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Differential Degradation of Extraplastidic and Plastidic Lipids during Freezing and Post-freezing Recovery in *Arabidopsis thaliana**[S]

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Changes in membrane lipid composition play important roles in plant adaptation to and survival after freezing. Plant response to cold and freezing involves three distinct phases: cold acclimation, freezing, and post-freezing recovery. Considerable progress has been made toward understanding lipid changes during cold acclimation and freezing, but little is known about lipid alteration during post-freezing recovery. We previously showed that phospholipase D (PLD) is involved in lipid hydrolysis and *Arabidopsis thaliana* freezing tolerance. This study was undertaken to determine how lipid species change during post-freezing recovery and to determine the effect of two PLDs, PLDα1 and PLDδ, on lipid changes during post-freezing recovery. During post-freezing recovery, hydrolysis of plastidic lipids, monogalactosyldiacylglycerol and plastidic phosphatidylglycerol, is the most prominent change. In contrast, during freezing, hydrolysis of extraplastidic phospholipids, phosphatidylcholine and phosphatidylethanolamine, occurs. Suppression of PLDα1 decreased phospholipid hydrolysis and phosphatidic acid production in both the freezing and post-freezing phases, whereas ablation of PLDδ increased lipid hydrolysis and phosphatidic acid production during post-freezing recovery. Thus, distinctly different changes in lipid hydrolysis occur in freezing and post-freezing recovery. The presence of PLDα1 correlates with phospholipid hydrolysis in both freezing and post-freezing phases, whereas the presence of PLDδ correlates with reduced lipid hydrolysis during post-freezing recovery. These data suggest a negative role for PLDα1 and a positive role for PLDδ in freezing tolerance.

Low temperature stress, such as freezing, causes great agricultural losses. The ability of plants to endure and survive at low temperatures is a major determinant of plant productivity and geographic distribution (1). Membranes are major injury sites during plant freezing (2, 3), and membrane lipids undergo substantial changes when plants are exposed to low temperatures (4). Plant response to cold and freezing can be divided into three distinct phases: cold acclimation, freezing, and post-freezing recovery. During cold acclimation, the degree of fatty acid unsaturation and the content of phospholipids increase (5). Such alterations are thought to enhance membrane fluidity and reduce the propensity of cellular membranes to undergo freezing-induced non-bilayer phase formation, thus enhancing membrane integrity and cellular function during freezing (4). During freezing, dramatic lipid alterations take place in both extraplastidic and plastidic membranes. In extraplastidic membranes, there are increases in PA[2] and lysophospholipids and decreases in phosphatidylcholine (PC) and phosphatidylethanolamine (PE); in plastidic membranes, there are decreases in monogalactosyldiacylglycerol (MGDG) (5). The recovery phase involves tissue thawing, cellular rehydration, restoration of cell structure, and resumption of cellular activities. The ability to successfully undergo these processes, many of which depend on membranes, is critical for cellular survival after freezing. However, little, if anything, is known about metabolism of the membrane lipid species, which were altered during cold acclimation and freezing, in the post-freezing phase.

In plants, several lipolytic enzymatic activities have been described, including PLD, PLA, PLC, nonspecific acylhydrolase, and galactolipases (6). PLDs are involved in the hydrolysis of phospholipids into PA and a head group. Recently, we have shown that PLDα1 and PLDδ, the two most abundant PLDs of the 12-member *Arabidopsis* PLD family, play important roles in freezing tolerance (5, 7). Comparative profiling of leaves from PLDα1-deficient and wild-type plants led to the conclusion

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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[2] The abbreviations used are: PA, phosphatidic acid; CA, cold acclimation; DGDG, digalactosyldiacylglycerol; ESI, electrospray ionization; lysoPC, lysophosphatidylcholine; lysoPG, lysophosphatidylglycerol; lysoPE, lysophosphatidylethanolamine; lysoPL, lysophospholipids; MS/MS, tandem mass spectrometry; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D; Col, Columbia ecotype; WS, Wassilewskija ecotype; PFR, post-freezing recovery.

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that freezing-induced PA is derived primarily from PC and that PLDα1 is responsible for ~50% of the PA formed during freezing. The lower ratio of PA to PC after freezing was proposed to be the basis for the greater freezing tolerance of PLDα1-deficient plants (5). In contrast, ablation of PLDδ rendered *Arabidopsis* more sensitive to freezing and caused just a subtle reduction in PA levels compared with wild type (7). It was proposed that PLDδ enhances freezing tolerance by mitigating post-freezing damage and cell death (7). The effects of PLDα1 and PLDδ on lipid changes during post-freezing recovery have not previously been described. This study was undertaken to determine: (i) how membrane glycerolipid species change during post-freezing recovery and (ii) the involvement of PLDα1 and PLDδ in the lipid changes.

EXPERIMENTAL PROCEDURES

Plant Materials—A PLDα1-deficient mutant was generated previously by antisense suppression from *Arabidopsis* (Columbia ecotype (Col)) (19). A PLDδ-knockout mutant was previously isolated from *Arabidopsis* (Wassilewskija ecotype (WS)) (18). The loss of *PLDα1* and *PLDδ* was confirmed by the absence of the transcript, protein, and activity of *PLDα*1 or *PLDδ* (18, 19).

Growth Conditions and Treatments—Four *Arabidopsis* genotypes were grown in Scotts Metromix soil. The pots with seeds were kept at 4 °C for 2 days and then moved to a growth chamber at 23 °C (day) and 19 °C (night) with fluorescent lighting of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 60% relative humidity, and a 12-h photoperiod. For cold acclimation, 4-week-old plants were placed at 4 °C for 3 days under light of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$. For freezing, cold-acclimated plants were subjected to a temperature drop from 4 to −2 °C at 3 °C/h in the growth chamber. The temperature was held at −2 °C for 2 h, and ice chips were placed on the soil to induce crystallization and prevent supercooling. The temperature was lowered to −8 °C at 1 °C/h and was held at −8 °C for 2 h before sampling. For post-freezing recovery, the temperature was raised to 4 °C at 1 °C/h and was held at 4 °C for 12 h before sampling. Different plants were used for each analysis.

