A sensitive approach based on electrospray ionization tandem mass spectrometry has been employed to profile membrane lipid molecular species in Arabidopsis undergoing cold and freezing stresses. Freezing at a sublethal temperature induced a decline in many molecular species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) but induced an increase in phosphatidic acid (PA) and lyso phospholipids. To probe the metabolic steps generating these changes, lipids of Arabidopsis deficient in the most abundant phospholipase D, PLDα, were analyzed. The PC content dropped only half as much, and PA levels rose only half as high in the PLDα-deficient plants as in wild-type plants. In contrast, neither PE nor PG levels decreased significantly more in wild-type plants than in PLDα-deficient plants. These data suggest that PC, rather than PE and PG, is the major in vivo substrate of PLDα. The action of PLDα during freezing is of special interest because Arabidopsis plants that are deficient in PLDα have improved tolerance to freezing. The greater loss of PC and increase in PA in wild-type plants as compared with PLDα-deficient plants may be responsible for destabilizing membrane bilayer structure, resulting in a greater propensity toward membrane fusion and cell death in wild-type plants.

Eukaryotic membranes contain diverse lipid molecular species, and the lipid composition changes in response to both internal and external cues. Knowing how lipid molecular species change and how the changes are generated is important to the understanding of membrane and cell functions. Detailed study of membrane lipid changes, however, has been technically challenging because of the complexity of lipid molecular species and analytical procedures. Recently, an approach based on electrospray ionization tandem mass spectrometry (ESI-MS/MS) has been developed to comprehensively analyze lipid composition in animal and yeast cells (1–9). It requires only simple sample preparation and small samples to identify and quantify lipid molecular species. Expansion of this approach to plants, which harbor unique lipids, such as galactosylglycerolipids, should greatly facilitate the understanding of lipid functions in plant growth, development, and stress responses.

Plant stress caused by freezing has been an area of intensive research for many years, but the molecular and cellular mechanisms of freezing injury and tolerance are not well understood (10–12). The best documented freezing injury occurs at the membrane level. One major form of freezing damage is due to the formation of lipid hexagonal II phase in regions where the plasma membrane and the chloroplast envelope are closely apposed (13, 14). Changes in membrane lipid composition occur when plants are exposed to freezing temperatures (15). Lipid hydrolysis has been proposed to be mainly responsible for the change, but the role of lipid hydrolysis in freezing injury and tolerance is not clear.

In plants, several lipolytic enzymatic activities have been described, including phospholipase D (PLD), phospholipase C, phospholipase A, nonspecific acyl hydrolase, and galactolipases. PLD, which hydrolyzes phospholipids to phosphatidic acid (PA) and free head groups, is particularly abundant. Arabidopsis has multiple PLD genes that are categorized as PLDα, β, γ, δ, and ϵ. PLDs display different requirements for Ca2+, phosphatidylinositol 4,5-bisphosphate, pH, and free fatty acids and also some difference in substrate selectivity (16–18). Thus, individual PLDs in the cell may be activated differently, hydrolyze different lipid species, and have unique functions. PLDs, the most common plant PLD, has been abrogated genetically in Arabidopsis by introducing a PLDα antisense gene (16, 19). The combination of lipid profiling and specific genetic alterations promises to be a powerful approach to defining the in vivo activity and cellular roles of specific hydrolytic enzymes.

In this study, an automated ESI-MS/MS strategy is established to profile quantitatively the molecular species of plant lipids and to determine the lipid changes in Arabidopsis after cold acclimation and freezing at sublethal temperature. Comparative lipid profiling of wild-type and PLDα-deficient plants led to definition of the in vivo substrate(s) and the
contribution of the most abundant PLD isoform to the freezing-induced lipid changes. PLDα-abrogated Arabidopsis displays an enhanced tolerance to freezing. Understanding the in vivo action of PLDα in freezing allows us to suggest a mechanism for the improved freezing tolerance of PLDα-deficient plants.

