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Quantitative profiling of polar glycerolipid species from organs of wild-type Arabidopsis and a *PHOSPHOLIPASE Dα1* knockout mutant

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Abstract

Lipid profiling is a targeted metabolomics platform that provides a comprehensive analysis of lipid species with high sensitivity. Profiling based on electrospray ionization tandem mass spectrometry (ESI-MS/MS) provides quantitative data and is adaptable to high throughput analyses. Here we report the profiling of 140 apparent molecular species of polar glycerolipids in Arabidopsis leaves, flower stalks, flowers, siliques, roots, and seeds. Considerable differences in lipid species occur among these organs, providing insights into the different lipid metabolic activities in a specific organ. In addition, comparative profiling between wild-type and a knockout mutant *pldα1* (locus ID: AT3G15730) provides insight into the metabolic function of phospholipase D (PLD) in different organs. PLDα1 contributes significantly to phosphatidic acid (PA) levels in roots, seeds, flowers, and flower stalks, but little to basal PA levels in siliques and leaves. In seeds of the *pldα1* mutant plants, levels of PA, lysophosphatidylcholine, and lysophosphatidylethanolamine were significantly lower than those of wild-type seeds, suggesting a role for PLDα1 in membrane lipid degradation in seeds.
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Keywords: *Arabidopsis thaliana*; Cruciferae; Electrospray ionization tandem mass spectrometry; Galactolipid species; Membrane lipids; Lipid profiling; Phospholipid species; Phospholipase D; Phospholipase A; Phosphatidic acid; Lipidomics

1. Introduction

Cellular lipids play pivotal roles in cell structure, metabolism, and regulation. Lipids are structurally diverse and metabolically dynamic. Changes in specific lipid metabolic steps or specific lipid species have been implicated in plant growth, development, and responses to biotic and abiotic stresses. Because lipids are major sources of biomaterials for various food, medicine, and industrial uses, lipid metabolism is a major target for manipulation by metabolic engineering. To facilitate the understanding of lipid metabolism and lipid functions in the cell, the development of comprehensive analytical strategies has gained increasing attention in recent years (Brügger et al., 1997; Welti et al., 2002; Forrester et al., 2004; Welti and Wang, 2004; Han and Gross, 2005; Wenk, 2005). This has promoted the emergence of lipidomics, which is becoming an integral part of functional genomics. Lipid profiling is a targeted metabolomics platform that aims at comprehensive analysis of lipid species. A profiling strategy based on electrospray tandem mass spectrometry (ESI-MS/MS) yields quantitative data, and is adaptable to high throughput analyses (Welti and Wang, 2004; Han and Gross, 2005). Lipid profiling data can potentially be utilized to define lipid metabolic pathways, roles of specific genes/enzymes in cellular and organismal functions, and lipid species that serve as substrates and products of specific enzymes (Welti et al., 2002; Li et al., 2004).

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Phospholipase D (PLD), which hydrolyzes phospholipids to generate phosphatidic acid (PA), is the major phospholipase activity in plants. Arabidopsis has 12 PLD genes, and the various PLD gene products are regulated differentially by Ca^{2+}, polyphosphoinositides and free fatty acids and display distinguishable substrate selectivities and specificities, suggesting that they are activated in response to different cues and may have unique cellular functions (Wang, 2002, 2004). The detailed cellular and physiological functions of the individual PLD gene products are beginning to be elucidated. Activation of PLD activity constitutes an important and early step in stress-induced phospholipid hydrolysis (Zien et al., 2001; Welti et al., 2002; Meijer and Munnik, 2003; Wang, 2002, 2004). PLDα1 (locus ID: AT3G15730) is the most abundant PLD in Arabidopsis, and this enzyme plays roles in cell signaling and membrane lipid metabolism in response to stresses, including wounding, freezing, water deficit, and pathogen elicitation. During wounding and freezing stresses, antisense suppression of PLDα1 has been shown to reduce the phospholipid hydrolysis that occurs in leaves by about half (Zien et al., 2001; Welti et al., 2002). In response to abscisic acid, PLDα1 is activated to produce PA that interacts with a protein phosphatase and G protein to mediate stomatal movements (Zhang et al., 2004; Mishra et al., 2006). However, it is unknown whether genetic abrogation of PLDα1 affects the basal membrane lipid composition.

This study was undertaken with two specific objectives. One is to analyze glycerolipid molecular species in different Arabidopsis organs to determine how the lipid species are quantitatively and qualitatively different among various plant organs. The other objective is to compare the glycerolipid profiles in different organs between wild-type plants and a *PHOSPHOLIPASE Dα1* knockout mutant, *pldα1*, to determine the effect of this enzyme on steady-state membrane lipid composition. Here we report quantitative analysis of 140 apparent molecular species of polar glycerolipids in several Arabidopsis organs of wild-type and *pldα1* plants.

2. Results and discussion

2.1. Lipid classes of various plant organs

Lipid profiling using ESI-MS/MS involves direct infusion of lipid extracts into a mass spectrometer and thus requires only simple sample preparation. Small amounts of samples were needed to identify and quantify membrane glycerolipid species. Routine profiling provides information on phospholipids and glycolipids speciated to the level of head group and number of carbon atoms and double bonds present in the acyl chains. The present analysis identified 140 polar plant membrane glycerolipid apparent molecular species of the major phospholipid classes [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylinositol (PI)], galactolipid classes [monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG)], and minor classes [phosphatidylserine (PS), phosphatidic acid (PA), lysophophatidylcholine (lyso PC), lysophosphatidylethanolamine (lyso PE), and lysophosphatidylglycerol (lyso PG)], as well as minor acyl species within each head group class. Table 1 shows the apparent molecular species in each of these classes. Anions corresponding to the molecular ions in leaf extracts were subjected to product ion analysis to identify the true molecular species and these are also indicated in Table 1. Although the Arabidopsis leaf molecular species of sulfoquinovosyldiacylglycerol (SQDG) are listed in Table 1, due to difficulty in obtaining internal standards, SQDG, a polar glycerolipid class present at levels of several nmol/mg dry weight, was not analyzed further in this study. Examples of the structures of lipids from each class are shown in Fig. 1.

Determination of total polar lipids in wild-type Arabidopsis organs indicated that leaves and flowers had the highest amounts of polar lipid, normalized to organ dry weight, while roots had the least (Table 2). The flowers were the only organ in which the wild-type genotype had a significantly higher level of total polar lipids than the mutant genotype (at $P < 0.05$) (Table 2). There were considerable differences in the patterns of lipid classes present in different plant organs (Fig. 2 and Supplementary data). The lipids that are largely synthesized and localized in plastids, PG including lyso PG, MGDG and DGDG, were most abundant in leaves (Fig. 2), accounting for 75% of the polar lipids analyzed in wild-type plants. PG, MGDG, and DGDG were also abundant in flower stalks (61% of total polar lipids), flowers (43%), and siliques (51%). In contrast, PG, MGDG, and DGDG in seeds (6%) and roots (6%) were much lower, as would be expected in these non-photosynthetic organs. DGDG has been detected at levels ranging from 1% to 5% of plasma membrane lipids in plants grown under normal (not nutrient-limited) conditions (Andersson et al., 2005 and references cited therein). Since DGDG-containing membranes represent only a fraction of the extra-plastidic membranes, DGDG is expected to be found in very small amounts outside the plastids. Although MGDG and DGDG are strongly localized in the plastid under most physiological conditions, in leaves, about 15% of the PG is extra-plastidic (Miquel et al., 1998). The level of PG as a percentage of PG + MGDG + DGDG is between 17% and 22% in leaves, flower stalks, flowers, and siliques, but PG represents 38% of PG + MGDG + DGDG in roots and 55% in seeds. If PG makes up a similar fraction of the plastidic lipids in different organs, these data would suggest that a larger fraction of the PG is located outside the plastid in roots and seeds than in leaves and other photosynthetic organs.