Lipid Extraction and ESI-MS/MS Analysis—The process of lipid extraction, ESI-MS/MS analysis, and quantification was performed as described previously with minor modifications (5, 8). Briefly, the above-ground rosettes of two or three different plants were cut at sampling time and, to inhibit lipolytic activities, were transferred immediately into 3 ml of isopropanol with 0.01% butylated hydroxytoluene at 75 °C. The tissue was extracted with chloroform/methanol (2:1) three additional times with 2 h of agitation each time. The remaining plant tissue was heated overnight at 105 °C and weighed. The weights of these extracted and dried tissues are described as "dry weight" of the plants. Lipid samples were analyzed on a triple quadrupole MS/MS equipped for ESI. Data processing was performed as previously described (5, 8). The lipids in each class were quantified in comparison to two internal standards of the class. Five replicates of each treatment for each genotype were analyzed. The Q-test was performed on the total amount of lipid in each head group class, and data from discordant samples were removed (5). Paired values were subjected to the *t* test to determine statistical significance. Hierarchal clustering analysis was performed using Genespring version 7.2 (Silicon Genetics).

RNA Extraction and Microarray Analysis—Total RNA was isolated from three independent *Arabidopsis* (Col) plants for each treatment using RNAiso reagent according to the manufacturer's instructions (TaKaRa). cRNA preparation and microarray hybridization were performed following the manufacturer's instructions (Affymetrix). The probe array scanning was performed with GCOS (Affymetrix GeneChip Operating Software, Version 1.4), and data were analyzed with Genespring version 7.2.

RESULTS

Large Changes in Lipid Profiles Occur during Freezing and Post-freezing Recovery—Using an ESI-MS/MS-based lipid profiling approach, this study identified and quantified 108 glycerolipid molecular species, during cold acclimation, freezing, and post-freezing recovery (Figs. 1–5 and Table 1). Two *Arabidopsis* ecotypes, Col and WS, were employed to examine the patterns of membrane lipid changes in different ecotypes in freezing responses.

To gain an overall appreciation of the effects of freezing and post-freezing recovery treatments in conjunction with PLD deficiency, hierarchal clustering of the lipid profiles was applied (Fig. 1). In the *left panel*, which shows the relationships among lipid profiles of wild-type (Col) and PLDα1-deficient plants subjected to various treatments, the major difference among the treatments and genotypes was between the cold-acclimated plants and plants that have been subjected to freezing, including those that have undergone post-freezing recovery. A close relationship between cold-acclimated wild-type and PLDα1-deficient plants (*first two columns*) implies that PLDα1 deficiency had little effect on lipid profile during cold acclimation. Among the plants subjected to freezing, the largest difference in lipid profile patterns was between wild-type (*third* and *fourth columns*) and PLDα1-deficient plants (*fifth* and *sixth columns*), indicating that wild-type and PLDα1-deficient plants responded differently to freezing. The closer relationship between the plants within each genotype subjected to freezing only in comparison to post-freezing recovery (*third versus fourth* and *fifth versus sixth columns*) indicates that the lipid compositional changes that occurred during post-freezing recovery were smaller than those that occurred during freezing.

In the *right panel*, which shows the relationships among lipid profiles of wild-type (WS) and PLDδ-deficient plants subjected to various treatments, the major difference among the treatments and genotypes was also between the cold-acclimated and the plants that were subjected to freezing, including those that underwent post-freezing recovery. Again, the close relationship between cold-acclimated wild-type and the PLD-deficient plants (this time PLDδ; *first two columns*) indicates that the deficiency had little effect on lipid profile during cold acclimation. Among the plants subjected to freezing, the largest differences were among plants within a genotype subjected to freezing only and those subjected to freezing plus post-freezing recovery (*third* compared with *fifth* and *fourth* compared with *sixth columns*). In comparison to the clustering with PLDα1-deficient plants and the corresponding wild-type (*left panel*),

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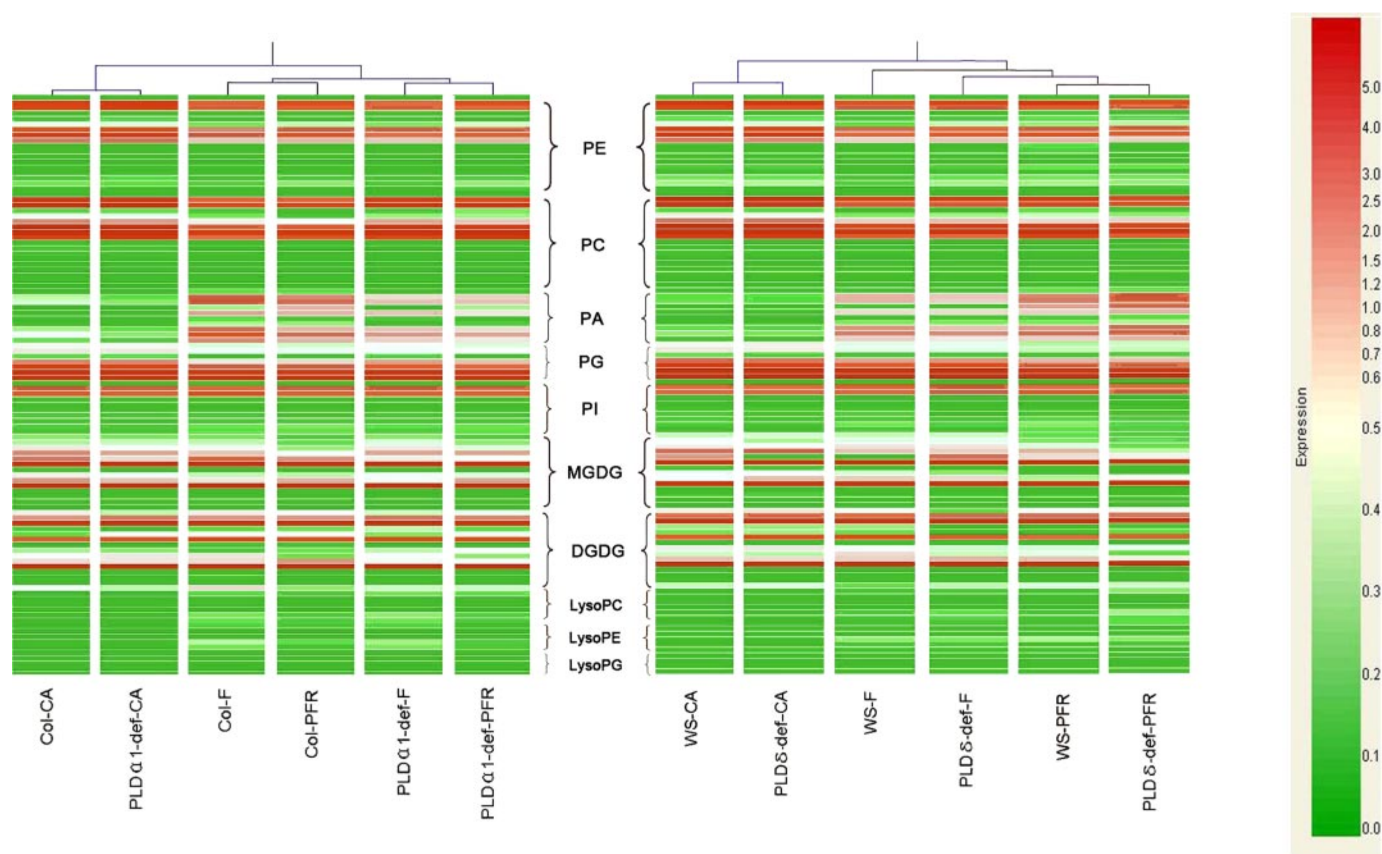

