Molecular species composition of plant cardiolipin determined by liquid chromatography mass spectrometry

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Abstract  Cardiolipin (CL), an anionic phospholipid of the inner mitochondrial membrane, provides essential functions for stabilizing respiratory complexes and is involved in mitochondrial morphogenesis and programmed cell death in animals. The role of CL and its metabolism in plants are less well understood. The measurement of CL in plants, including its molecular species composition, is hampered by the fact that CL is of extremely low abundance, and that plants contain large amounts of interfering compounds including galactolipids, neutral lipids, and pigments. We used solid phase extraction by anion exchange chromatography to purify CL from crude plant lipid extracts. LC/MS was used to determine the content and molecular species composition of CL. Thus, up to 23 different molecular species of CL were detected in different plant species, including Arabidopsis, mung bean, spinach, barley, and tobacco. Similar to animals, plant CL is dominated by highly unsaturated species, mostly containing linoleic and linolenic acid. During phosphate deprivation or exposure to highly unsaturated species, including Arabidopsis, accompanied with an increased degree in unsaturation, the amount of CL decreased in Arabidopsis, accompanied with an increased degree in unsaturation. The mechanism of CL remodeling during stress, and the function of highly unsaturated CL molecular species, remains to be defined.—Zhou, Y., H. Peisker, and P. Dörmann. Molecular species composition of plant cardiolipin determined by liquid chromatography mass spectrometry. J. Lipid Res. 2016. 57: 1308–1321.

Supplementary key words  Arabidopsis • anion exchange chromatography • solid phase extraction • lipidomics • mitochondria • phospholipids

Cardiolipin (CL) is an anionic membrane phospholipid with bipartite structure containing four acyl groups bound to two glycerol moieties, which are linked via two phosphate groups to a third glycerol. CL is distributed in the cytoplasmic membrane in prokaryotes and almost exclusively located in the inner mitochondrial membrane of eukaryotes. A growing body of evidence indicates that CL is required for different mitochondrial functions. CL is involved in maintaining the mitochondrial membrane potential, which is important for protein import (1, 2). CL is also required for the activity and structural integrity of complexes of the electron transport chain, including NADH-ubiquinone reductase (complex I), ubiquinone-cytochrome c reductase (complex III), and cytochrome c oxidase (complex IV) and the mitochondrial F$_{1}$F$_{0}$-ATP synthase (complex V) (3–5). CL binds to the ADP/ATP carrier protein influencing its catalytic and structural properties (6, 7). Moreover, CL plays an essential role in the regulation of apoptotic cell death (8–12). CL serves as an anchor for cytochrome c on the outer leaflet of the inner mitochondrial membrane. Stress signals induce the peroxidation of CL by cytochrome c, and peroxidized CL releases cytochrome c from inner membrane. CL also interacts with B-cell lymphoma 2 (Bcl-2) family proteins, thereby increasing the permeability of the outer mitochondrial membrane. The increased permeability facilitates cytochrome c release into the cytosol and stimulates the activation of caspase (8–12). Although not all of the regulatory proteins are conserved in animals and plants, a number of molecular characteristics including the caspase-like protease activity, Bcl-2-like family members, and mitochondrial proteins are highly related (13).

In plants, CL is crucial for maintaining mitochondrial ultrastructure and for stabilizing the respiratory complex I/complex III supercomplexes, as well as the major mitochondrial fission factor DRP3 complex (14, 15). CL depletion in the Arabidopsis cls mutant leads to defects in mitochondrial morphogenesis and stress responses, resulting in mitochondrial ultrastructure and mitochondrial function.

Abbreviations:  CDP-DAG, cytidine diphosphate diacylglycerol; CL, cardiolipin; DGDG, digalactosyldiacylglycerol; DW, dry weight; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; Q-TOF, quadrupole time-of-flight; SPE, solid phase extraction; SQDG, sulfoquinovosyldiacylglycerol.

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in enhanced sensitivity to programmed cell death effectors including UV light, heat shock, and darkness (14–16).

The de novo biosynthesis pathway of CL in plants is similar to other eukaryotes but is different from most prokaryotes. In Escherichia coli, CL synthase transfers a phosphatidyl group from phosphatidylglycerol (PG) or phosphatidyl ethanolamine (PE) to another PG to form CL (17, 18). In Actinobacteria (19), yeast (20), mammals (21), and plants (16), CL is synthesized from cytidine diphosphate diacylglycerol (CDP-DAG) and PG, using CDP-DAG as a phosphatidyl donor. Microscopic analysis of Arabidopsis leaf cells stained with the CL-specific fluorescent dye 10-N-nonyl-acridine orange showed that CL is enriched in the so-called CL-enriched membrane domains. Arabidopsis CL synthase is targeted to the inner mitochondrial membrane with its C terminus facing the intermembrane space (14). In vitro assays showed that plant CL synthases prefer both CDP-DAG and PG esterified to oleic acid (18:1) over CDP-DAG and PG esterified to palmitic acid (16:0) (22, 23). It is possible that the enzymatic specificity of plant CL synthase leads to the enrichment of specific CL species, which could play an essential role in mitochondria. However, this scenario remains elusive because the molecular species composition of CL in plants is unknown.

Previously, CL from plants was determined by chromatographic methods, including TLC, GC, or HPLC. TLC separation, in some cases combined with radiochemical labeling, leads to determination of the relative abundance of CL in comparison with other lipids (15, 22). Combination of TLC and GC or GC/MS provides the means for quantification of individual fatty acids in CL (24). HPLC was used to separate and quantify major lipid classes in plants, and trace amount of CL can be detected in some plant tissues (25). MS/MS with LC separation or direct infusion was developed for profiling plant membrane lipids, providing detailed information on most lipids except CL (26, 27). LC/MS was used to analyze CL in Arabidopsis, but only the relative abundance for a single selected molecular species was obtained (14).