Although the percentage of the total polar lipids represented by the extra-plastidically synthesized lipid classes (PC, lyso PC, PE, lyso PE, PI, and PS) in different organs varied from 23% to 93% of the measured lipids, the absolute

Table 1
Complex polar lipids of Arabidopsis

Apparent lipid molecular species (total acyl carbons: total double bonds)	Compound number	Formula	Nominal mass used in lipid profiling	Mass used for product ion analysis	Lipid molecular species identified in Arabidopsis leaves
			Mass of [M+H]$^+$	*Mass of [M+OAc]$^-$*	
32:0 PC	1	$C_{40}H_{80}0_8PN$	734	792	n.d.
34:4 PC	2	$C_{42}H_{76}O_8PN$	754	812	16:1–18:3 (2a)
34:3 PC	3	$C_{42}H_{78}O_8PN$	756	814	16:0–18:3 (3a)
34:2 PC	4	$C_{42}H_{80}O_8PN$	758	816	16:0–18:2 (4a)
34:1 PC	5	$C_{42}H_{82}O_8PN$	760	818	n.d.
36:6 PC	6	$C_{44}H_{76}O_8PN$	778	836	18:3–18:3 (6a)
36:5 PC	7	$C_{44}H_{78}O_8PN$	780	838	18:3–18:2 (7a)
36:4 PC	8	$C_{44}H_{80}O_8PN$	782	840	18:2–18:2 (8a) > 18:1–18:3 (8b)
36:3 PC	9	$C_{44}H_{82}O_8PN$	784	842	18:1–18:2 (9a) > 18:0–18:3 (9b)
36:2 PC	10	$C_{44}H_{84}O_8PN$	786	844	18:0–18:2 (10a) > 18:1–18:1 (10b)
36:1 PC	11	$C_{44}H_{86}O_8PN$	788	846	n.d.
38:6 PC	12	$C_{46}H_{80}O_8PN$	806	864	n.d.
38:5 PC	13	$C_{46}H_{82}O_8PN$	808	866	20:2–18:3 (13a)
38:4 PC	14	$C_{46}H_{84}O_8PN$	810	868	20:1–18:3 (14a) > 20:2–18:2 (14b)
38:3 PC	15	$C_{46}H_{86}O_8PN$	812	870	20:1–18:2 (15a) > 20:0–18:3 (15b)
38:2 PC	16	$C_{46}H_{88}O_8PN$	814	872	n.d.
40:5 PC	17	$C_{48}H_{86}O_8PN$	836	894	n.d.
40:4 PC	18	$C_{48}H_{88}O_8PN$	838	896	n.d.
40:3 PC	19	$C_{48}H_{90}O_8PN$	840	898	22:0–18:3 (19b)
40:2 PC	20	$C_{48}H_{92}O_8PN$	842	900	22:1–18:1 (20a), 22:0–18:2 (20b)
			Mass of [M+H]$^+$	*Mass of [M−H]$^-$*	
34:4 PE	21	$C_{39}H_{70}O_8PN$	712	710	16:1–18:3 (21a)
34:3 PE	22	$C_{39}H_{72}O_8PN$	714	712	16:0–18:3 (22a) > 16:1–18:2 (22b)
34:2 PE	23	$C_{39}H_{74}O_8PN$	716	714	16:0–18:2 (23a)
34:1 PE	24	$C_{39}H_{76}O_8PN$	718	716	16:0–18:1(24a)
36:6 PE	25	$C_{41}H_{70}O_8PN$	736	734	18:3–18:3 (25a)
36:5 PE	26	$C_{41}H_{72}O_8PN$	738	736	18:3–18:2 (26a)
36:4 PE	27	$C_{41}H_{74}O_8PN$	740	738	18:2–18:2 (27a) > 18:1–18:3 (27b)
36:3 PE	28	$C_{41}H_{76}O_8PN$	742	740	18:0–18:3 (28a), 18:1–18:2 (28b)
36:2 PE	29	$C_{41}H_{78}O_8PN$	744	742	18:0–18:2 (29a), 18:1–18:1 (29b)
36:1 PE	30	$C_{41}H_{80}O_8PN$	746	744	n.d.
38:6 PE	31	$C_{43}H_{74}O_8PN$	746	744	n.d.
38:5 PE	32	$C_{43}H_{76}O_8PN$	766	764	20:2–18:3 (32a)
38:4 PE	33	$C_{43}H_{79}O_8PN$	768	766	20:1–18:3 (33a), 20:2–18:2 (33b)
38:3 PE	34	$C_{43}H_{80}O_8PN$	770	768	20:1–18:2 (34a) > 20:0–18:3 (34b)
38:2 PE	35	$C_{43}H_{82}O_8PN$	772	770	20:0–18:2 (35a)
40:3 PE	36	$C_{45}H_{84}O_8PN$	798	796	22:0–18:3 (36a)
40:2 PE	37	$C_{45}H_{86}O_8PN$	800	798	22:0–18:2 (37a)
42:4 PE	38	$C_{47}H_{86}O_8PN$	824	822	24:1–18:3 (38a)
42:3 PE	39	$C_{47}H_{88}O_8PN$	826	824	24:1–18:2 (39a) > 24:0–18:3 (39b)
42:2 PE	40	$C_{47}H_{90}O_8PN$	828	826	24:0–18:2 (40a)
			Mass of [M−H]$^-$	*Mass of [M−H]$^-$*	
34:4 PI	41	$C_{43}H_{75}O_{13}P$	829	829	16:1–18:3 (41a)
34:3 PI	42	$C_{43}H_{77}O_{13}P$	831	831	18:3–16:0 (42a)
34:2 PI	43	$C_{43}H_{79}O_{13}P$	833	833	16:0–18:2 (43a)
34:1 PI	44	$C_{43}H_{81}O_{13}P$	835	835	16:0–18:1 (44a)
36:6 PI	45	$C_{45}H_{75}O_{13}P$	853	853	18:3–18:3 (45a)
36:5 PI	46	$C_{45}H_{77}O_{13}P$	855	855	18:2–18:3 (46a)
36:4 PI	47	$C_{45}H_{79}O_{13}P$	857	857	18:2–18:2 (47a), 18:1–18:3 (47b)
36:3 PI	48	$C_{45}H_{81}O_{13}P$	859	859	18:1–18:2 (48a), 18:0–18:3 (48b)
36:2 PI	49	$C_{45}H_{83}O_{13}P$	861	861	18:2–18:0 (49a)
36:1 PI	50	$C_{45}H_{85}O_{13}P$	863	863	18:0–18:1 (50a)
			Mass of [M−H]$^-$	*Mass of [M−H]$^-$*	
34:6 PA	51	$C_{37}H_{61}O_8P$	663	663	18:3–16:3
34:4 PA	52	$C_{37}H_{65}O_8P$	667	667	18:3–16:1 (52a)
34:3 PA	53	$C_{37}H_{67}O_8P$	669	669	18:3–16:0 (53a) > 18:2–16:1 (53b)
34:2 PA	54	$C_{37}H_{69}O_8P$	671	671	n.d.
34:1 PA	55	$C_{37}H_{71}O_8P$	673	673	n.d.
36:6 PA	56	$C_{39}H_{65}O_8P$	691	691	18:3–18:3 (56a)