FIGURE 1. **Hierarchal clustering analysis of *Arabidopsis* lipid molecular species during CA, freezing (*F*), and PFR.** *Left panel*, wild-type Col plants and PLDα1-deficient plants; *right panel*, wild-type WS plants and PLDδ-deficient plants. Each *colored bar* within a *column* represents a lipid molecular species in the indicated plants and treatments. The *color* of *each bar* represents the levels of corresponding lipid species. Expression is $\log_2$ lipid amount (nanomoles/mg dry weight). A total of 108 lipid species in the indicated lipid classes was organized using class (as indicated), total acyl carbons (in ascending order within a class), and total double bonds (in ascending order with class and total acyl carbons). The dry weight is dry weight minus lipid (*i.e.* dry weight after lipid extraction).

this arrangement suggests that PLDδ deficiency affects plant leaf response to freezing less than PLDα1 deficiency. Assessing the role of PLDδ deficiency in post-freezing recovery from the hierarchal clustering is difficult; whereas the relationships indicate that PLDδ-deficient plants undergoing post-freezing recovery were slightly less changed than wild-type plants undergoing post-freezing recovery, it is clear that various lipid species were affected differentially and more detailed analysis, as included below, is required.

Lipid Changes during Post-freezing Recovery Differ from Changes during Freezing—Detailed analysis of the lipid profiles indicates that changes in phospholipids and galactolipids in the two ecotypes were similar during cold acclimation, freezing, and post-freezing recovery (Figs. 2–5, *left panels*, and Table 1), except for minor differences in PA species 36:4, 36:5, and 36:6 (total acyl chains: double bonds; Figs. 2 and 3), and in lysophospholipid and lysoPE species 18:2 and 18:3 (Figs. 4 and 5).

Compared with the lipid levels of cold-acclimated *Arabidopsis*, at −8 °C, a sublethal-freezing temperature for cold-acclimated *Arabidopsis*, the amount of almost every molecular species of PG, PC, and PE decreased, with the 36:4 and 36:5 species contributing the most to the decline in PE and PC levels. The level of PC decreased 46%, PE 37%, and PG 36% in Col plants (Table 1). In contrast, the levels of the lipid metabolites, PA and lysophospholipids, increased, and changes during freezing in galactolipids MGDG and DGDG were small. Similar trends were observed in WS plants (Table 1).

Changes in lipid species during post-freezing recovery differed from those that occurred during freezing. In the post-freezing phase there was no decrease in PC and PE; in fact the amounts of these lipids tended to increase, suggesting net synthesis of these lipid classes. On the other hand, there was a decrease in the plastidic galactolipid MGDG; in particular, the level of MGDG decreased 35% in Col plants and 31% in WS plants. There also tended to be a decrease in the plastidic components, DGDG and PG. The decline indicates lipid degradation in this compartment during tissue thawing. Thus, plastidic lipids were declining while synthesis of extraplastidic lipids was occurring during post-freezing recovery.

Plastidic PG Is Hydrolyzed to PA by PLDα1 during Post-freezing Recovery—Analysis of molecular species in Col, WS, and *PLDδ*-knockout mutant plants (PLDδ-deficient) reveals that the levels of 34:4 PA during post-freezing recovery were 3- to 15-fold higher than during freezing (Figs. 2 and 3), whereas other PA species were nearly unchanged, or in some cases were decreased (Figs. 2 and 3). Product ion analysis of the 34:4 PA showed that this species contains 18:3 and 16:1 acyl groups (8). 18:3–16:1 PG is most prevalent PG species, but an 18:3/16:1 combination is found in only minor amounts in other glycerolipids (5, 8, 9). Thus the 34:4 diacylglycerol moiety serves as a

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TABLE 1

Total amount of lipid in each head group class during CA, freezing, and PFR in Col, PLDα1-deficient (def), WS, and PLDδ-def plants

The dry weight is dry weight minus lipid.

