 plant compared to Col0 plant at the adult stage in greenhouse.
The Importance of Cardiolipin Synthase for Mitochondrial Ultrastructure, Respiratory Function, Plant Development, and Stress Responses in Arabidopsis

Bernard Pineau, Mickaël Bourge, Jessica Marion, Caroline Mauve, Francoise Gilard, Lilly Maneta-Peyret, Patrick Moreau, Béatrice Satiat-Jeunemaître, Spencer C. Brown, Rosine De Paepe and Antoine Danon

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