In contrast to plants, MS has successfully been used to determine the CL molecular species in bacteria and animals. Direct infusion MS (“shotgun lipidomics”) resulted in the quantification of CL molecular species from mouse (myocardium, liver, skeletal muscle) (28). Multidimensional MS-based shotgun lipidomics was also used to analyze membrane lipids including CL in purified mitochondria (29). Normal-phase LC/ESI/MS led to the quantification of CL molecular species in lipid extracts of E. coli (30). More LC/MS methods have been developed for CL analysis from animal or human samples (31–35).

In the present work, we present a new strategy for the quantification of a full set of molecular CL species from plants. This method is based on the enrichment of acidic glycerolipids including CL via anion exchange chromatography and its quantification by LC/MS. Using this method, it was for the first time possible to identify and quantify ~23 molecular species of CL in different plant tissues and to observe changes in absolute amounts and composition of CL during different environmental stress conditions.
Quantification of CL by LC/MS

The HPLC system was composed of an Agilent 1200 Series quaternary pump with an autosampler and a degasser (1200 Series, Agilent Technologies). Solvents for LC/MS analysis, including chloroform and methanol, were HPLC grade. Tetrahydrofuran (LiChrosolv, Merck Millipore) for LC/MS was gradient grade. The lipid extract purified by DEAE chromatography (30 µl) was injected onto a Nucleosil C18 Gravity analytical column (50 mm × 4.6 mm, 1.8 µm particle size; Macherey-Nagel). The binary gradient consisted of solvent A (tetrahydrofuran-methanol-5 mM ammonium acetate, 3:2:5, v/v/v) and solvent B (tetrahydrofuran-methanol-0.5 mM ammonium acetate, 7:2:1, v/v/v) (40). The solvent gradient was formulated as follows: 40% solvent B for 0.5 min, 40% to 75% in 9.5 min, hold at 75% for 5 min, 75% to 100% in 15 min, hold at 100% for 5 min, 100% to 40% in 2 min, hold at 40% for 10 min. The chromatography was performed at room temperature and at a flow rate of 200 µl/min.

A 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS instrument (Agilent) was used in the negative electrospray ionization mode. The instrument parameters were set as described (41). The energy for collision-induced dissociation was 60 V. Mass spectra were recorded at a rate of 5 spectra s⁻¹. The extracted ion chromatograms (“MS only mode” of unfragmented ions) were used for quantification after integration using the Agilent MassHunter Qualitative Analysis software. The data were further processed in Microsoft Office Excel 2013. Values are given in nmol mg⁻¹ DW. Because the CL molecular species were separated by chromatography, a correction for isotopic overlap was not required.

Validation studies

To study within-day precision, lipids were extracted from spinach leaves with three different fresh weights of (250, 500, and 750 mg; 3–5 replications each), and CL molecular species were quantified by LC/MS. To determine between-day precision, the lipid extraction and quantification was repeated in the same way 7 days later.

An add-back study was performed by adding different amounts of (18:1)₄ CL (1.1 to 5.5 nmol) to 500 mg of spinach leaf tissue at the beginning of the lipid extraction. The amounts of (18:1)₄ CL were determined by LC/MS and compared with the amounts of (18:1)₄ CL quantified by GC.

The recovery was determined by adding 5.00 nmol of (14:0)₄ CL to 500 mg of spinach leaf tissue. After lipid extraction and DEAE column chromatography, the peak area of (14:0)₄ CL was determined by LC/MS. The peak area of (14:0)₄ CL was also directly measured by LC/MS without lipid extraction and DEAE column chromatography. Because a large loss of (14:0)₄ CL in the strongly diluted solution was observed in the latter experiment, (14:0)₄ CL was mixed with spinach leaf lipids (as a “carrier”) immediately before LC/MS measurement. The recovery was determined as the ratio of the peak areas of (14:0)₄ CL extracted with spinach leaf lipids versus the peak area of (14:0)₄ CL directly measured by LC/MS.

RESULTS

CL from plants can be purified by anion exchange chromatography

Many different glycerolipids can be analyzed by MS of total lipids extracted from plants with the exception of CL (42). Attempts to analyze CL from total leaf lipids by LC/MS or LC/MS/MS using a variety of solvents and C18 or a C8 reverse phase columns were not successful. The signal intensity for CL was too low, presumably due to the low abundance and interference with other lipids, and therefore this method was not suitable for quantitative analysis of CL. Solid phase extraction (SPE) was used to purify CL prior to LC/MS analysis. SPE with anion exchange columns was previously used to separate neutral and basic phospholipids (e.g., PE, PC) from anionic lipids including CL from rats (43). After testing several SPE columns and eluents, including normal and reverse phases and anion exchange columns, SPE on a DEAE cellulose column was selected because this method resulted in a satisfactory separation with the enrichment of CL in the acidic lipid fraction, accompanied with the removal of neutral and basic lipids (Fig. 1). Figure 2A shows the separation of total lipids from Arabidopsis callus and leaves by DEAE column chromatography. The neutral (MGDG and DGDG) and basic lipids (PE and PC), as well as some unidentified lipids, were removed in the neutral/basic fraction F1. CL together with PI, PG, SQDG, and other unidentified anionic lipids were eluted in acidic fraction F2.