Lipid class	Genotype	CA	Freezing	PFR
		nmol/mg dry weight		
PG	Col	23.9 ± 1.3	15.5 ± 2.4[a]	14.3 ± 1.5[a]
	PLDα1-def	24.2 ± 0.8	17.5 ± 1.4[a]	16.8 ± 2.5[a]
	WS	25.8 ± 2.1	16.7 ± 4.6[a]	13.7 ± 2.8[a]
	PLDδ-def	26.4 ± 0.7	15.5 ± 1.9[a]	14.0 ± 3.7[a]
PC	Col	26.8 ± 1.8	15.7 ± 3.0[a]	17.7 ± 6.1[a]
	PLDα1-def	28.3 ± 2.0	21.4 ± 1.6[a,b]	21.3 ± 4.8[a]
	WS	30.0 ± 1.7	17.1 ± 5.2[a]	20.8 ± 2.9[a]
	PLDδ-def	28.8 ± 1.0	15.9 ± 4.1[a]	17.2 ± 3.5[a]
PE	Col	15.5 ± 0.8	9.8 ± 1.7[a]	12.1 ± 1.1[a]
	PLDα1-def	16.5 ± 1.4	10.1 ± 2.2[a]	12.3 ± 2.3[a]
	WS	17.5 ± 1.74	11.2 ± 2.1[a]	13.5 ± 3.1[a]
	PLDδ-def	16.7 ± 1.5	14.3 ± 5.7[a]	12.0 ± 1.8[a]
PI	Col	5.4 ± 0.3	5.9 ± 0.6	5.1 ± 0.3
	PLDα1-def	4.8 ± 0.3	5.1 ± 0.4	5.2 ± 0.1
	WS	5.1 ± 0.4	5.5 ± 0.5	5.5 ± 0.9
	PLDδ-def	5.1 ± 0.8	4.7 ± 0.5	5.0 ± 0.6
PA	Col	1.9 ± 0.3	10.4 ± 0.9[a]	8.2 ± 1.3[a]
	PLDα1-def	1.2 ± 0.1	4.9 ± 0.3[a,b]	4.9 ± 0.5[a,b]
	WS	1.2 ± 0.3	6.2 ± 2.1[a]	8.3 ± 2.0[a]
	PLDδ-def	1.0 ± 0.3	6.0 ± 0.8[a]	11.2 ± 2.4[a,c]
MGDG	Col	77.0 ± 2.5	71.5 ± 8.3	46.8 ± 4.0[a,c]
	PLDα1-def	77.2 ± 1.3	68.2 ± 4.0[a]	51.0 ± 5.2[a,c]
	WS	81.3 ± 5.0	67.4 ± 1.5[a]	46.2 ± 7.7[a,c]
	PLDδ-def	90.0 ± 5.0	57.5 ± 3.3[a]	33.7 ± 9.1[a,b]
DGDG	Col	35.9 ± 0.9	40.2 ± 4.9	36.6 ± 1.6
	PLDα1-def	36.9 ± 3.2	43.4 ± 8.0	36.1 ± 2.1
	WS	37.8 ± 4.3	42.3 ± 4.3	34.0 ± 3.5[b]
	PLDδ-def	40.8 ± 1.4	30.6 ± 1.6[c]	32.9 ± 4.2

The values are means ± S.D. ($n = 4$ or 5).
[a] The value is different from that of cold acclimation ($p < 0.05$).
[b] The value is different from that of freezing ($p < 0.05$).
[c] The value is different from that of wild type under the same condition ($p < 0.05$).

marker for PG, and its presence in PA suggests that the hydrolysis of PG to PA is a specific response of *Arabidopsis* to post-freezing recovery. Furthermore, because 16:1-containing PG species are specifically produced in plastidic membranes (10), the hydrolysis of 34:4 PG to 34:4 PA indicates that lipid degradation during post-freezing recovery occurs in plastids.

The conclusion that 34:4 PA is derived from 34:4 PG hydrolysis is also supported by quantitative changes in the two lipids during post-freezing recovery (Table 2). Decreases in 34:4 PG were 0.8, 1.5, and 1.6 nmol/mg in Col, WS, and PLDδ-deficient mutant plants, respectively. The decline was matched with increases in 34:4 PA of 0.7, 1.0, and 1.7 nmol/mg for the three genotypes, respectively. However, such changes in 34:4 PG and 34:4 PA levels did not occur in PLDα1-deficient plants. There was no significant change in 34:4 PG in PLDα1-deficient plants (Fig. 2). The increase of 34:4 PA in PLDα1-deficient plants was 0.2 nmol/mg, much lower than that in Col plants (Figs. 2 and 3). These data suggest that the hydrolysis of 34:4 PG to 34:4 PA induced by post-freezing recovery was inhibited by suppression of PLDα1.

These and previous data show that ablation of PLDα1 reduced the loss of PC during freezing and reduced the increase in PA (Fig. 2 and Table 1) (5). PLDα1 was responsible for >50% of the PA produced during freezing (Table 1) (5). Here we show that, during post-freezing recovery, PLDα1 is most likely responsible for the hydrolysis of 34:4 PG to 34:4 PA and that plastidic PG may be an important *in vivo* substrate of PLDα1 during this period.