**EXPERIMENTAL PROCEDURES**

Plant Growth and Treatments—Arabidopsis wild-type (Columbia ecotype) and PLDα-deficient plants (19) were sown in Scotts Metromix soil. The PLDα-deficiency was confirmed by assaying the PLDα activity and immunoblotting with a PLDα-specific antibody, following the procedure described previously (16). The pots were kept at 4 °C for 2 days and then moved to a growth chamber at 23 °C (day) and 19 °C (night) with a 12-h day length, daytime fluorescent lighting at 120 μmol m⁻² s⁻¹, and 58% relative humidity.

For cold acclimation, 35-day-old, preflowering plants were placed at 4 °C for 3 days in a growth chamber with a light intensity of 30 μmol m⁻² s⁻¹. Nonacclimated plants remained in the 23 °C growth chamber until they were harvested on the same day that the cold-acclimated plants were harvested. For freezing, cold-acclimated plants at 4 °C were subjected to a temperature drop from 4 to −2 °C at 3 °C/hour in the growth chamber. The frozen tissue was subjected to −8 °C overnight. The frozen tissue was placed on the soil to induce crystallization and prevent supercooling. After 2 h at −2 °C, the temperature was lowered to −8 °C/hour. After 2 h at −8 °C, the plants were harvested for lipid analysis.

**Lipid Extraction and Lipid Standards**—For each sample, the above-ground rosettes of two or three plants were cut at the sampling time and transferred to 50 ml of methanol/propionic acid with 0.01% butylated hydroxytoluene at 75 °C. After 15 min, 1.5 ml of chloroform and 0.6 ml of water were added. The tubes were shaken for 1 h, followed by removal of the extract. The plants were re-extracted with chloroform/methanol (2:1) with 0.01% butylated hydroxytoluene five times with 30 min of agitation each time, until all of the remaining tissue appeared white. The extract was filtered through a 0.45 μm filter. The extract was heated overnight at 105 °C to complete the extraction. The weights of these dried, extracted tissues are the "weights at 75 °C".

For cold acclimation, 35-day-old, preflowering plants were placed at 4 °C for 3 days in a growth chamber with a light intensity of 30 μmol m⁻² s⁻¹. Nonacclimated plants remained in the 23 °C growth chamber until they were harvested on the same day that the cold-acclimated plants were harvested. For freezing, cold-acclimated plants at 4 °C were subjected to a temperature drop from 4 to −2 °C at 3 °C/hour in the growth chamber. The frozen tissue was subjected to −8 °C overnight. The frozen tissue was placed on the soil to induce crystallization and prevent supercooling. After 2 h at −2 °C, the temperature was lowered to −8 °C/hour. After 2 h at −8 °C, the plants were harvested for lipid analysis.

Phospholipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), and except for di24:1-PC and di24:1-PE, which were prepared by transphosphatidylation of di24:1-PC (20). Phospholipids were quantified by phosphate assay (21), except for the hydrogenated PI components, which were quantified, using pentadecanoic acid as an internal standard, as fatty acid methyl esters by gas-liquid chromatography.

**ESI-MS/MS**—From 1 ml of plant lipid extract, two aliquots were taken for mass spectrometry analysis: one sample for phospholipid scanning modes were described by Brügger et al. (2). The samples were analyzed on a triple quadrupole tandem mass spectrometer (Micromass Ltd.). Each head group-specific scan was collected as one continuum spectrum. In each spectrum, the background was subtracted, the data were smoothed, and then peak height was determined with the centroiding routine. Identification of the peaks of interest and calculation of lipid species amounts were performed using Microsoft Excel. Corrections for overlap of isotopic variants (A = 2 peaks) in higher mass lipids were applied.

For acyl analysis, the data from that replicate sample for that head group class was averaged. The mean and standard deviation were calculated. Paired values were subjected to t tests to determine statistical significance.