Different amounts of CL standards [i.e., (14:0)₄ CL, (18:0)₄ CL, and (18:1)₄ CL] were mixed and chromatographed on the DEAE cellulose column as described above. Then the relative amounts of CL standards were measured by quantification of the acyl groups by GC. The presence of different acyl chain lengths and degrees of unsaturation in CL did not affect the isolation via the DEAE column (Fig. 2B). In a second experiment, CL was purified from total spinach leaf lipids by DEAE chromatography. After additional purification by TLC, a single band for CL was obtained and the acyl composition was quantified by GC (Fig. 2C). Approximately 90% of the acyl groups in spinach CL have a chain length of 18:X. The most abundant fatty acids are α-linolenic (18:3) and linoleic acid (18:2), amounting to 40% and 37%, respectively. About 10% of the fatty acids are 16:X, mostly palmitic acid (16:0). The acyl chain composition of

![Figure 1](Image 372x133 to 491x310) Flow chart for CL analysis from plants. Total lipids are extracted from ~500 mg plant material with chloroform-methanol. Lipids are fractionated by anion exchange chromatography on a DEAE column. Neutral and basic lipids are eluted with chloroform-methanol-water (3:7:1, v/v/v), and acidic lipids are eluted with chloroform-methanol-0.8 M ammonium acetate (3:7:1, v/v/v).
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Identification of CL molecular species by LC/MS

Previous studies indicated that due to the presence of two phosphate groups, CL molecular species can be observed in the negative mode by MS as CL anions carrying one or two negative charges (molecular ions [M-H]⁻ or [M-2H]²⁻). Indeed, MS direct infusion experiments with CL standards confirmed that CL signals are higher in the negative mode as compared with the positive mode. Because CL contents in plant tissues are considered to be extremely low, we selected LC/MS in the negative mode as a means to quantify the different CL molecular species. The LC/MS chromatogram of (18:1)₄ CL shows a peak at 19.4840 min for the extracted ion of m/z 1456.0281 (Fig. 3A) corresponding to the singly negatively charged molecular ion [M-H]⁻. The MS spectrum at 19.4840 min reveals the presence of a second ion at m/z 727.5095, which corresponds to the double negative ion [M-2H]²⁻ (Fig. 3B). The ratio of the [M-H]⁻ and [M-2H]²⁻ signals was similar for the different standards (14:0)₄ CL, (18:0)₄ CL, and (18:1)₄ CL, and the singly negative signal was always higher.

The MS/MS spectrum of the [M-H]⁻ anion of (18:1)₄ CL is depicted in Fig. 3C. Fragmentation of (18:1)₄ CL results in a number of anions, including m/z 152.9945 for glycerolphosphate, m/z 281.2483 for the 18:1 free fatty acid anion, m/z 417.2387 for monoacylglycerolphosphate, and m/z 699.4941 for diacylglycerolphosphate. The fragmentation pattern of the parental ion of (18:1)₄ CL can be explained by dissociation of the ester bond of the fatty acids (1) or of the phosphate ester linkages to glycerol (2–4) (Fig. 3D). Based on the presence of the ions for free fatty acid anions and of mono- and diacylglycerolphosphates, the acyl composition of CL molecular species can be exactly determined via MS/MS experiments (Tables 1, 2).

Separation and quantification of CL molecular species

Figure 4A shows the extracted ion chromatograms for the [M-H]⁻ ions of a mixture of the three CL standards containing 150 pmol each of (14:0)₄ CL, (18:0)₄ CL, and (18:1)₄ CL. All three peaks were eluted from the reverse phase column in the range of 75% to 100% solvent B (between 14 and 26 min), in the order of (14:0)₄ CL, (18:1)₄ CL, and (18:0)₄ CL. The exact amounts of the three CL standards in the mixture were determined by GC of fatty acid methyl esters. The ratios of the LC/MS [M-H]⁻ peak areas to the amounts of CL quantified by GC were calculated (Fig. 4A). These ratios were very similar for (14:0)₄ CL, (18:0)₄ CL, and (18:1)₄ CL, indicating that the chain length and degree of unsaturation had no large impact on the amounts of CL detected as [M-H]⁻. Therefore, no correction factor for the different CL molecular species was required, and (14:0)₄ CL was selected as an internal standard because its retention time was expected not to overlap with authentic plant CL molecular species.

Next, CL from bovine heart was analyzed by LC/MS to address the questions whether isotopologic and isomeric forms of CL can be separated. CL in spinach was similar to that of purified spinach mitochondria (24). Based on the acyl chain composition, we predicted that 72:X [i.e., (18:X)₄ CL] represents the most abundant CL molecular species in spinach with a high degree of unsaturation.
overlap with molecular species differing by the presence of one double bond in one of the acyl chains. Figure 4B shows the extracted ion chromatograms of bovine heart CL for $m/z$ 1,447.9650 (black), 1,449.9806 (light gray), and 1,451.9963 (dark gray). The different peaks eluting at 16.7615, 17.8781, 18.8049, and 19.5842 min were further analyzed by MS/MS experiments to determine their acyl composition. The most abundant molecular species of bovine heart CL is 72:8 with four 18:2 fatty acids, followed by 72:7 and 72:6 molecular species. MS/MS analysis of the peak eluting at 16.7615 min revealed that it contains 72:8 CL with four 18:2 fatty acids [i.e., (18:2)$_4$ CL]. In addition to the $m/z$ of 1,447.9650 corresponding to [M-H]$^-$ containing $^{12}$C atoms ($^{12}$C-72:8 CL), two isotopologs eluting at the same time with two (1,449.9806) or four (1,451.9963) $^{13}$C carbons can be identified. The peak areas of the different isotopologs of 72:8 CL eluting at 16.7615 min correspond to the calculated isotope distribution of (18:2)$_4$ CL. The isobaric peaks of $m/z$ 1,449.9860 at 16.7615 min (containing $^{13}$C$_2$-72:8 CL) and at 17.8781 min ($^{12}$C-72:7 CL) are clearly separated. Similarly, the peaks of $m/z$ 1,451.9963 at 16.7615 min ($^{13}$C$_4$-72:8 CL), at 17.8781 min ($^{13}$C$_2$-72:7 CL) and at 18.8049 min ($^{12}$C-72:6 CL) are separated by LC/MS. Therefore, isotopologs with two $^{13}$C carbon atoms are separated from CL molecular species differing by one double bond, and thus a correction for isotopic overlap was not required.