(*continued on next page*)

Table 1 (*continued*)

Apparent lipid molecular species (total acyl carbons: total double bonds)	Compound number	Formula	Nominal mass used in lipid profiling	Mass used for product ion analysis	Lipid molecular species identified in Arabidopsis leaves
36:5 PA	57	$C_{39}H_{67}O_8P$	693	693	18:2–18:3 (57a)
36:4 PA	58	$C_{39}H_{69}O_8P$	695	695	18:2–18:2 (58a) > 18:3–18:1 (58b)
36:3 PA	59	$C_{39}H_{71}O_8P$	697	697	18:2–18:1 (59a), 18:0–18:3 (59b)
36:2 PA	60	$C_{39}H_{73}O_8P$	699	699	18:0–18:2 (60a) > 18:1–18:1 (60b)
			Mass of $[M-H]^-$	*Mass of* $[M-H]^-$	
34:4 PS	61	$C_{40}H_{70}O_{10}PN$	754	754	n.d.
34:3 PS	62	$C_{40}H_{72}O_{10}PN$	756	756	n.d.
34:2 PS	63	$C_{40}H_{74}O_{10}PN$	758	758	n.d.
34:1 PS	64	$C_{40}H_{76}O_{10}PN$	760	760	n.d.
36:6 PS	65	$C_{42}H_{70}O_{10}PN$	778	778	n.d.
36:5 PS	66	$C_{42}H_{72}O_{10}PN$	780	780	n.d.
36:4 PS	67	$C_{42}H_{74}O_{10}PN$	782	782	n.d.
36:3 PS	68	$C_{42}H_{76}O_{10}PN$	784	784	n.d.
36:2 PS	69	$C_{42}H_{78}O_{10}PN$	786	786	n.d.
36:1 PS	70	$C_{42}H_{80}O_{10}PN$	788	788	n.d.
38:6 PS	71	$C_{44}H_{74}O_{10}PN$	806	806	n.d.
38:5 PS	72	$C_{44}H_{76}O_{10}PN$	808	808	n.d.
38:4 PS	73	$C_{44}H_{78}O_{10}PN$	810	810	n.d.
38:3 PS	74	$C_{44}H_{80}O_{10}PN$	812	812	n.d.
38:2 PS	75	$C_{44}H_{82}O_{10}PN$	814	814	n.d.
38:1 PS	76	$C_{44}H_{84}O_{10}PN$	816	816	n.d.
40:4 PS	77	$C_{46}H_{82}O_{10}PN$	838	838	n.d.
40:3 PS	78	$C_{46}H_{84}O_{10}PN$	840	840	n.d.
40:2 PS	79	$C_{46}H_{86}O_{10}PN$	842	842	n.d.
40:1 PS	80	$C_{46}H_{88}O_{10}PN$	844	844	n.d.
42:4 PS	81	$C_{48}H_{86}O_{10}PN$	866	866	24:1–18:3 (81a)
42:3 PS	82	$C_{48}H_{88}O_{10}PN$	868	868	24:0–18:3 (82a), 24:1–18:2 (82b)
42:2 PS	83	$C_{48}H_{90}O_{10}PN$	870	870	24:0–18:2 (83a)
42:1 PS	84	$C_{48}H_{92}O_{10}PN$	872	872	n.d.
44:3 PS	85	$C_{50}H_{92}O_{10}PN$	896	896	26:0–18:3 (85a)
44:2 PS	86	$C_{50}H_{94}O_{10}PN$	898	898	26:0–18:2 (86a)
			Mass of $[M+H]^+$	*Mass of* $[M+OAc]^-$	
16:1 LysoPC	87	$C_{24}H_{48}O_7PN$	494	552	16:1
16:0 LysoPC	88	$C_{24}H_{50}O_7PN$	496	554	16:0
18:3 LysoPC	89	$C_{26}H_{48}O_7PN$	518	576	18:3
18:2 LysoPC	90	$C_{26}H_{50}O_7PN$	520	578	18:2
18:1 LysoPC	91	$C_{26}H_{52}O_7PN$	522	580	18:1
18:0 LysoPC	92	$C_{26}H_{54}O_7PN$	524	582	n.d.
			Mass of $[M+H]^+$	*Mass of* $[M-H]^-$	
16:1 LysoPE	93	$C_{21}H_{42}O_7PN$	452	450	n.d.
16:0 LysoPE	94	$C_{21}H_{44}O_7PN$	454	452	16:0
18:3 LysoPE	95	$C_{23}H_{42}O_7PN$	476	474	18:3
18:2 LysoPE	96	$C_{23}H_{44}O_7PN$	478	476	18:2
18:1 LysoPE	97	$C_{23}H_{46}O_7PN$	480	478	18:1
			Mass of $[M-H]^-$	*Mass of* $[M-H]^-$	
16:1 LysoPG	98	$C_{22}H_{43}O_9P$	481	481	n.d.
16:0 LysoPG	99	$C_{22}H_{45}O_9P$	483	483	16:0
18:3 LysoPG	100	$C_{24}H_{43}O_9P$	505	505	18:3
18:2 LysoPG	101	$C_{24}H_{45}O_9P$	507	507	18:2
18:1 LysoPG	102	$C_{24}H_{47}O_9P$	509	509	18:1
			Mass of $[M-H]^-$	*Mass of* $[M-H]^-$	
32:1 PG	103	$C_{38}H_{73}O_{10}P$	719	719	16:1–16:0 (103a)
32:0 PG	104	$C_{38}H_{75}O_{10}P$	721	721	16:0–16:0 (104a)
34:4 PG	105	$C_{40}H_{71}O1_0P$	741	741	16:1–18:3 (105a)
34:3 PG	106	$C_{40}H_{73}O_{10}P$	743	743	18:3–16:0 (106a) > 16:1–18:2 (106b)
34:2 PG	107	$C_{40}H_{75}O_{10}P$	745	745	18:2–16:0 (107a), 16:1–18:1 (107b)
34:1 PG	108	$C_{40}H_{77}O_{10}P$	747	747	18:1–16:0 (108a) > 16:1–18:0 (108b)
34:0 PG	109	$C_{40}H_{79}O_{10}P$	749	749	18:0–16:0 (109b)

Table 1 (*continued*)