**Protein Extraction and PLD Activity Assay**—Total protein from Arabidopsis leaves was extracted by grinding in an ice-chilled mortar and pestle with buffer A as described previously (19). Equal amounts of protein were separated by an 8% gel and transferred onto polyvinylidene difluoride filters. The membranes were blotted with a PLDα-specific antibody, followed by incubation with a second antibody conjugated to alkaline phosphatase, as described previously (20). PLDs activity was determined using egg yolk (PC) mixed with dipalmitoylglycerol-3-phospho[methyl-³H]choline as described previously (19).

**RNA Isolation and Blotting**—Total RNA was isolated from Arabidopsis rosettes. Equal amounts of total RNA (10 μg/lane) were separated by 1% formaldehyde-agarose denaturing gel electrophoresis and transferred onto nylon membranes. The full-length RNA-Pb-RNA was labeled with [α-³²P]CTP by random priming. The isolation and hybridization were performed as described previously (25).

**Ion Leakage and Freezing Tolerance**—To measure ionic leakage, Arabidopsis rosettes were collected 1 h after freezing at indicated temperatures and then incubated at 4 °C for 24 h. Deionized water was added, and conductivity of the solution was measured after...
gentle agitation at 23 °C for 1 h. Total ionic strength was measured after the solution was heated in 100 °C water bath for 10 min and cooled to 23 °C. Leaked ions were calculated as described previously (19). To test freezing tolerance, wild-type and PLDα-deficient plants were grown aseptically on agar containing Murashige and Skoog basal medium for 4 weeks. The plants were cold acclimated at 2 °C for 24 h, and the temperature was then lowered 1 °C/h to −10 °C. After being at −10 °C for 2 h, the plants were placed at 4 °C for 4 h and then grown at 23 °C.

RESULTS

**ESI-MS/MS Profiling and Quantitation of Plant Lipids**—Electrospray ionization of crude lipid extracts of Arabidopsis

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**FIG. 1.** Cold acclimation-induced changes in lipid molecular species in Arabidopsis as revealed by ESI-MS/MS. The double-hatched bars represent nonacclimated plants, and the black bars represent cold-acclimated plants. The values are the means ± S.D. (n = 4 or 5). An L indicates that the value is lower than that of the cold-acclimated plants of the same genotype (p < 0.05). An H indicates that the value is higher than that of cold-acclimated plants of the same genotype (p < 0.05). A, phospholipids and galactolipids of wild-type plants. B, phospholipids and galactolipids of PLDα-deficient plants.

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**TABLE I**

| Lipid(s) analyzed | Ion analyzed         | Scan mode       | Polarity | Collision energy | Time |
|-------------------|----------------------|-----------------|----------|------------------|------|
| PC                | [M+H]^+              | Precursors of m/z 184^+ | +        | 28               | 2    |
| Lysophosphatidylethanolamine (LPE) | [M+H]^+              | Precursors of m/z 184^+ | +        | 25               | 2    |
| PE/LysoPE         | [M+H]^+              | Neutral loss of 141 amu | +        | 20               | 2    |
| PALysoPG          | [M+H]^+              | Precursors of m/z 153^- | -        | 40               | 7    |
| PI                | [M+H]^+              | Precursors of m/z 241^- | -        | 43               | 3    |
| PS^-              | [M+H]^+              | Neutal loss of 87 amu | -        | 25               | 5    |
| MGDG              | [M+Na]^+             | Precursors of m/z 243^- | +        | 45               | 5    |
| DGDG              | [M+Na]^+             | Precursors of m/z 243^- | +        | 66               | 5    |

^a Neutral loss of 87 scanning in the negative mode for PS demonstrated that Arabidopsis contains about 0.4 nmol of PS/mg dry weight and that 34:2 and 34:3 are the major PS species, but the sensitivity of this scan mode did not allow detailed analysis.
Membrane Lipid Profiling Shows PLDα Role

**TABLE II**

Major acyl species of the most common lipid molecular species in Arabidopsis leaves

If one acyl species fragment gave a stronger signal, that species is indicated second. A stronger signal suggests that the species is in the 2-position, but this is not an unequivocal designation of position (24).