Different isomeric molecular species of CL can have the same $m/z$, for example 72:6 CL with the two isomers (18:2)$_2$(18:1)$_2$ CL and (18:2)$_3$(18:0) CL. Indeed, two isomers of 72:6 CL were identified by LC/MS of bovine heart CL, (18:2)$_2$(18:1)$_2$ CL eluting at 18.8049 min and (18:2)$_3$(18:0) CL at 19.5842 min. Therefore, isomeric CL molecular species carrying acyl groups with different degrees of unsaturation can be separated by LC/MS but oftentimes elute at very similar retention times.

To determine the limits of detection for CL, different amounts of (18:1)$_4$ CL were quantified using (14:0)$_4$ CL as internal standard. Figure 4C shows the double logarithmic plot of the amount of (18:1)$_4$ CL measured by LC/MS versus the amount of (18:1)$_4$ CL quantified by GC of acyl groups after methylation. The quantification of (18:1)$_4$ CL by LC/MS was linear in the range of $\sim$5 to $\sim$1,000 pmol. Taken together, the method of choice for the determination of CL from plants encompasses the purification of CL from crude lipid extracts by DEAE column chromatography and subsequent quantification by LC/MS of the extracted ion chromatogram for [M-H]$^-$ using (14:0)$_4$ CL as internal standard. For exact identification, the acyl compositions of all molecular CL species detected by LC/MS need to be verified by MS/MS spectra (Table 2).

Validation of CL quantification from spinach leaves

Anionic lipids including CL were isolated from spinach leaves using DEAE column chromatography and separated by LC/MS analysis. As the DEAE column fraction F2 contains PG, PI, and SQDG, in addition to CL (Fig. 2A), it was of interest to compare the retention times of the different lipids on the LC/MS column. Figure 5A shows that the...
The table below shows the CL molecular species:

| Acyl Composition | Molecular Species | Formula (M) | [MH]⁻ (Calculated Mass) | Retention Time (min) | Occurrence |
|------------------|-------------------|-------------|------------------------|----------------------|------------|
| 1 14:0 14:0 14:0 14:0 | C₉₀H₁₄₀O₂₁P₂ | 1,239.8397 | 15.3483 | St |
| 2 18:3 18:3 18:3 18:3 | C₉₀H₁₄₀O₂₁P₂ | 1,439.9023 | 14.5967 | AC, AL, AR, BL, MH, SL, TL |
| 3 18:3 18:3 18:3 18:2 | C₉₀H₁₄₀O₂₁P₂ | 1,441.9178 | 15.1124 | AC, AL, AR, BL, MH, SL, TL |
| 4 18:3 18:3 18:2 18:2 | C₉₀H₁₄₀O₂₁P₂ | 1,443.9337 | 15.7630 | AC, AL, AR, BL, MH, SL, TL |
| 5 18:3 18:2 18:2 18:2 | C₈₉H₁₄₀O₂₁P₂ | 1,445.9493 | 16.0538 | AC, AL, AR, BL, MH, SL, TL |
| 6 18:3 18:3 18:2 18:1 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 7 18:3 18:2 18:1 18:1 | C₉₀H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 8 18:3 18:2 18:2 18:2 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 9 18:3 18:2 18:3 18:1 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 10 18:3 18:3 18:3 18:2 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 11 18:3 18:3 18:3 18:2 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 12 18:3 18:2 18:3 18:1 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 13 18:3 18:2 18:3 18:1 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 14 18:3 18:2 18:3 18:1 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 15 18:3 18:2 18:3 18:1 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 16 18:3 18:2 18:3 18:1 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 17 18:3 18:2 18:3 18:1 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |
| 18 18:3 18:2 18:3 18:1 | C₈₉H₁₄₀O₂₁P₂ | 1,447.9650 | 16.9618 | AC, AL, AR, BL, MH, SL, TL |

All CL molecular species were characterized by LC/MS/MS analysis. Occurrence of the CL molecular species: AC, Arabidopsis callus; AL, Arabidopsis leaf; AR, Arabidopsis root; BH, bovine heart (commercial CL); BL, barley leaf; MH, mung bean hypocotyl; SL, spinach leaf; St, standards (14:0)₄ CL, (18:1)₄ CL, (18:0)₄ CL; TL, tobacco leaf.

main molecular species of the lipids (34:4 PG, 34:3 PI, 34:3 SQDG) are eluted at 6 to 8 min, well before 72:12 CL (14 min). The other molecular species of PG, PI, and SQDG elute at ~5 to 10 min (not shown). Therefore, LC/MS resulted in the separation of CL from other anionic lipids, thereby contributing to the enhanced sensitivity of CL quantification.