Apparent lipid molecular species (total acyl carbons: total double bonds)	Compound number	Formula	Nominal mass used in lipid profiling	Mass used for product ion analysis	Lipid molecular species identified in Arabidopsis leaves
			Mass of [M+Na]$^+$	*Mass of [M+OAc]$^-$*	
34:6 MGDG	110	$C_{43}H_{70}O_{10}$	769	805	18:3–16:3 (110a)
34:5 MGDG	111	$C_{43}H_{72}O_{10}$	771	807	18:3–16:2 (111a), 18:2–16:3 (111b)
34:4 MGDG	112	$C_{43}H_{74}O_{10}$	773	809	18:3–16:1 (112a), 18:2–16:2 (112b), 18:1–16:3 (112c)
34:3 MGDG	113	$C_{43}H_{76}O_{10}$	775	811	16:0–18:3 (113a), 18:2–16:1 (113b), 18:0–16:3 (113c), 18:1–16:2 (113d)
34:2 MGDG	114	$C_{43}H_{78}O_{10}$	777	813	18:1–16:1 (114a) > 16:0–18:2 (114b)
34:1 MGDG	115	$C_{43}H_{80}O_{10}$	779	815	18:0–16:1 (115a) > 16:0–18:1 (115b)
36:6 MGDG	116	$C_{45}H_{74}O_{10}$	797	833	18:3–18:3 (116a)
36:5 MGDG	117	$C_{45}H_{76}O_{10}$	799	835	18:2–18:3 (117a)
36:4 MGDG	118	$C_{45}H_{78}O_{10}$	801	837	n.d.
36:3 MGDG	119	$C_{45}H_{80}O_{10}$	803	839	n.d.
36:2 MGDG	120	$C_{45}H_{82}O_{10}$	805	841	n.d.
36:1 MGDG	121	$C_{45}H_{84}O_{10}$	807	843	n.d.
38:6 MGDG	122	$C_{47}H_{78}O_{10}$	825	861	n.d.
38:5 MGDG	123	$C_{47}H_{80}O_{10}$	827	863	n.d.
38:4 MGDG	124	$C_{47}H_{82}O_{10}$	829	865	n.d.
38:3 MGDG	125	$C_{47}H_{84}O_{10}$	831	867	n.d.
			Mass of [M+Na]$^+$	*Mass of [M+OAc]$^-$*	
34:6 DGDG	126	$C_{49}H_{80}O_{15}$	931	967	18:3–16:3 (126a)
34:5 DGDG	127	$C_{49}H_{82}O_{15}$	933	969	18:3–16:2 (127a) > 18:2–16:3 (127b)
34:4 DGDG	128	$C_{49}H_{84}O_{15}$	935	971	n.d.
34:3 DGDG	129	$C_{49}H_{86}O_{15}$	937	973	16:0–18:3 (129a) > 18:2–16:1 (129b)
34:2 DGDG	130	$C_{49}H_{88}O_{15}$	939	975	18:2–16:0 (130a) > 18:1–16:1 (130b)
34:1 DGDG	131	$C_{49}H_{90}O_{15}$	941	977	18:1–16:0 (131a)
36:6 DGDG	132	$C_{51}H_{84}O_{15}$	959	995	18:3–18:3 (132a)
36:5 DGDG	133	$C_{51}H_{86}O_{15}$	961	997	18:3–18:2 (133a)
36:4 DGDG	134	$C_{51}H_{88}O_{15}$	963	999	18:2–18:2 (134a), 18:1–18:3 (134b)
36:3 DGDG	135	$C_{51}H_{90}O_{15}$	965	1001	18:3–18:0 (135a), 18:2–18:1 (135b)
36:2 DGDG	136	$C_{51}H_{92}O_{15}$	967	1003	18:2–18:0 (136a), 18:1–18:1 (136b)
36:1 DGDG	137	$C_{51}H_{94}O_{15}$	969	1005	n.d.
38:6 DGDG	138	$C_{53}H_{88}O_{15}$	987	1023	n.d.
38:5 DGDG	139	$C_{53}H_{90}O_{15}$	989	1025	n.d.
38:4 DGDG	140	$C_{53}H_{92}O_{15}$	991	1027	n.d.
38:3 DGDG	141	$C_{53}H_{94}O_{15}$	993	1029	n.d.
				Mass of [M−H]$^-$	
34:6 SQDG	142	$C_{43}H_{70}O_{12}S$		809	18:3–16:3 (142a)
34:5 SQDG	143	$C_{43}H_{72}O_{12}S$		811	16:2–18:3 (143a)
34:4 SQDG	144	$C_{43}H_{74}O_{12}S$		813	18:3–16:1 (144a)
34:3 SQDG	145	$C_{43}H_{76}O_{12}S$		815	18:3–16:0 (145a)
34:2 SQDG	146	$C_{43}H_{78}O_{12}S$		817	16:0–18:2 (146a)
34:1 SQDG	147	$C_{43}H_{80}O_{12}S$		819	16:0–18:1 (147a)
36:6 SQDG	148	$C_{45}H_{74}O_{12}S$		837	18:3–18:3 (148a)
36:5 SQDG	149	$C_{45}H_{76}O_{12}S$		839	18:3–18:2 (149a)
36:4 SQDG	150	$C_{45}H_{78}O_{12}S$		841	18:2–18:2 (150a) > 18:1–18:3 (150b)
36:3 SQDG	151	$C_{45}H_{80}O_{12}S$		843	18:2–18:1 (151a) > 18:3–18:0 (151b)
36:2 SQDG	152	$C_{45}H_{82}O_{12}S$		845	18:0–18:2 (152a)

Apparent lipid molecular species, designated by total acyl carbons and total acyl double bonds, are listed and numbered. Lipid molecular species, identified by product ion analysis of the molecular ions in extracts from Arabidopsis leaves and designated by individual acyl chains, are labeled with small letters in addition to the number designation. The mass spectral data do not allow accurate designation of acyl chain position and, thus, no positional information is implied by these data. n.d., not determined.

amounts of these classes in the leaves (51 nmol/mg of dry weight), flower stalks (25 nmol/mg), siliques (46 nmol/mg), roots (31 nmol/mg), and seeds (53 nmol/mg) of wild-type plants were within approximately a factor of 2. In flowers, however, the absolute amount of extra-plastidically produced lipids was much higher, 106 nmol/mg of dry weight. Lyso PC and lyso PE amounts were highest in seeds, followed by flowers, roots, siliques, leaves, and flower stalks. Lyso PG levels were very low, amounting to less than 20% of the total lysophospholipids (lyso PC + lyso PE + lyso PG) determined in all organs. Absolute amounts of PA, which is made both plastidically and extra-plastidically, were highest in roots, followed by flowers, seeds, flower stalks, siliques, and leaves, in that order.