| Acyl chain | PC       | PE       | PA       | PI       | PG       | MGDG     | DGDG     |
|------------|----------|----------|----------|----------|----------|----------|----------|
| 34:2       | 16:0–18:2| 16:0–18:2| 18:2–16:0| 18:2–16:0| 18:2–16:0| 16:0–18:2| 16:0–18:2|
| 34:3       | 16:0–18:2| 16:0–18:2| 18:2–16:0| 18:2–16:0| 18:2–16:0| 16:0–18:2| 16:0–18:2|
| 34:4       | 16:0–18:2| 16:0–18:2| 18:2–16:0| 18:2–16:0| 18:2–16:0| 16:0–18:2| 16:0–18:2|
| 34:6       | 18:0–18:2| 18:0–18:2| 18:0–18:2| 18:0–18:2| 18:0–18:2| 18:0–18:2| 18:0–18:2|
| 36:2       | 18:1–18:2| 18:1–18:2| 18:1–18:2| 18:1–18:2| 18:1–18:2| 18:1–18:2| 18:1–18:2|
| 36:3       | 18:2–18:2| 18:2–18:2| 18:2–18:2| 18:2–18:2| 18:2–18:2| 18:2–18:2| 18:2–18:2|
| 36:4       | 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3|
| 36:5       | 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3|
| 36:6       | 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3| 18:3–18:3|

**TABLE III**

Total amount of lipid in each head group class in cold acclimation and freezing

| Lipid        | Cold acclimation experiment | Freezing experiment |
|--------------|-----------------------------|---------------------|
|              | In non-acclimated plants    | In cold-acclimated plants | In cold-acclimated plants | In plants subjected to –8 °C treatment |
|              | nmol/mg                     |                      |                      |                              |
| PI           | 5.1 ± 0.7                   | 5.5 ± 0.4            | 5.4 ± 0.3            | 5.9 ± 0.6                    |
| PG           | 22.3 ± 1.9                  | 24.5 ± 1.7           | 24.0 ± 1.2           | 18.8 ± 2.8                   |
| PE           | 11.7 ± 1.4                  | 14.3 ± 2.6           | 15.5 ± 0.8           | 9.7 ± 1.7                    |
| PC           | 22.8 ± 0.7                  | 23.6 ± 2.3           | 26.0 ± 0.2           | 14.4 ± 1.1                   |
| PA           | 1.2 ± 0.1*                  | 1.9 ± 0.5            | 1.9 ± 0.3            | 11.5 ± 1.0                   |
| MGDG         | 71.4 ± 5.1*                 | 87.8 ± 6.0           | 77.0 ± 2.5           | 71.5 ± 8.3                   |
| DGDG         | 30.7 ± 2.8*                 | 41.0 ± 3.5           | 35.9 ± 0.9           | 40.2 ± 4.9                   |
| LysoPE       | 0.12 ± 0.03*                | 0.20 ± 0.01          | 0.06 ± 0.02          | 0.80 ± 0.11                   |
| LysoPC       | 0.14 ± 0.01*                | 0.20 ± 0.02          | 0.09 ± 0.03          | 0.71 ± 0.14                   |
| PI           | 4.7 ± 0.5                   | 5.6 ± 0.7            | 4.8 ± 0.3            | 5.1 ± 0.4                    |
| PG           | 20.7 ± 2.9                  | 23.5 ± 2.6           | 24.2 ± 0.8           | 20.2 ± 2.0                   |
| PE           | 10.9 ± 1.2*                 | 15.0 ± 1.7           | 16.5 ± 1.4           | 10.1 ± 2.2                   |
| PC           | 23.1 ± 0.4*                 | 25.0 ± 0.6           | 27.4 ± 0.7           | 21.4 ± 1.6                   |
| PA           | 0.8 ± 0.3*                  | 1.4 ± 0.3            | 1.2 ± 0.1            | 5.8 ± 0.2                    |
| MGDG         | 64.8 ± 7.2                  | 78.2 ± 12.0          | 77.5 ± 6.6           | 68.2 ± 4.9                   |
| DGDG         | 26.2 ± 5.6*                 | 34.9 ± 3.5           | 36.9 ± 3.1           | 39.9 ± 0.4                   |
| LysoPE       | 0.15 ± 0.05                 | 0.21 ± 0.01          | 0.08 ± 0.01          | 0.80 ± 0.24                   |
| LysoPC       | 0.17 ± 0.03                 | 0.18 ± 0.04          | 0.10 ± 0.01          | 0.84 ± 0.12                   |