Similar to the separation of CL molecular species from bovine heart (Fig. 4B), the molecular species from spinach leaves were separated by LC/MS (Fig. 5B; Table 1). In addition, the molecular species from spinach were also separated from isotopologs (see Fig. 4B). For most molecular species, only one acyl composition could be attributed (depicted by black traces/MS only in Fig. 5B). For some molecular species, different acyl compositions were detected. Therefore, the MS/MS traces for three different fragments (acyl anions of 18:1, 18:2, or 18:3) are shown for 72:9 CL, 72:8 CL, 72:7 CL, 72:6 CL, and 70:9 CL. For clarity, in the following these molecular species were quantified based on their MS-only chromatograms, disregarding the presence of molecules with different acyl compositions.

To test the within-day and between-day precision, the main molecular species of CL from spinach leaf were quantified on day 1, and again 7 days later, by LC/MS. Three different amounts of fresh weight (250, 500, or 750 mg) were used for CL extraction. Extraction from all three amounts of fresh weight gave similar CL contents, but 500 mg fresh weight provided the most reproducible results (Fig. 6A). The within-day precision can be deduced from the relative SD (ratio of SD to the mean; n = 5; in %) of the CL contents measured on day 1 (Fig. 6A). The relative SDs (for 500 mg fresh weight, day 1) for the two most abundant molecular species were 14.3% for 72:11 CL and 13.8% for 72:10 CL. For the between-day precision, we calculated the ratio of the difference between the means measured at day 1 and day 8, to the mean of day 1. These values were 8.7% for 72:11 CL and 9.1% for 72:10 CL. Therefore, measurements of CL molecular species via LC/MS are characterized both by high within-day and between-day precision.

Next, an add-back study was performed by adding different amounts of (18:1)₄ CL to 500 mg of spinach leaves. The amounts of (18:1)₄ CL were quantified by LC/MS and plotted versus the amounts determined by GC of fatty acid methyl esters. Figure 6B shows that the (18:1)₄ CL amounts measured by LC/MS in a spinach extract are very similar to those determined by GC. Linear regression indicates that the values are highly correlated (R² = 0.967) and that the regression line is close to the diagonal.

The recovery was determined as the ratio of the peak area of (14:0)₄ CL added to 500 mg of spinach leaf tissue prior to lipid extraction, versus the peak area of (14:0)₄ CL directly measured by LC/MS. The recovery for (14:0)₄ CL was 83.7 ± 5.4% (n = 3).
Characterization of CL molecular species from plant tissues

Quantification of the most abundant molecular species of CL from spinach leaf is depicted in Fig. 7A. Spinach CL is dominated by 72:X molecular species with four 18:X acyl chains, amounting to 96 mol% of total CL. A minor amount of 70:X with three 18:X and one 16:0 was also present, as well as 68:X molecular species with two 18:X and two 16:0 acyl groups. Among the 72:X, species, 72:10 and 72:9 are the most abundant molecules, followed by other highly unsaturated molecular species. Calculation of the acyl composition of spinach CL based on its molecular species composition (Fig. 7A) revealed that it contains 44% 18:3, 42% 18:2, 9% 18:1, and 4% 16:0. This is in agreement with the acyl composition of spinach CL as determined by GC (Fig. 2C).

Lipids were extracted from different plants including tobacco leaves, mung bean hypocotyls, and barley leaves, in addition to spinach leaves (Fig. 7A–D). The total CL content or composition is also changed. We tested the impact of phosphate deprivation on CL in leaves or roots by growing Arabidopsis plants in hydroponic culture in the presence or absence of phosphate (Fig. 8A, B). The total CL content in leaves decreases by 17%, from 0.208 ± 0.032 to 0.174 ± 0.014 nmol mg⁻¹ DW after phosphate deprivation, while in roots it decreases by 67%, from 0.399 ± 0.005 to 0.132 ± 0.006 nmol mg⁻¹ DW (mean ± SD, n = 3). The degree of unsaturation of CL in leaves is higher than in roots, regardless of phosphate availability, because CL in leaves (+P) contains an average of 9.91 ± 0.02 double bonds per CL molecule, while CL in roots (+P) it contains 9.46 ± 0.06 (mean ± SD, n = 3). The average numbers of double bonds per CL molecule are increased in both leaves and roots during growth on low phosphate medium. The 72:12 CL shows the strongest change in leaves where it increases 2-fold during phosphate deprivation. Apart from 72:12 and 72:11, other 72:X molecules are decreased in leaves (−P). The increase in the degree of unsaturation of CL in roots is reflected by the finding that highly unsaturated molecular species 72:12 and 72:11 show a moderate decrease, while the more saturated molecules 72:10 and 72:9 are more strongly reduced.