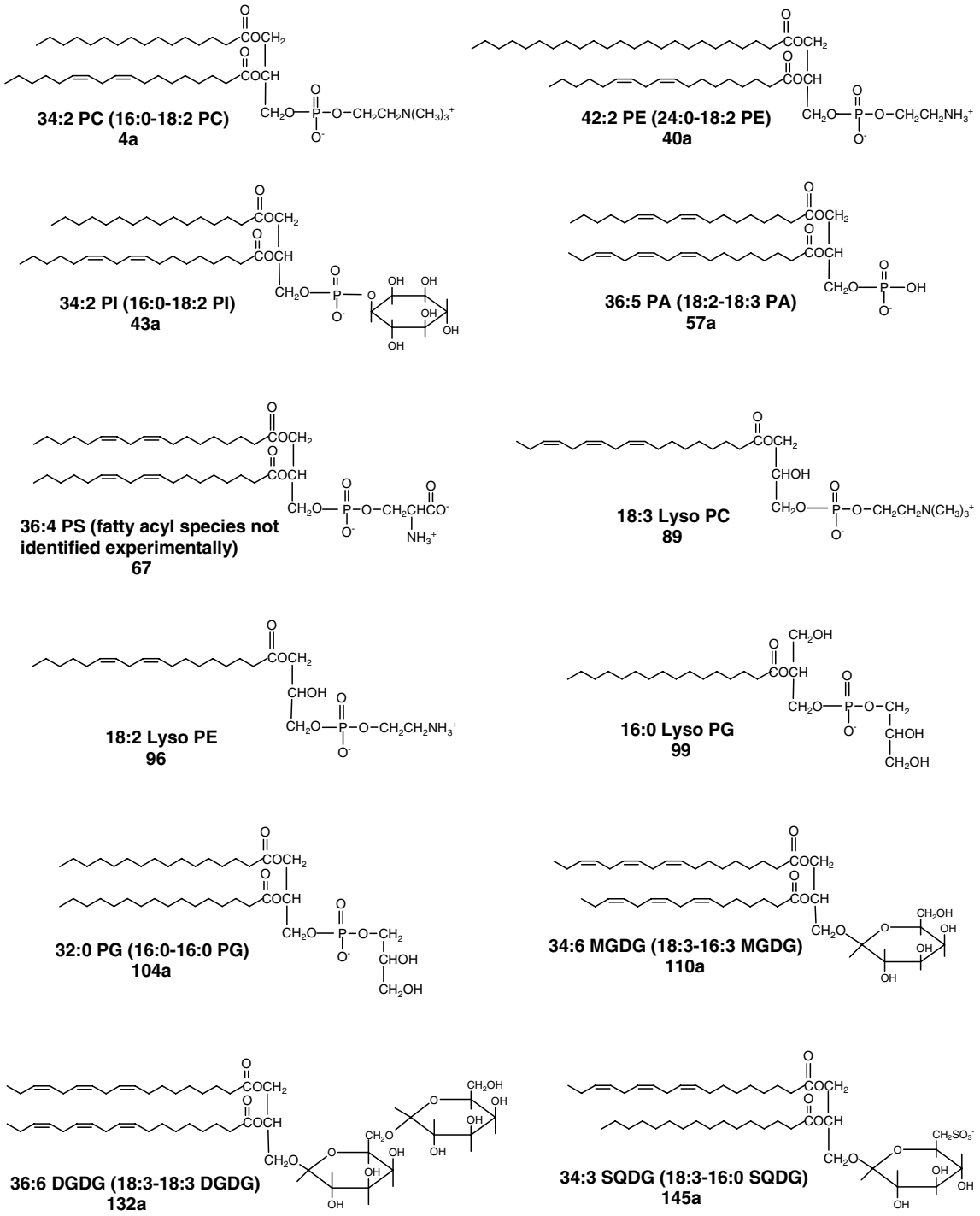

Fig. 1. Representative structures from each lipid class. Provided for each compound is the total acyl carbons: total double bonds and lipid class (individual acyl species, when known and not obvious from the total acyl carbons, and lipid class), and the compound number (Table 1).

Table 2
Total polar lipids in different organs of wild-type and PLDα1-knockout (*pldα1*) *Arabidopsis* plants

Total polar lipids	Leaves	Flower stalks	Flowers	Siliques	Roots	Seeds
	Lipid amount (nmol/mg; means ± SD)					
Wild-type	204 ± 22	67 ± 25	193 ± 52	96 ± 17	39 ± 12	58 ± 31
pldα1	218 ± 10	66 ± 35	139[L] ± 46	86 ± 24	36 ± 10	59 ± 23

Each value is expressed in nmol of polar lipids per milligram of dry plant organ (nmol/mg) and is a mean of 10 samples, except for leaves ($n = 5$). [L]The value for the *pldα1* plant is significantly lower than the value for wild-type plants at $P < 0.05$.

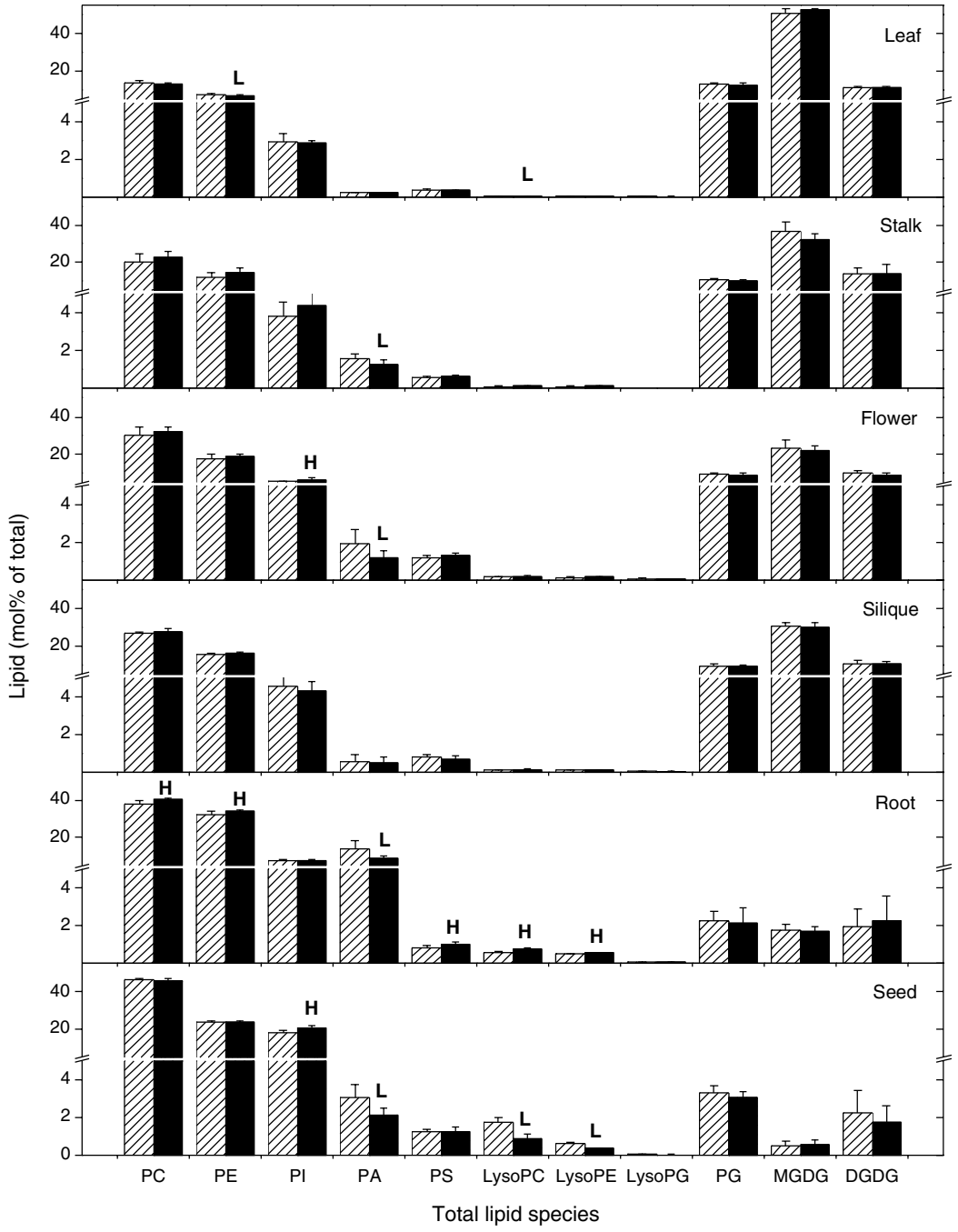

Fig. 2. Polar lipid classes (mol% of total polar glycerolipids analyzed) in organs of wild-type (stipled bars) and PLDα1-knockout (solid bars) plants. Wild-type and mutant plants were grown at the same time and in the same growth chamber. Organs of the two genotypes were sampled at the same times and at the same developmental stages. Except for seeds, all organs, including roots, siliques, leaves, flower stalks, and flowers, were collected from flowering plants. Flower samples include all parts of fully open flowers. Siliques were collected between 10 and 15 days post-flowering. Seeds were dry seeds stored at room temperature for eight weeks after harvest from plants grown under the same conditions. Values are means ± SD ($n = 5$ for leaves and 10 for other organs). The data in tabular form can be found in the Supplementary data. [L]The value for the PLDα1-deficient plant is lower than the value for wild-type plants at $P < 0.05$ (t-test). [H]The value for the PLDα1-deficient plant is higher than the value for wild-type plants at $P < 0.05$.