* The value is lower than that of the cold-acclimated plants of the same genotype in the same experiment (p < 0.05).

b The value is higher than that of cold-acclimated plants of the same genotype in the same experiment (p < 0.05).

c The value for the PLDα-deficient plant is different from that of wild-type plants treated in the same way in the same experiment (p < 0.05).

rosettes results in the generation of singly charged molecular ions with excellent concentration sensitivity. The molecular species of phospholipids PC, PE, PG, PI, PS, PA, lysoPC, and lysoPE were identified by precursor or neutral loss scanning (Table I). To develop the ESI-MS/MS analysis of MGDG and DGDG, which are major plant lipids and unique to photosynthetic membranes, the fragmentation pattern of Arabidopsis galactosylglycerolipids was interpreted based on the analysis of cyanobacterial lipid species using fast atom bombardment tandem mass spectrometry (Ref. 22 and Table I). The major molecular species of MGDG are 34:6 (16:3–18:3) and 36:6 (di18:3) (Fig. 1 and Table II). This is consistent with the fatty acid composition of MGDG, which is mainly hexadecatrienoic (16:3) and linolenic (18:3) acids (26, 27). The major molecular species of DGDG are 34:3 (16:0–18:3) and 36:6 (di18:3), which is consistent with the fatty acid composition of DGDG, which is largely palmitic (16:0) and linolenic (18:3) acids (26, 27).

The lipids in each class were quantified in comparison with two internal standards of that class. To verify the quantitativeness of the MS/MS data, the amount and fatty acid composition of each head group class (Table III), as determined by ESI-MS/MS, were compared with those determined previously using traditional lipid analyses of Arabidopsis leaves (26, 27). They are in good agreement. Compared with the traditional analysis, however, the ESI-MS/MS approach requires simple sample preparation, small samples, and short analysis time to identify and quantify lipid molecular species. Profiling of the molecular species of PC, PE, PG, PI, PA, lysoPC, lysoPE, MGDG, and DGDG takes 26 min (Table I). ESI-MS/MS can detect the major and also minor molecular species, such as PA and lysophospholipids (Figs. 1 and 2). ESI-MS/MS also allowed detection of minor PE and PC species containing long chain fatty acyl groups (Fig. 1). Signals can be easily observed from phospholipid components as di- and tri-acyl PE and PC (17).
the negative mode allowed identification of fatty acyl groups and tentative assignment of these groups to the sn-1 and sn-2 positions (Table II).

Changes of Lipid Molecular Species during Cold Acclimation and Freezing—The ESI-MS/MS analysis was used to probe changes in lipid molecular species of Arabidopsis as a result...
Membrane Lipid Profiling Shows PLDα Role

Fig. 4. Freezing-induced changes in lysolipid molecular species in Arabidopsis as revealed by ESI-MS/MS. The black bars represent cold-acclimated plants, and the hatched bars represent plants subjected to −8 °C. The values are the means ± S.D. (n = 4 or 5). An L indicates that the value is lower than that of the cold-acclimated plants of the same genotype (p < 0.05). An H indicates that the value is higher than that of cold-acclimated plants of the same genotype (p < 0.05). A, lysoPC and lysoPE of wild-type plants. B, lysoPC and lysoPE of PLDα-deficient plants.

of temperature manipulation. Compared with plants grown at 19–23 °C, cold acclimation of Arabidopsis plants for 3 days at 4 °C produced significant changes in membrane lipid molecular species (Figs. 1 and 2 and Table III). Many species that contain two polyunsaturated acyl species, such as 36:5- and 36:6-PC (di18:3 PC), 36:5- and 36:6-PE, 34:6-MGDG, and 36:6-DGDG, increased (Fig. 1A). Polysaturated lysophospholipid species, such as 18:3-lysoPC and 18:3-lysoPE, tended to increase. 16:0-LysoPC and 16:0-lysoPE also tended to increase (Fig. 2A). On the other hand, the levels of more saturated species, such as 36:2- and 36:3-PC (Fig. 1A) and 18:1-lysoPC (Fig. 2A) declined significantly. There was a general trend toward an increase in the total amount of each lipid class in cold acclimation (Table III).