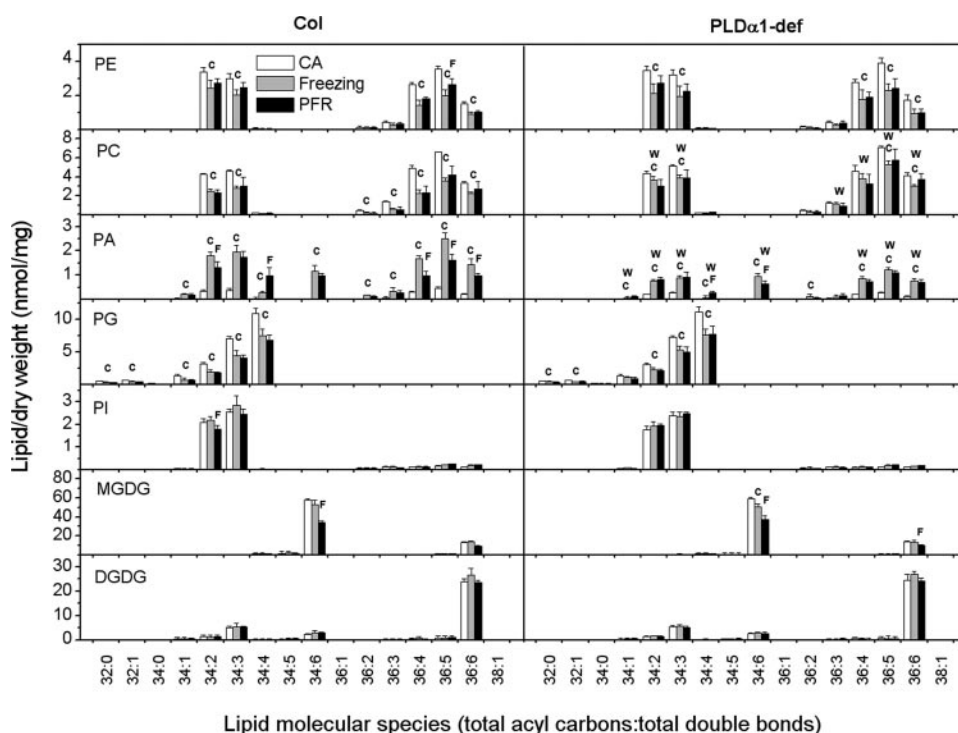

FIGURE 2. **Changes in lipid molecular species during CA, freezing, and PFR in *Arabidopsis*.** *Left panel*, phospholipids and galactolipids of wild-type Col plants; *right panel*, phospholipids and galactolipids of PLDα1-deficient plants. The dry weight is dry weight minus lipid (*i.e.* dry weight after lipid extraction). *White bars* represent cold-acclimated plants, *gray bars* represent frozen plants, and *black bars* represent post-freezing recovery plants. Values are means ± S.D. ($n = 4$ or 5). *C*, the value is different from that of cold acclimation ($p < 0.05$). *F*, the value is different from that of freezing ($p < 0.05$). *W*, the value is different from that of wild type under the same condition ($p < 0.05$).

Ablation of PLDδ Increases Phospholipid Hydrolysis during Post-freezing Recovery—Lipid profiling, combined with genetic manipulation, shows that PLDα1 and -δ have different functions in lipid metabolism during freezing and post-freezing recovery. Although PLDα1 is active in both freezing and thawing phases, hydrolyzing membrane lipids under subzero temperature stress, ablation of PLDδ had only a small effect on PA levels during freezing (Fig. 3 and Table 1) (7). However, ablation of PLDδ led to a large increase in PA levels during post-freezing recovery (Fig. 3 and Table 1). From freezing to post-freezing recovery, the PA level in PLDδ-deficient plants increased >85%, from 6.0 to 11.2 nmol/mg, whereas the change in the PA level of WS plants was insignificant (Table 1). The 34- and 36-carbon acyl composition is consistent with PG, PC, and/or PE as sources of the PA formed in PLDδ-deficient plants. These data show that lipid degradation continued during post-freezing recovery and suggest that PLDδ is a negative regulator of PA production.

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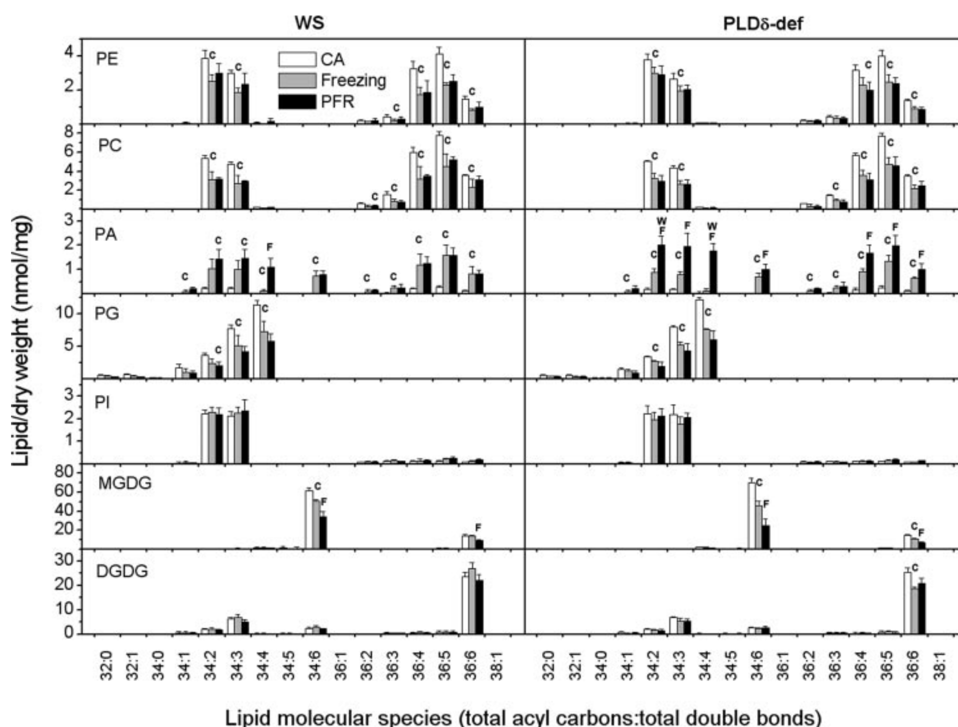

FIGURE 3. **Changes in lipid molecular species during CA, freezing, and PFR in *Arabidopsis*.** *Left panel*, phospholipids and galactolipids of wild-type WS plants; *right panel*, phospholipids and galactolipids of PLDδ-deficient plants. The dry weight is dry weight minus lipid (*i.e.* dry weight after lipid extraction). *White bars* represent cold-acclimated plants, *gray bars* represent frozen plants, and *black bars* represent post-freezing recovery plants. Values are means ± S.D. ($n = 4$ or 5). *C*, the value is different from that of cold acclimation ($p < 0.05$). *F*, the value is different from that of freezing ($p < 0.05$). *W*, the value is different from that of wild type under the same condition ($p < 0.05$).