The leaf lipid amounts determined with the current method were compared to those obtained using conventional techniques (Zien et al., 2001). Values for phospholipids are similar, but the values in this work for galactolipids are higher. It is our experience that phospholipid amount per dry weight in leaves is quite constant, but the amount of galactolipid per dry weight is more variable and dependent on growth conditions. PA levels in leaves, measured in this work (0.43 nmol/mg dry weight), were somewhat lower than in a previous study using mass-spectrometry-based lipid profiling [1.2 nmol/mg dry weight (Welti et al., 2002)] or in previous studies using traditional analytical techniques [ca. 0.5 nmol/mg dry weight (Wang et al., 2000) and ca. 1.6 nmol/mg dry weight (Zien et al., 2001)].

It is difficult to discern whether PA levels might reflect, to some extent, PA generated during harvest, but in each study care was taken to immediately place harvested tissues in hot isopropanol to avoid generation of PA via PLD action during and after harvest. Plant PA levels do vary depending on growth conditions (S. Maatta, M.R. Roth, and R. Welti, unpublished data). The ability of ESI-MS/MS lipid profiling to detect small changes in lipid molecular species has been demonstrated recently by adding small, known amounts of specific lipid species to an Arabidopsis lipid extract (Welti et al., 2005).

2.2. Principal component analysis of the lipid molecular species of plant organs

Differences among the samples (organs and genotypes) were assessed using principal component analysis (PCA). The results of this analysis call attention to key statistically significant differences among the plant organs analyzed. The analysis of the dataset allowed extraction of 20 principal components that explained 90% of the variance in the system. The first three components, which are used for plotting the scores and loadings, explained 63% of the variance. Fig. 3 depicts two scores plots. The PCA data show clearly that for each organ, the scores for the genotypes *pldα1* and wild-type are closely associated in comparison to the scores for different organs, indicating that the compositions of the genotypes are relatively similar in comparison to the various organs.

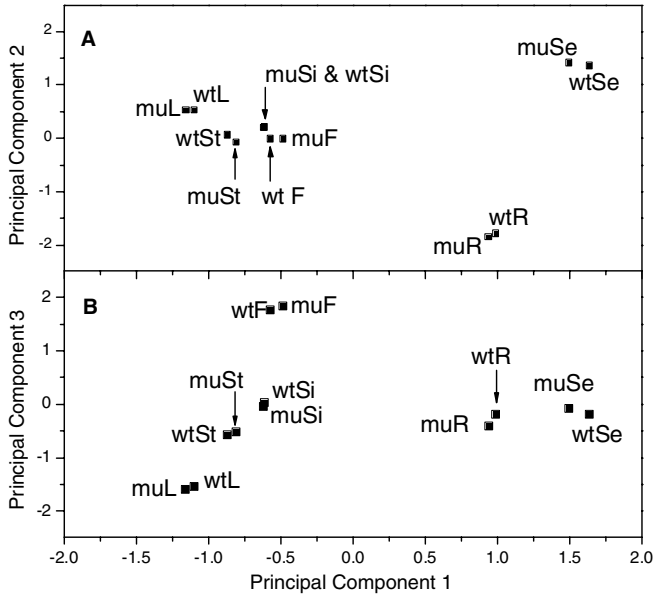

Fig. 3. Principal component analysis (PCA) of polar glycerolipid species in 110 samples among six organs. The first three components, explaining 63% of the variance, were used for plotting the scores. For each point, organ per genotype is the mean of corresponding replicates' principal component scores. (A) The scores plot of principal component 1 (36% of variance) vs. principal component 2 (18%). (B) The scores plot of principal component 1 (36%) vs. principal component 3 (9% of variance). wt, wild-type; mu, *PLDα1*-knockout mutant; F, flower; Si, silique; R, root; L, leaf; St, flower stalk; L, leaf; and Se, seed.

Principal component 1 describes the separation of the two non-photosynthetic organs, seed and root, from the photosynthetic organs (leaves, siliques, flower stalks, and flowers), which are clustered together with negative principal component 1 scores (Fig. 3a). Principal component 2, in contrast, is the main component that describes the difference between seed and root samples. The highest and lowest loadings values (Table 3) are the lipid species that are most important in assignment of each principal component. Examination of loadings (Table 3) reveals that the separation of roots and seeds from other organs along the principal component 1 axis results primarily from a higher content of plastidic lipids (MGDG, DGDG, and PG species) in the leaf, flower stalk, flower, and siliques and higher levels of phospholipids and lysophospholipids in the roots and seeds, indicating that these are the most important lipid species for differentiating roots and seeds from the other organ types. The separation of roots from seeds along the principal component 2 axis results most obviously from the higher levels of many very-long-chain PE species and PA species in roots, indicating that these species are most characteristic of the compositional differences between roots and seeds. Principal component 2 is also defined by high 38:3 and 38:4 PC (**15**, **14**), PE (**34**, **33**), and PS (**74**, **73**) species in seeds; these species probably represent combinations of 20:1 with 18:2 or 18:3 acyl chains, as 20:1 is much more common in seeds than in other organs (Katavic et al., 1995).

Principal component 3 describes differences among the four photosynthetic organs (Fig. 3b). Leaf and flower are opposed along the principal component 3 axis. Examination of loadings (Table 3) reveals that the separation between flowers and leaves along the principal component 3 axis results primarily from the higher levels of PS species in flowers and higher levels of MGDG and DGDG in leaves, indicating that these species are important in statistically differentiating flowers from leaves.

2.3. Differences in acyl species among lipid classes and plant organs

PC and PE contain primarily species with totals of 34 carbons and 36 carbons, while PI contains mostly combinations totaling 34 carbons (Fig. 4, Table 4, and Supplementary data). PE contains more species with greater than 36 carbons than PC or PI. These differing acyl compositions of the classes within an organ illustrate the well-demonstrated principle that acyl chains are non-randomly distributed in lipid species, due to the specificities of acyltransferases and desaturases (Somerville et al., 2000). The non-randomness of the acyl distribution is also demonstrated by the small amounts of 32-carbon species in comparison with levels of 16-carbon fatty acids in all organs and classes. For example, more than one-third of the fatty acids in Arabidopsis leaves are 16-carbon fatty acids (Zien et al., 2001; Miquel et al., 1998), but 32-carbon species represent less than 1% of the lipid species in every lipid class,