More dramatic changes in lipid molecular species occurred when cold-acclimated plants were subjected to freezing at −8 °C (Figs. 3 and 4). The freezing study was conducted using plants grown at a different time from those used in the cold acclimation experiments. The data on lipids in the cold-acclimated plants in the two experiments are very similar, with a slight discrepancy in the lysoPC and lysoPE levels (Table III). Upon freezing at −8 °C, massive declines in the levels of PC, PE, and PG occurred, but PA and lysoPC and lysoPE amounts increased dramatically (Figs. 3A and 4A and Table III). These changes suggest a rise in lipolytic activities during freezing. The loss occurred to almost all molecular species of PG, PC, and PE, with the 36:4 and 36:5 species contributing the most to the decline in PE and PC levels. On the other hand, there was no loss of PI, and the polysaturated 36:5- and 36:6-PI species actually increased. Although MGDG levels tended to decline, no loss of DGDG occurred in Arabidopsis exposed to −8 °C (Fig. 3A and Table III). The large decline in major membrane phospholipids but not galactolipids suggests that at this sublethal freezing temperature, phospholipases were activated to a greater extent than galactolipases. The low level of the phospholipase A products, lysophospholipids, in comparison with the PLD product, PA, indicates that PLD is more active than phospholipase A in frozen plant tissue. We also profiled the lipid changes in plants subjected to freezing at −4 °C (data not shown). The increase in PA and lysophospholipid levels was smaller than that of those subjected to −8 °C, indicating that the hydrolytic activity increased as the temperature decreased.

Comparison of the molecular species of the phospholipids in cold-acclimated plants with those subjected to freezing also was performed by examining the data as mole percentages (data not shown). Small but significant differences were slightly higher mole percentages of 34:2 PC and 34:2 PE species in the −8 °C-treated plants, suggesting that these species, with an acyl composition of 16:0–18:2, were slightly resistant to hydrolysis.

Role of PLD in Freezing-induced Lipid Changes—To determine the metabolic steps generating the freeze-induced lipid changes, the lipid species of wild-type and PLDα-deficient Arabidopsis plants were profiled comparatively (Figs. 1–4, A versus B). Arabidopsis plants with less than 3% of the wild-type level of PLDα activity (Fig. 5A) were produced by antisense abrogation of PLDα (16). The loss of activity in the antisense plants resulted from a decreased expression of PLDα, as shown by the virtual absence of PLDα mRNA and protein (Fig. 5, A and B). The abrogation of PLD activity is specific to PLDα; the expression and activity of other PLDs are not affected (16, 28).

Cold acclimation led to an increase in PLDα mRNA levels in wild-type plants, whereas expression in PLDα-deficient plants remained low (Fig. 5B). However, wild-type and PLDα-deficient plants underwent similar alterations in lipid composition in 3 days at 4 °C (Figs. 1 and 2). PA levels did tend to be lower in PLDα-deficient than in wild-type plants both before and after cold acclimation (Fig. 1 and Table III). Overall, these results indicate that PLDα did not play a major role in the alterations of lipid molecular species that occurred in cold acclimation and that the differences between wild-type and PLDα-deficient plants in freezing tolerance are unlikely to be due to lipid compositional differences that occur in cold acclimation.