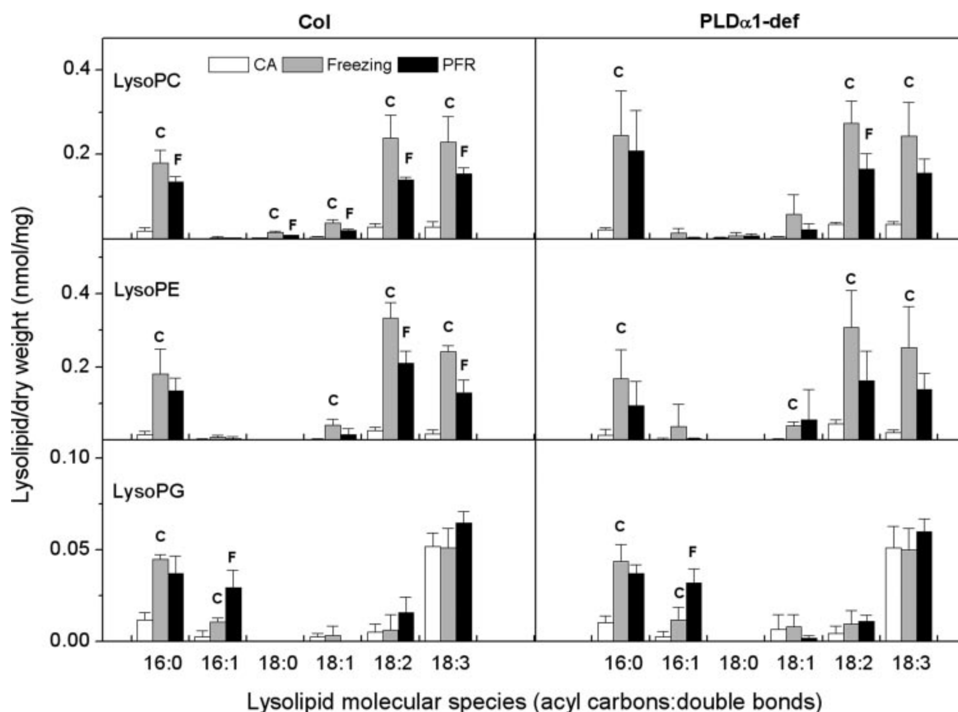

FIGURE 4. **Changes in lysophospholipid molecular species during CA, freezing, and PFR in *Arabidopsis*.** *Left panel*, phospholipids and galactolipids of wild-type Col plants; *right panel*, lysophospholipids of PLDα1-def plants. The dry weight is dry weight minus lipid (*i.e.* dry weight after lipid extraction). *White bars* represent cold-acclimated plants, *gray bars* represent frozen plants, and *black bars* represent post-freezing recovery plants. Values are means ± S.D. ($n = 4$ or 5). *C*, the value is different from that of cold acclimation ($p < 0.05$). *F*, the value is different from that of freezing ($p < 0.05$). *W*, the value is different from that of wild type under the same condition ($p < 0.05$).

Lipids Other Than Diacylphospholipids Are Also Altered in Post-freezing Recovery—The level of MGDG decreased substantially in all genotypes during post-freezing recovery. Ablation of PLDα1 did not affect the extent of decrease. The loss of MGDG in PLDδ-deficient plants tended to be more severe than that in other genotypes. The result is consistent with the notion that there is more lipid hydrolysis overall in the PLDδ-deficient genotype. However, although MGDG is apparently converted to PA during freezing as evidenced by the formation of otherwise non-existent 34:6 PA (Figs. 2 and 3) (5), net formation of PA from hydrolyzed MGDG was not apparent during post-freezing recovery (Figs. 2 and 3). Changes in levels of DGDG were smaller than those of MGDG and were not significantly affected by deficiency of either PLD (Table 1 and Fig. 2).

Lysophospholipids are minor phospholipid species in *Arabidopsis*. LysoPC and lysoPE levels increase severalfold during freezing in Col and PLDα1-deficient plants (5). In this study, lysoPG, as well as lysoPC and lysoPE, was quantified during cold acclimation, freezing, and post-freezing recovery (Figs. 4 and 5 and Table 3). During freezing, all molecular species of lysophospholipids except 18:3 lysoPG increased significantly in Col, WS, PLDα1-deficient, and PLDδ-deficient plants (Figs. 4 and 5). Ablation of PLDδ tended to result in greater lysophospholipid increases during post-freezing recovery, with significant increases occurring in several lysoPC species and in 16:1 lysoPG (Fig. 5). 16:1 LysoPG may be a second hydrolysis product of 34:4 PG, formed by PLA action.

Expression of PLDs and Lipid Biosynthetic Genes during Post-freezing Recovery—The transcript levels of 12 members of *PLD* family in *Arabidopsis* and 34 genes involved in lipid biosynthesis were profiled by microarray during cold acclimation and post-freezing recovery (Table 4 and supplemental materials (Table

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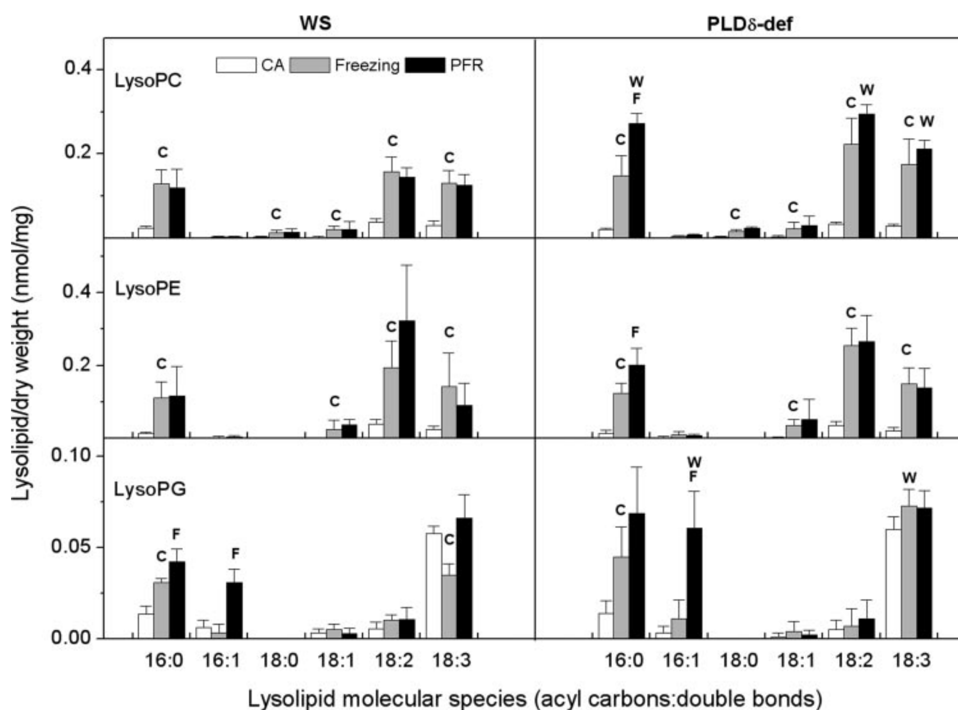

FIGURE 5. **Changes in lysophospholipid molecular species during CA, freezing, and PFR in *Arabidopsis*.** *Left panel*, lysophospholipid of wild-type WS plants; *right panel*, lysophospholipids of PLDδ-deficient plants. The dry weight is dry weight minus lipid (*i.e.* dry weight after lipid extraction). *White bars* represent cold-acclimated plants, *gray bars* represent frozen plants, and *black bars* represent post-freezing recovery plants. Values are means ± S.D. ($n = 4$ or 5). *C*, the value is different to that of cold acclimation ($p < 0.05$). *F*, the value is different from that of freezing ($p < 0.05$). *W*, the value is different from that of wild type under the same condition ($p < 0.05$).