Changes in CL composition during phosphate deprivation

Under phosphate deprivation, the amounts of the non-phosphorous galactolipid DGDG and of the sulfolipid SQDG increase, while the contents of phospholipids decrease, to save phosphate for other essential cellular processes including DNA and RNA synthesis (44). The extra pool of DGDG that increases during phosphate deprivation was located to the chloroplast, plasma membrane, and tonoplast, and it was even detected in the mitochondrion (44–46). The contents of PC and PG strongly decrease under phosphate deprivation, but it was unclear whether the CL content or composition is also changed. We tested the impact of phosphate deprivation on CL in leaves or roots by growing Arabidopsis plants in hydroponic culture in the presence or absence of phosphate (Fig. 8A, B). The total CL content in leaves decreases by 17%, from 0.208 ± 0.032 to 0.174 ± 0.014 nmol mg⁻¹ DW after phosphate deprivation, while in roots it decreases by 67%, from 0.399 ± 0.005 to 0.132 ± 0.006 nmol mg⁻¹ DW (mean ± SD, n = 3). The degree of unsaturation of CL in leaves is higher than in roots, regardless of phosphate availability, because CL in leaves (+P) contains an average of 9.91 ± 0.02 double bonds per CL molecule, while CL in roots (+P) it contains 9.46 ± 0.06 (mean ± SD, n = 3). The average numbers of double bonds per CL molecule are increased in both leaves and roots during growth on low phosphate medium. The 72:12 CL shows the strongest change in leaves where it increases 2-fold during phosphate deprivation. Apart from 72:12 and 72:11, other 72:X molecules are decreased in leaves (−P). The increase in the degree of unsaturation of CL in roots is reflected by the finding that highly unsaturated molecular species 72:12 and 72:11 show a moderate decrease, while the more saturated molecules 72:10 and 72:9 are more strongly reduced.

The phosphate-deficient Arabidopsis pho1-2 mutant represents an alternative, genetic model to study phosphate
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in pho1-2 is also reflected by the higher average number of double bonds per CL molecule and is consistent with the results obtained with phosphate-deprived WT leaves (Fig. 8A).

CL response to heat shock and darkness

In animals, CL is known to play an important role during programmed cell death (11), but its function in plant cell death is less well understood. Cell death in plants can be induced by applying a short heat stress or by growing the plants in complete darkness (14). Exposure to a short heat shock, as well as the growth in darkness, resulted in senescence and cell death in the CL-deficient cls mutant of Arabidopsis, while the WT was not affected (14). The responses of CL content and composition to heat shock and growth under dark conditions were studied to unravel the effect of programmed cell death on CL.

First, Arabidopsis plants were exposed to 65°C for 10 min and subsequently grown at room temperature under short day conditions for 1, 2, or 3 days. The CL content and pattern was compared with control plants not exposed to heat stress. No significant changes in CL content or composition were observed in the different plants (Fig. 9A). The number of double bonds per CL molecule was 10.12 ± 0.01 in control plants and did not change after the heat shock (mean ± SD, n = 3). Therefore, the heat shock of 10 min did not affect CL content or composition.

Next, CL was measured in Arabidopsis plants exposed to darkness for 2, 4, or 6 days, to observe the changes in CL during dark-induced cell death. The plants were grown on soil under short day conditions. The total CL content decreased from 0.252 ± 0.011 at 0 days to 0.200 ± 0.008 at 4 days and 0.177 ± 0.001 nmol mg⁻¹ DW (mean ± SD, n = 3) at 6 days of growth under dark conditions. The degree of unsaturation of CL increased continuously, from 9.74 ± 0.03 double bonds per molecule at day 0 to 10.18 ± 0.03 nmol mg⁻¹ DW (mean ± SD, n = 3) after 6 days. This result is in line with the finding that the highly unsaturated molecular species 72:12 and 72:11 increased, while other more saturated molecular species decreased (72:10, 72:9, 72:8) (Fig. 9B).

DISCUSSION

CL, a crucial phospholipid in bacterial cells and in mitochondria of eukaryotes, has previously been analyzed in plants by chromatographic methods, including TLC, GC, and HPLC (15, 22, 24, 25). These techniques provided data on the content of plant CL but failed to give the information of molecular species composition. In contrast to plants, the total amount and molecular species composition of CL from animal tissues has previously been studied by MS in great detail. In most studies, crude lipid extracts were obtained from tissue samples, cell cultures, or isolated mitochondria using methanol/chloroform/aqueous buffer system (48, 49). Subsequently, CL was measured by separation of lipid extracts via LC/MS using different tandem mass spectrometers (30, 31, 34, 35, 50) (Table 3).

The determination of CL molecular species from animal limitation, because the leaves of pho1-2 are permanently phosphate deprived due to the block in the root phosphate transporter PHO1 (47). As a consequence, the contents of phospholipids are decreased in pho1-2 leaves while the amounts of DGDG and SQDG are increased (44). CL was measured by LC/MS in leaves of Arabidopsis WT and pho1-2 (Fig. 8C). The total content of CL in pho1-2 (0.237 ± 0.020 nmol mg⁻¹ DW) was slightly reduced as compared with WT (0.252 ± 0.001 nmol mg⁻¹ DW; mean ± SD, n = 3). Differences in the degree of CL desaturation were detected because CL in pho1-2 is more highly unsaturated, as the contents of 72:12 and 72:11 are increased, while the amounts of 72:10, 72:9, and 72:8 are decreased (Fig. 8C). This increase in highly unsaturated molecular CL species...
cells is straightforward, as the relative proportion of mitochondrial membranes in animal cells is considerably higher than in plant cells, which contain the chloroplast as an additional organelle. Plastids in plant cells harbor large amounts of membrane lipids and pigments, which interfere with LC/MS quantification of minor lipid classes like CL. Therefore, detailed information on the molecular species composition of plant CL was lacking. Here, we report an LC/MS approach for the determination of CL from plants. Anion exchange chromatography on a DEAE column was essential for measuring CL by LC/MS. After removing the major basic and neutral lipids (MGDG, DGDG, PE, PC), separation and identification of a large number of molecular CL species, including minor molecular species, became feasible (Fig. 1). Only acidic lipids remained present in the purified CL fraction, and they were separated by the subsequent LC/MS step (Fig. 5A). DEAE column separation did not lead to the differential enrichment of CL molecular species carrying acyl groups with different chain lengths or degrees of unsaturation (Fig. 2B, C). Previously, CL was enriched from animal lipids before quantification by MS using silica/normal phase chromatography (32, 33, 51) (Table 3).