PLDα-deficient plants displayed more freezing tolerance than wild-type plants, as demonstrated by decreased membrane leakage and increased survival during freezing (Fig. 5C and D). After being at −10 °C for 2 h, wild-type plants and the transgenic control (data not shown) did not survive, whereas PLDα-deficient plants survived (Fig. 5D). During freezing, the lipid composition of plants with and without PLDα differed significantly. Although PC levels declined 45% in wild-type plants, the decrease was only 22% in PLDα-deficient plants at −8 °C (Fig. 3 and Table III). On the other hand, PE and PG...
levels declined to a similar extent in wild-type and PLDα-deficient plants, suggesting the PLDα preferentially hydrolyzed PC. The total amounts of PA increased in both genotypes, with wild-type amounts increasing more than 5-fold, but PLDα-deficient plants accumulated only 48% as much PA as wild-type plants (Fig. 3 and Table III). Thus, under these freezing conditions, PLDα is responsible for approximately half of PC hydrolysis to PA and about half of the PA production. On the other hand, other enzymes seem to be responsible for PE hydrolysis and most of the PG hydrolysis. These data suggest that PC is the major in vivo substrate of PLDα.

The molecular species profile of PA generated upon freezing...
in wild-type plants (Fig. 3A) is consistent with the conclusion that PA is derived primarily from PC. However, upon freezing, both wild-type and PLD\(\alpha\)-deficient plants gained similar amounts of one PA molecular species, 34:6 PA, that was absent from the plants before the freezing treatment (Fig. 3). The acyl composition, 16:3–18:3 (Table II), suggests that this PA species is likely to have been derived from MGDG. The similar amounts of 34:6 PA formed in wild-type and PLD\(\alpha\)-deficient plants also suggest that the formation of this PA species was independent of PLD\(\alpha\) action.

**DISCUSSION**

This study shows that ESI-MS/MS allows rapid analysis of the composition of plant membrane lipids that include MGDG and DGDG, the unique and abundant glycolipids in photosynthetic membranes. In combination with analysis of the acyl distribution by fragment analysis, a very complete picture of the composition of membrane glycerolipids was obtained. The easy sample preparation and relatively short analysis time permit massive profiling of membrane lipid changes triggered by external or internal challenges.

Because PLD\(\alpha\) activity depends on pH, calcium concentration, and the presence of nonsubstrate lipids, questions have arisen about the identity of the in vivo substrates of PLD\(\alpha\). PLD\(\alpha\) is active in vitro either at neutral pH with millimolar levels of Ca\(^{2+}\) or under acidic conditions with micromolar Ca\(^{2+}\) and the substrate in vesicles containing PE and polyphosphoinositides (29). Comparative lipid profiling of wild-type and PLD\(\alpha\)-deficient *Arabidopsis* demonstrates that PC is the major in vivo substrate for PLD\(\alpha\) during freezing-induced activation. This confirms in vitro data that showed that PC is hydrolyzed preferentially as compared with PE (17).

PLD is a major family of lipolytic enzymes in plants, and five types of PLD activities have been described. The loss of PLD\(\alpha\) reduced the freezing-induced increase of PA by more than 50% (5.0 nmol/mg dry weight) and reduced the freezing-induced loss of PC to a similar extent (5.6 nmol/mg dry weight), demonstrating that PLD\(\alpha\) is a key enzyme catalyzing the decline of PC during freezing. These data show that application of ESI-MS/MS-based lipid profiling to a biological system in which there are defined alterations of gene affecting lipid metabolism is a particularly powerful approach to defining the metabolic processes that generate the lipid changes.

The detailed ESI-MS/MS-based analysis also provides information as to whether any specific molecular lipid molecular species are preferentially metabolized and as to whether particular reactions occur. We found a slight tendency in both wild-type and PLD\(\alpha\)-deficient plants to leave 34:2 PC and PE species unhydrolyzed. It is unclear whether this represents a preference of a hydrolytic enzyme(s) for more unsaturated species or whether these species are somewhat inaccessible to hydrolytic enzymes, perhaps because of an asymmetric distribution of acyl species across the lipid bilayers of the plant cell membranes. The appearance in the frozen plants of 34:6 PA, which contains acyl species that are found otherwise only in galactolipids, particularly in MGDG, suggests that some PA is formed by phosphorylation of diacylglycerol produced by hydrolysis of MGDG.