S1)). Ratios of expression in post-freezing recovery *versus* cold acclimation range from 0.7 to 2.0, except for *PLDβ1* (ratio of 5.4). Specifically for *PLDα1* and *PLDδ*, the ratios were 1.7 and 2.0, respectively (Table 4). Of the genes involved in the biosynthesis of phospholipids and galactolipids examined, CTP:phosphocholine cytidylyltransferase, which catalyzes a regulatory step in PC synthesis exhibited the largest increase (4.6-fold). This indicates an increase in PC production during the post-freezing recovery. The result is consistent with lipid analysis showing that the amounts of phospholipids increased in the post-freezing phase (supplemental Table S1).

DISCUSSION

The biochemical and molecular processes by which plants respond to cold acclimation have been extensively investigated. Changes in a large number of genes and metabolites have been observed in *Arabidopsis* (12, 13). Membranes are initial sites of temperature sensing and major sites of freezing injury (4, 14). During cold acclimation, membrane phospholipids accumulate (5); this is speculated to enhance membrane fluidity and is thought to be an important strategy in increasing plant freezing tolerance (2, 3, 4, 14). During freezing, more drastic lipid changes take place, perhaps in response to severe injury caused by the formation of extracellular and intercellular ice (4). In *Arabidopsis*, PC, PE, and PG decrease, whereas their metabolites PA and lysophospholipids increase (5). During post-freezing recovery, the degradation of extraplastidic lipids PC and PE ceases but the loss of plastidic lipids, particularly MGDG and plastidically localized PG, increases. DGDG tends to be more stable than MGDG and plastidic PG, which could mean that thylakoid lipids are more susceptible to degradation than chloroplast envelope lipids. These distinctive changes point to specific temporal and spatial activation of lipolytic enzymes during freezing and post-freezing recovery. Whereas phospholipases, including PLDs and PLAs, are activated during freezing, a large increase in galactolipase activity occurs during post-freezing recovery.

The damage to plastidic membranes during thawing could negatively impact plant recovery and survival, because thylakoid membranes are the site of the light-dependent reactions of photosynthesis. Photoinhibition in chilling-sensitive plants, such as cotton, soybeans, and cucumbers, is closely related to the lipid composition of chloroplast membranes, especially to the composition and amount of PG species (15). Suppression of PG synthesis leads to impairment of photosynthesis (16). In this study, we observed that the plastidic 34:4 PG species appears to be degraded by PLDα1-mediated hydrolysis and, to a lesser

TABLE 2

Amount of 34:4 PG and 34:4 PA species and comparison of amount of 34:4 PG and 34:4 PA species between freezing and PFR

The dry weight is dry weight minus lipid.

Genotype	Lipid species	Lipid/dry weight		
		Lipid after freezing	Lipid after PFR	Lipid change
		nmol/mg dry weight		
Col	34:4 PG	7.5 ± 1.0	6.8 ± 0.8	−0.8
	34:4 PA	0.30 ± 0.05	0.98 ± 0.34	+0.7
PLDα1-def	34:4 PG	7.6 ± 0.9	7.7 ± 1.3	+0.1
	34:4 PA	0.07 ± 0.08	0.29 ± 0.05	+0.2
WS	34:4 PG	7.3 ± 1.6	5.7 ± 1.1	−1.5
	34:4 PA	0.15 ± 0.04	1.1 ± 0.36	+1.0
PLDδ-def	34:4 PG	7.6 ± 0.1	6.0 ± 1.3	−1.6
	34:4 PA	0.12 ± 0.09	1.8 ± 0.3	+1.7

TABLE 3

Total amount of lysophospholipids in each head group class during CA, freezing, and PFR in Col, PLDα1-deficient, WS, and PLDδ-KO plants

The dry weight is dry weight minus lipid.

Lipid class	Genotype	CA	Freezing	PFR
			nmol/mg dry weight	
LysoPC	Col	0.09 ± 0.03	0.71 ± 0.14[a]	0.50 ± 0.09[a,b]
	PLDα1-def	0.10 ± 0.01	0.84 ± 0.12[a]	0.56 ± 0.12[a,b]
	WS	0.10 ± 0.02	0.57 ± 0.28[a]	0.43 ± 0.10[a]
	PLDδ-def	0.09 ± 0.01	0.79 ± 0.07[a]	0.84 ± 0.06[a,c]
LysoPE	Col	0.06 ± 0.02	0.80 ± 0.10[a]	0.5 ± 0.09[a,b]
	PLDα1-def	0.08 ± 0.01	0.80 ± 0.24[a]	0.45 ± 0.22[a,b]
	WS	0.09 ± 0.02	0.46 ± 0.10[a]	0.57 ± 0.13[a]
	PLDδ-def	0.07 ± 0.01	0.6 ± 0.17[a]	0.67 ± 0.09[a]
LysoPG	Col	0.07 ± 0.01	0.12 ± 0.02[a]	0.15 ± 0.03[a]
	PLDα1-def	0.07 ± 0.02	0.12 ± 0.01[a]	0.14 ± 0.01[a]
	WS	0.09 ± 0.01	0.09 ± 0.03[a]	0.15 ± 0.02[a,b]
	PLDδ-def	0.08 ± 0.01	0.16 ± 0.05[a]	0.21 ± 0.05[a,c]

[a] The value is different from that of cold acclimation ($p < 0.05$).
[b] The value is different from that of freezing ($p < 0.05$).
[c] The value is different from that of wild type under the same condition ($p < 0.05$).

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TABLE 4

The ratio of *PLD* expression in PFR *versus* CA in *Arabidopsis* (Col)

Expression profiling used microarrays from Affymetrix. Data were processed by using Genespring version 7.2. The values are the average of three replicates from independent plants.