CL molecular species from animal tissues can be measured using a MALDI ion source or by direct infusion into the mass spectrometer with an electrospray ion source (ESI) (28, 32). An increase in sensitivity can be obtained after separation of CL molecular species by LC (e.g., using a normal phase column) (30, 34, 35). We used reversed phase LC for separation as this method is highly robust and reproducible (Figs. 4, 5). The elution of CL molecular species from the reverse phase column depended on the acyl chain length and degree of unsaturation as previously described (31, 33, 50, 51). CL molecular species with shorter acyl chains or with a higher degree of unsaturation were eluted earlier (Figs. 4, 5).

CL can lose one or two protons, producing singly and double negatively charged ions during ionization in the negative mode. During LC/MS analysis, singly charged parental ions \([\text{M-H}]^-\) were the main products and were therefore selected for MS/MS fragmentation and quantification (Fig. 3). The higher abundance of \([\text{M-H}]^-\) versus \([\text{M-2H}]^{2-}\) ions might depend on the solvent system and the characteristics of the ionization source and is in agreement with other studies (33, 34, 50, 51) (Table 3). On the other hand, double negative anions of CL, \([\text{M-2H}]^{2-}\), were used for quantification in alternative approaches (28, 30, 31, 35). CL can also be recorded in the positive mode (\([\text{M}+\text{Na}]^+\) or \([\text{M}+\text{NH}_4]^+\)). CL molecules show a wide range of isotope distribution with isotopologs containing only $^{12}$C atoms or one, two, three, or more $^{13}$C atoms. All isotopologs arrows point to the respective molecular species, with isotopolog peaks of another molecular species carrying two $^{13}$C and harboring one additional double bond eluting $\approx$1 min earlier. For the molecular species that consist of molecules with different fatty acid combinations, the MS/MS trace data for the different acyl anions are shown (18:1, red; 18:2, green; 18:3, yellow).
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Previously, CL was quantified in Arabidopsis leaves by TLC combined with densitometry. The total amount of CL was found to be $\sim 34 \pm 12$ ng mg$^{-1}$ fresh weight (15). This amounts to $\sim 0.24$ nmol mg$^{-1}$ DW (molecular mass of CL $\sim 1,400$ ng nmol$^{-1}$; 1 mg fresh weight $= 0.1$ mg DW), in agreement with data presented here. The total content of glycerolipids in Arabidopsis leaves and roots was previously determined to be $\sim 165$ and $64$ nmol mg$^{-1}$ DW, respectively (52). Therefore, CL amounts to only 0.12% and 0.62% of total lipids in leaves and roots, respectively.

of a given molecular species of CL elute at the same retention time, and the peak containing only $^{12}$C carbons is the most abundant one. No isotopic correction was required because the $^{12}$C peak with the $m/z$ of [M-H]$^-$ could clearly be distinguished from ions containing one or more $^{13}$C atoms (Figs. 4, 5).

Because the peak areas during LC/MS of equimolar mixtures of (14:0)$_4$ CL, (18:0)$_4$ CL, and (18:1)$_4$ CL are similar, no correction factor was used for the different molecular species of CL, and (14:0)$_4$ CL was selected as internal standard with single-point calibration, similar to previous studies on quantification of animal CL (28, 31, 34, 35, 50). On the other hand, mass-dependent correction factors were used to quantify CL from E. coli (30) (Table 3).

The total amount of CL determined in different plant species and organs varied between 0.064 and 0.320 nmol mg$^{-1}$ DW in tobacco and spinach leaves, respectively (Fig. 7). Arabidopsis leaves and roots contain $\sim 0.2$ and 0.399 nmol mg$^{-1}$ DW of total CL (Fig. 8). Previously, CL was quantified in Arabidopsis leaves by TLC combined with densitometry. The total amount of CL was found to be $\sim 34 \pm 12$ ng mg$^{-1}$ fresh weight (15). This amounts to $\sim 0.24$ nmol mg$^{-1}$ DW (molecular mass of CL $\sim 1,400$ ng nmol$^{-1}$; 1 mg fresh weight $= 0.1$ mg DW), in agreement with data presented here. The total content of glycerolipids in Arabidopsis leaves and roots was previously determined to be $\sim 165$ and $64$ nmol mg$^{-1}$ DW, respectively (52). Therefore, CL amounts to only 0.12% and 0.62% of total lipids in leaves and roots, respectively.

Fig. 6. Validation for the quantification of CL molecular species. A: Lipids were isolated from spinach leaves by DEAE chromatography and quantified by LC/MS. The bar diagram shows the molecular species of 72:12 CL, 72:11 CL, 72:10 CL, 72:9 CL, 72:8 CL, and 72:7 CL as quantified on day 1 (left three bars) and day 8 (right three bars). On each day, lipids were isolated from three different fresh weights of spinach leaves (250, 500, and 750 mg tissue; from left to right). The bars show mean and SD of at least three measurements. B: Add-back study for (18:1)$_4$ CL. The amount of (18:1)$_4$ CL added to 500 mg spinach leaf tissue before lipid extraction and purification was compared with the amount of that measured by GC. Data points show mean and SD of five measurements. The diagonal indicating perfect matches is shown as dashed line.