Table 3
Loadings of the first three principal components

Lipid species	Principal component 1 loadings	Lipid species	Principal component 2 loadings	Lipid species	Principal component 3 loadings
Twelve lowest loading values					
34:6 MGDG (**110**)	−0.976	40:2 PE (**37**)	−0.910	34:2 DGDG (**130**)	−0.353
36:6 MGDG (**116**)	−0.974	42:2 PE (**40**)	−0.899	34:1 PS (**64**)	−0.297
34:3 DGDG (**129**)	−0.972	34:3 PE (**22**)	−0.884	34:2 MGDG (**114**)	−0.283
34:4 PG (**105**)	−0.967	42:3 PE (**39**)	−0.882	34:6 DGDG (**126**)	−0.281
36:6 DGDG (**132**)	−0.951	40:3 PE (**36**)	−0.862	36:5 MGDG (**117**)	−0.258
34:3 PG (**106**)	−0.945	42:4 PE (**38**)	−0.849	36:3 DGDG (**135**)	−0.258
34:3 MGDG (**113**)	−0.908	38:2 PE (**35**)	−0.847	36:5 DGDG (**133**)	−0.258
36:4 MGDG (**118**)	−0.848	18:3 Lyso PG (**100**)	−0.845	34:4 MGDG (**112**)	−0.256
32:1 PG (**103**)	−0.813	34:3 PA (**53**)	−0.833	34:4 DGDG (**128**)	−0.242
38:4 MGDG (**124**)	−0.688	34:3 PC (**3**)	−0.794	42:2 PS (**83**)	−0.219
34:4 MGDG (**112**)	−0.639	36:6 PA (**56**)	−0.782	34:1 PG (**108**)	−0.207
38:2 PS (**75**)	−0.582	34:2 PA (**54**)	−0.754	34:4 PA (**52**)	−0.190
Twelve highest loading values					
36:5 PI (**46**)	0.912	36:3 PI (**48**)	0.569	34:2 PC (**4**)	0.538
36:2 PC (**10**)	0.914	38:3 PE (**34**)	0.623	38:5 PE (**32**)	0.549
40:3 PC (**19**)	0.922	34:1 PE (**24**)	0.626	36:6 PE (**25**)	0.555
34:2 PI (**43**)	0.923	34:1 PC (**5**)	0.630	38:6 PC (**12**)	0.595
18:1 Lyso PE (**97**)	0.927	40:4 PS (**77**)	0.642	36:6 PS (**65**)	0.600
36:3 PE (**28**)	0.935	38:3 PC (**15**)	0.665	16:1 Lyso PG (**98**)	0.638
16:0 Lyso PC (**88**)	0.940	38:4 PS (**73**)	0.668	38:2 PS (**75**)	0.753
36:4 PE (**27**)	0.953	38:4 PE (**33**)	0.678	38:3 PS (**74**)	0.798
18:2 LysoPE (**96**)	0.958	38:5 PC (**13**)	0.686	34:2 PS (**63**)	0.849
36:3 PC (**9**)	0.967	34:6 DGDG (**126**)	0.689	36:2 PS (**69**)	0.854
34:4 PC (**2**)	0.969	34:2 PG (**107**)	0.690	34:3 PS (**62**)	0.872
34:4 PE (**21**)	0.977	38:4 PC (**14**)	0.699	36:3 PS (**68**)	0.914

The 12 highest and 12 lowest loading values are indicated.

instead of more than 10% of 32-carbon species, as would be expected if the acyl species were randomly distributed. There is a tendency toward longer chains in all the phospholipids of seeds as compared to other organs; only 21% of the PC of seeds contains 34 carbons, while 34-carbon species make up 40–45% of the PC of leaves, flower stalks, flowers, siliques, and roots. In particular, 36:4 species are more prominent in seeds than in other organs. For example, 36:4 PC (**8**) comprises 37% of seed PC, but only 13% of leaf PC. Product ion analysis of leaf extract indicated that 36:4 PC (**8**) was mostly 18:2–18:2 PC (**8a**) (Welti et al., 2002; Table 1); the high levels of 36:4 lipids in seeds likely reflect high levels of 18:2 acyl chains.

Among all of the phospholipid classes, the PA species patterns among the various organs are the most varied (Figs. 4 and 5, and Supplementary data). In leaves of wild-type plants, 34-carbon PA species represent 81% of the total PA, but in seeds, 34-carbon species represent only 37% of the total PA; the 34-carbon species in other organs represent 56–63% of the total PA. In particular, 34:3 PA (**53**) represents 41% of the PA in leaves and only 13% in seeds. 34:4 PA (**52**) is 10% of the PA in leaves, but no more than 2% of the PA in any other organ. Like 36:4 PC (**8**) and 36:4 PE (**27**), 36:4 PA (**58**) is enriched in seeds, accounting for 31% of seed PA, as compared to the one-third to one-half that percentage in the other organs, except leaves, where 36:4 accounted for only 1% of the total PA. Recent studies have indicated that PA is an important class of signaling lipids in plants and other organisms, and the intracellular levels of PA change considerably in plants (Testerink and Munnik, 2005; Wang et al., 2006). The varied PA species patterns in different organs could play a role in the specificity to PA functions in different cellular processes.

The molecular species composition of PS is particularly notable in that, in all organs, this lipid class contains a high proportion of very-long-chain fatty acids (Fig. 5). PS species contain up to 44 carbons in the two acyl chains. Product ion analysis of these lipid species from leaves demonstrated that these very-long-chain PS species are composed mostly of 18-carbon species, combined with a very-long-chain fatty acyl species, containing up to 24, and to a small extent, 26 carbons (Table 1). As in the PC, PE, and PI classes, the variety of PS molecular species is qualitatively similar among the various organs, but roots and seeds contain a slightly larger fraction of very-long-chain species, and seeds have relatively more PS species with a total of four double bonds [36:4 (**67**), 38:4 (**73**), 40:4 (**77**), and 42:4 (**81**) PS] than the other organs. The PS species data are consistent with earlier fatty acid compositional analyses showing PS comprises more very-long-chain molecular species than any other lipid class in other plant species (Murata et al., 1984; Bohn et al., 2001). Murata and co-workers found that 14–45% of PS fatty acids in 18 different flowering plant species contain 20–26 carbons. If these data are translated into diacyl species composition by assuming that each of the very-long chains were paired with a shorter species, then 28–90% of the diacyl species would contain a very-long