Gene name	Ratio of expression in PFR *versus* CA	Transcript ID (array design)
PLDα1	1.7	At3g15730
PLDα2	1.0	At1g52570
PLDα3	1.2	At5g25370
PLDα4	0.9	At1g55180
PLDβ1	5.4	At2g42010
PLDβ2	0.9	At4g00240
PLDγ1	2.1	At4g11850
PLDγ2	1.1	At4g11830
PLDγ3	1.6	At4g11840
PLDδ	2.0	At4g35790
PLDζ1	1.3	At3g16785
PLDζ2	0.7	At3g05630

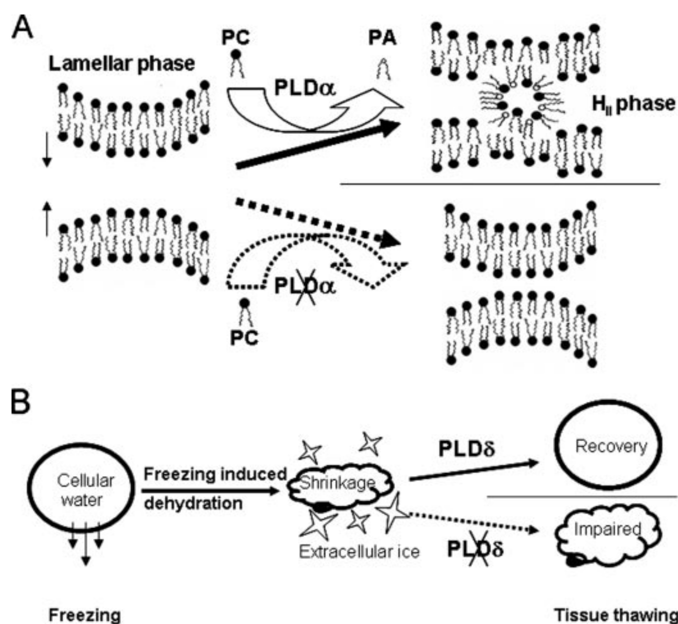

FIGURE 6. **Working model for freezing tolerance in PLDα1-deficient plants and for freezing sensitivity in PLDδ-deficient plants.** *A*, the molecular shape of PC is a *cylinder*; the molecular shape of PA is a *cone*. Suppression of PLDα1 results in a low ratio of PA to PC. A low ratio of PA to PC reduces the propensity for formation of non-lamellar phase, hexagonal II phase, and thus enhances plant tolerance to freezing. *B*, the negative effects of the PA increase and reactive oxygen species in PLDδ-deficient plants may reduce the recovery of cells from freezing-induced damage.

extent, by PLA action. PLDα1-catalyzed degradation occurred only during the post-freezing recovery phase, indicating that chloroplasts lose their membrane integrity during the thawing process, exposing PG to the lipolytic activity. Previously, *in vitro* studies showed that PLDα1 used PG as substrate (20).

PLDα1 and PLDδ have distinctively different biochemical properties, subcellular associations, and gene expression patterns (11). *Arabidopsis* abrogated of *PLDα1* and *Arabidopsis* abrogated of *PLDδ* display opposite responses to freezing: Whereas PLDα1-deficient plants are more tolerant to freezing, PLDδ deficiency renders plants more sensitive to freezing (5, 7). It has been proposed that high PLDα1 activity destabilizes membranes and increases membrane leakage (Fig. 6*A*), whereas PLDδ produces signaling PA and mitigates stress damage inflicted by reactive oxygen species (7, 18). *PLDδ* alterations do not result in changes in the expression of the cold-regulated genes *COR47* or *COR78*, nor in any change in cold-induced increases in the levels of compatible osmolytes, proline, or soluble sugars, which are known to play a role in plant freezing tolerance. These results suggest that PLDs and associated membrane lipid hydrolysis are components of a signaling pathway involved in mediating plant freezing tolerance. However, the precise mechanism by which PLDδ positively regulates the freezing response remains to be elucidated.

Lipid profiling data indicate that PLDδ is not a major contributor to the large decline of membrane lipids during freezing. PLDδ-deficient plants produced almost the same amount of PA as did the WS control during freezing. During post-freezing recovery, however, the loss of PLDδ was associated with an increase in PA production. The increased amount of PA formed includes the 34:4 species, which is likely to originate from plastidic PG. PE and/or PC may be the source of 36-carbon PA species observed. The increase in PA levels may produce non-lamellar phase membrane lipid and/or might reduce programmed cell death induced by reactive oxygen species H_2O_2; this has been demonstrated to occur when PA formation is catalyzed directly by PLDδ (7, 18). In addition, the higher levels of lysophospholipids in PLDδ-deficient plants could cause physical damage to the membranes because of their detergent-like properties. It is interesting to note that lysoPE has been reported to be an inhibitor of PLD (21). It might be possible that PLDδ-derived PA inhibits the function of PLA. Based on the lipid changes, we propose that the increased freezing sensitivity of PLDδ-deficient plants is due to the higher level of lipid hydrolysis in PLDδ-deficient plants. Other data suggest that an additional mechanism of PLDδ action might be membrane stabilization. PLDδ may stabilize cell membranes through its interaction with the cytoskeleton (22). PLDδ binds to tubulin, and activation of PLD is thought to be critical in triggering microtubule reorganization (23). The dynamics of microtubules are important in plant response to a variety of stresses, including temperature (24). The loss of the role of PLDδ in cytoskeletal reorganization may contribute to increased lipid hydrolysis and decreased membrane stability, thus reducing freezing tolerance in PLDδ-deficient plants (Fig. 6).

In summary, the present data reveal that, in plant response to freezing, distinctively different changes occur in freezing and post-freezing recovery. During freezing, most lipid hydrolysis occurs in extraplastidic phospholipids, but during post-freezing recovery, lipid hydrolysis occurs mainly in plastidic lipids. In addition, this study indicates that the presence of PLDα1 is correlated with phospholipid hydrolysis during both freezing and post-freezing phases, but the presence of PLDδ is not. In contrast, the presence of PLDδ is associated with a positive effect on freezing tolerance and reduced hydrolysis of both plastidic and non-plastidic lipids during post-freezing recovery.

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Differential Degradation of Extraplastidic and Plastidic Lipids during Freezing and Post-freezing Recovery in *Arabidopsis thaliana*

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