Fig. 7. The CL composition in green and nongreen plant tissues. CL composition in spinach leaves (A), tobacco leaves (B), mung bean hypocotyls (C), and green and etiolated barley leaves (D). CL in total lipid extract was isolated by DEAE column chromatography and quantified by LC/MS using (14:0)$_4$ CL as internal standard. The bars on the right indicate the total amounts of CL. Numbers depict the average numbers of double bonds calculated per CL molecule. Data represent mean and SD of at least three measurements ($n = 3$).
from *Arabidopsis*. The two CL synthase preparations displayed a preference for both CDP-DAG and PG esterified with unsaturated 18:X acyl groups (22, 23). The high degree of unsaturation renders plant CL distinct from bacterial and yeast CL, but similar to animal CL. The most abundant CL species in *E. coli* were 64:2, 66:2, and 68:2, and no CL molecular species containing more than four total double bonds were detected (30). Similarly, CL from yeast harbored four or fewer double bonds, and the major species from yeast were 66:4, 68:4, 70:4, and 72:4 (29). Mammalian CL (e.g., from bovine heart) is rich in 72:8 with four 18:2 acyl groups (33) (Fig. 4B).

Under phosphate deprivation, a certain proportion of phospholipids is replaced with DGDG in the chloroplast and in extraplastidial membranes (44). Mitochondrial lipids also participate in the phosphate response because the amounts of PC and PE decrease, while the DGDG content increases in mitochondria during phosphate starvation (45). Previous measurements in *Arabidopsis* cell cultures and in isolated mitochondria by TLC in combination with fatty acid quantification by GC suggested that CL slightly increases after phosphate deprivation (45). We measured CL in extracts from whole *Arabidopsis* leaves and roots.

The molecular species composition of CL in plants is conserved, as 72:X CL species with four 18:X fatty acids are predominant in the different plants, including spinach, tobacco, mung bean, barley, and *Arabidopsis* leaves and roots (Figs. 7, 8, 9). The most abundant molecular species (72:12, 72:11, 72:10, 72:9, 72:8) contain mostly 18:2 or 18:3 fatty acids, and the average number of double bonds per CL molecule is in the range of 9 to 10 (Fig. 7).

The molecular species composition of CL from green tissues (leaves of spinach, tobacco, *Arabidopsis*) or non-green tissues (mung bean hypocotyls, *Arabidopsis* roots) are very similar. Furthermore, monocots (barley leaf) have a similar set of highly unsaturated CL molecules as dicots, and the molecular species composition is also similar in green and etiolated barley leaves. Therefore, plant CL is highly unsaturated.

These results are consistent with in vitro CL synthase assays with mung bean mitochondrial inner membranes or with a protein extract containing CL synthase purified from *Arabidopsis*. The two CL synthase preparations displayed a preference for both CDP-DAG and PG esterified with unsaturated 18:X acyl groups (22, 23). The high degree of unsaturation renders plant CL distinct from bacterial and yeast CL, but similar to animal CL. The most abundant CL species in *E. coli* were 64:2, 66:2, and 68:2, and no CL molecular species containing more than four total double bonds were detected (30). Similarly, CL from yeast harbored four or fewer double bonds, and the major species from yeast were 66:4, 68:4, 70:4, and 72:4 (29). Mammalian CL (e.g., from bovine heart) is rich in 72:8 with four 18:2 acyl groups (33) (Fig. 4B).

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mitochondria might be due to the fact that CL is essential for mitochondrial functions and therefore cannot be removed. On the other hand, the CL content was strongly decreased in roots after phosphate deprivation (Fig. 8B), in accordance with the decrease in other phospholipids (PC, PE) in roots (52). These changes might be caused by the strong increase in root biomass caused by root proliferation during phosphate deprivation. The degree of desaturation of CL under phosphate deprivation was increased (Fig. 8). In leaves, the total amounts of 72:12 and 72:11 were increased, while 72:10, 72:9, and 72:8 were decreased. In roots, the amounts of 72:12 and 72:11 were only slightly increased, while 72:10 and 72:9 were strongly decreased during phosphate deprivation. These results point toward the remodeling of CL under phosphate starvation.

CL plays a critical role in programmed cell death in animal cells (8–12). Previous results indicated that CL also plays a role in programmed cell death in Arabidopsis because CL deficient els mutant plants displayed a higher sensitivity to cell death-inducing stress factors including heat shock and exposure to prolonged darkness (14). We used the same two stress conditions to record the impact of cell death on the CL content or composition. A short heat shock of 10 min at 65°C had no effect on the total CL amount or degree of unsaturation in Arabidopsis plants (Fig. 9A), possibly because this stress was rather mild. However, growth under darkness resulted in the decrease in CL content and a shift toward higher unsaturated molecular species in Arabidopsis leaves. Therefore, the latter results suggest that changes in the content and composition of the molecular species of CL might be relevant for cell death.

Several enzymes have been proposed to be involved in remodeling of CL in yeast and animals, including tafazzin (21, 53), acyl-CoA:lyso-CL-acyltransferase (54), calcium-independent phospholipase A2 (55), and the trifunctional enzyme (56). The high abundance of highly unsaturated molecular species of CL, and their increase during phosphate deficiency and growth in darkness, point toward a critical role of these unsaturated CL forms in the inner mitochondrial membranes. It is possible that highly unsaturated CL molecules are required to maintain the activity of the respiratory chain complexes, and this might become even more relevant during phosphate deficiency stress or growth under darkness. The establishment of the LC/MS method for CL measurements from plants along with the results on the shift in CL molecular species composition under phosphate deprivation and growth under darkness provide the means to investigate CL remodeling mechanisms in plants in more detail [4].

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