The difference in membrane lipid compositions in response to freezing of wild-type and PLD\(\alpha\)-deficient *Arabidopsis* and the change in freezing tolerance caused by this alteration indicate that cellular membranes are important in stress responses. Indeed, the best documented freezing injury occurs at the membrane level (10, 13, 14, 30–32). Workers in the Steponkus laboratory have demonstrated three types of freezing injury. In expansion-induced lysis, which typically occurs at \(-2\) to \(-4\) °C, water leaves the cells, resulting in dehydration and high osmolality inside the cells. When thawing occurs, water quickly enters the cell, resulting in lysis. At about \(-4\) to \(-10\) °C, many plants undergo damage caused by freeze-induced lamellar-to-hexagonal II phase transitions between the plasma membrane and the chloroplast envelope membranes; this damage is brought on by dehydration of the membrane lipids. Below \(-10\) °C, the damage is typically due to other mechanisms, including fracture jump lesions in membranes. In *Arabidopsis*, the incidence of expansion-induced lysis has been shown to be very low, and injury in nonacclimated *Arabidopsis* is primarily associated with formation of the hexagonal II phase (33).

Typically, under field conditions, plants acclimate to the cold at low temperatures above freezing before they are subjected to freezing temperatures. As demonstrated in this work and by others, in cold acclimation, the amount of unsaturated fatty acyl groups in membrane lipids is increased (33–35), and the amount of most membrane glycerolipids typically is increased (36–38). Such lipid changes reduce, and in some cases may eliminate, the propensity of cellular membranes to undergo freezing-induced nonbilayer phase formation (33, 39). Sugawara and Steponkus (40) also demonstrated the importance of lipid composition to freezing tolerance by showing that the addition of d18:2-PC to the plasma membranes of nonacclimated rice protoplasts by fusion with liposomes resulted in a reduced propensity of the plasma membrane to participate in freeze-induced formation of the hexagonal phase. These data indicate that membrane lipid composition can have a profound impact on the tendency of a membrane lipid bilayer to undergo a transition to the nonbilayer hexagonal II phase.

Our data demonstrate that the increase in lipolytic activities during freezing alters membrane lipid composition. Although early studies suggested that PLD activity could be involved in freezing-induced lipid hydrolysis (15), the role of PLD in freezing injury and tolerance is unknown. The present results show that PLD\(\alpha\) causes PC hydrolysis, and that upon freezing of wild-type plants, PC is hydrolyzed to a greater extent than PE or PG. The molar ratio of PC/PE in wild-type plants tends to drop (from 1.68 ± 0.05 to 1.48 ± 0.19) after freezing, whereas the amount of PA is increased 6-fold. Because unsaturated PEs have a strong propensity to form hexagonal phases (41) and PA has tendency to form the hexagonal II phase in the presence of Ca\(^{2+}\) (42), these compositional alterations are likely to lead to the formation of a nonbilayer lipid phase. Localized transformation of a membrane lipid bilayer into a nonbilayer structure compromises membrane integrity and cell function. In contrast, in PLD\(\alpha\)-deficient plants, PC, a bilayer-stabilizing lipid, is hydrolyzed to a much lesser extent than in wild-type plants, and the PC/PE ratio increases from 1.66 ± 0.09 to 2.11 ± 0.23 during freezing, whereas PA levels increase to about half the level of frozen wild-type plants. Combined, the alterations that occur in PLD\(\alpha\)-deficient plants are less likely to induce nonbilayer phase formation than those that occur in wild-type plants. Indeed, measurements of membrane competency show that more ion leakage occurred in leaves of wild-type plants than in the leaves of PLD\(\alpha\)-deficient plants. We propose that the reduced levels of PC and increased levels of PA in wild-type *Arabidopsis* membranes promote nonbilayer phase formation that leads to cell death. These results suggest that PLD\(\alpha\) and PC hydrolysis play key roles in freezing injury and point to a new avenue for improving freezing tolerance.

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