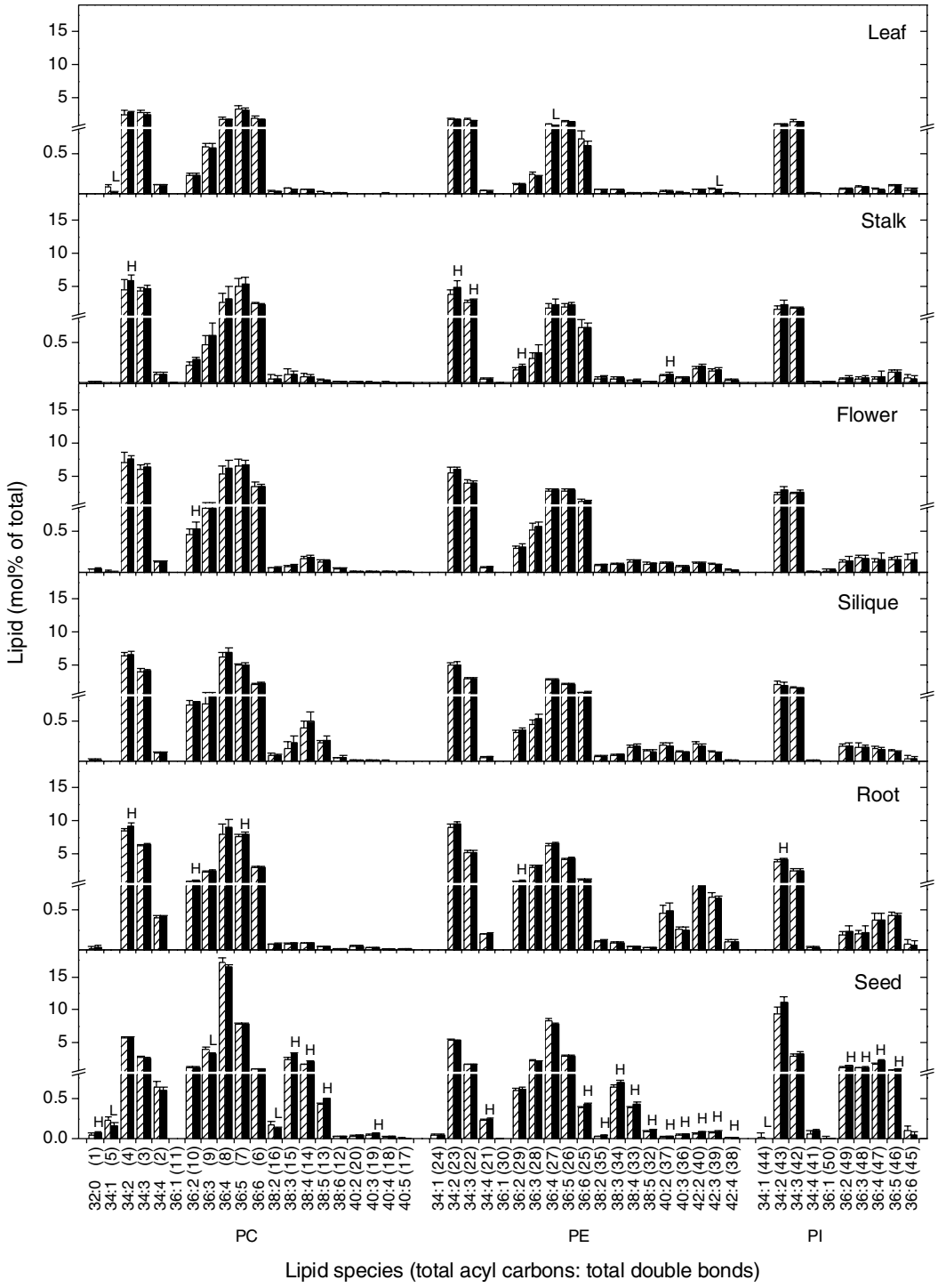

Fig. 4. Molecular species of PC, PE, and PI (mol% of total polar glycerolipids analyzed) in organs of wild-type (stippled bars) and PLDα1-knockout (solid bars) plants. Values are means ± SD ($n = 5$ for leaves and 10 for other organs). The data in tabular form can be found in the Supplementary data. [L]The value for the PLDα1-deficient plant is lower than the value for wild-type plants; $P < 0.05$. [H]The value for the PLDα1-deficient plant is higher than the value for wild-type plants; $P < 0.05$.

chain. Thus, Arabidopsis is quite typical of higher plant PS composition, with 49–72% of its total PS in various organs containing more than 36 total acyl carbons. The PS molecular species with very-long chains arise through the action of a serine exchange enzyme that catalyzes the reaction, serine + (PC or PE) → PS + (choline or ethanolamine) (Vincent et al., 1999, 2001). Indeed, PC and especially PE are the only other phospholipids that contain significant amounts of very-long-chain species. Recently, it has been suggested that very-long-chain fatty acids in polar membrane lipids may play a role in stabilizing highly curved membrane domains (Schneiter et al., 2004).

Lysophospholipids contain mostly 16:0, 18:2, and 18:3 species, although 16:1 is also a significant molecular species

Table 4
Phospholipid chain lengths in wild-type Arabidopsis organs (mol% of class)

	Leaves	Flower stalks	Flowers	Siliques	Roots	Seeds
PC (% of PC)						
With 34 acyl carbons	40	45	43	40	40	21
With 36 acyl carbons	58	53	55	56	59	68
With >36 acyl carbons	2	1	2	4	1	11
PE (% of PE)						
With 34 acyl carbons	48	54	53	51	44	31
With 36 acyl carbons	47	41	43	42	48	63
With >36 acyl carbons	5	5	5	7	8	6
PI (% of PI)						
With 34 acyl carbons	88	92	87	85	83	70
With 36 acyl carbons	12	8	13	15	17	30
PA (% of PA)						
With 34 acyl carbons	81	61	56	63	56	37
With 36 acyl carbons	19	39	44	37	44	63
PS (% of PS)						
With 34 acyl carbons	32	28	32	19	21	17
With 36 acyl carbons	17	14	19	15	10	11
With >36 acyl carbons	51	58	49	67	69	72

in lyso PG (**98**) (Fig. 6). The molecular species patterns of lyso PC and lyso PE are similar in the various organs, but 18:2 lyso PC (**90**) and 18:2 lyso PE (**96**) are relatively more abundant in seeds than in other organs, accounting for about half of the lyso PC and lyso PE in seeds.

The typically plastidic diacyl lipid species are shown in Fig. 7. In leaves, flower stalks, flowers, and siliques, where MGDG is abundant, the major molecular species are 34:6 (**110**) and 36:6 (**116**), which were demonstrated in leaves (Table 1) to be, respectively, the plastidically produced species, 18:3–16:3 (**110a**), and the species 18:3–18:3 (**116a**), which has acyl chains that originate extra-plastidically (Somerville et al., 2000; Welti et al., 2002; Table 1). These MGDG species are also detectable in roots, and 34:6 (**110**) is detectable in seeds, although the level is very low. In DGDG, the 36:6 (**132**) species, also comprised of 18:3–18:3 (**132a**), is also prominent, but the second most common species is 34:3 DGDG (**129**), which in leaves was demonstrated to be a combination of 18:3 and 16:0 (**129a**) (Welti et al., 2002; Table 1). In roots and seeds, 36:6 DGDG (**132**) is also detectable, but very low. In leaves, flower stalks, flowers, and siliques, where PG is plentiful, the most common PG molecular species are 34:4 (**105**), 34:3 (**106**), and 34:2 (**107**). In leaves, 34:4 (**105**) and 32:1 PG (**103**) have been shown to be 18:3–16:1 PG (**105a**) and 16:0–16:1 PG (**103a**), respectively (Welti et al., 2002; Table 1); these acyl combinations are consistent with these species being plastidically produced (Somerville et al., 2000). The abundances of 34:4 PG (**105**) and 32:1 (**103**) PG correlate well with the overall prevalence of plastidically produced lipids, with leaves having the most plastidically produced lipids, flower stalks and siliques having the second most, flowers having the next most, and roots and seeds having very few plastidically produced lipids and virtually no 34:4 (**105**) nor 32:1 (**103**) PG (Fig. 7).

2.4. Comparison of lipid molecular species between wild-type and PLDα1-knockout plants

The role of the most abundant PLD gene product, PLDα1, in the composition of polar membrane lipid species in leaves, flower stalks, flowers, siliques, seeds, and roots of Arabidopsis was investigated by comparing the lipid profiles of wild-type and *pldα1* plants. A T-DNA insertion about one-third of the way into the gene sequence rendered the *PLDα1* knockout plant *pldα1* devoid of PLDα1 activity (Fig. 8A), protein (Fig. 8B), and mRNA (Fig. 8C). Under normal growth conditions in growth chambers or greenhouses, Arabidopsis plants without PLDα1 do not exhibit an overt alteration in growth and development. However, *pldα1* leaves lose more water than wild-type leaves because PLDα1 mediates the abscisic acid-promoted stomatal closure (Zhang et al., 2004; Mishra et al., 2006). PLDα1 activity is highest in roots and lowest in flower stalks and siliques (Fig. 8A). The pattern of activity correlates closely with both immunoblot and expression data (Fig. 8B and C, top rows). In roots, a broad band of protein that was immunoreactive with the PLDα1 antibody was visible in the *PLDα1* knockout plants, but not in the wild-type plants. The origin of the band is unclear. It is possible that a closely related PLD, most likely PLDα2, may be increased in roots of *pldα1* plants as compared to wild-type plants (Fig. 8B).

Polar lipid amounts (nmol/mg dry weight) of *pldα1* plants were similar to wild-type plants, except for the level in flowers, which was significantly lower in *pldα1* plants (Table 2). Comparison of the lipid class composition of organs from wild-type and *pldα1* plants showed two major differences. First, in flower stalks, flowers, roots, and seeds, there was significantly less PA in *pldα1* than in the

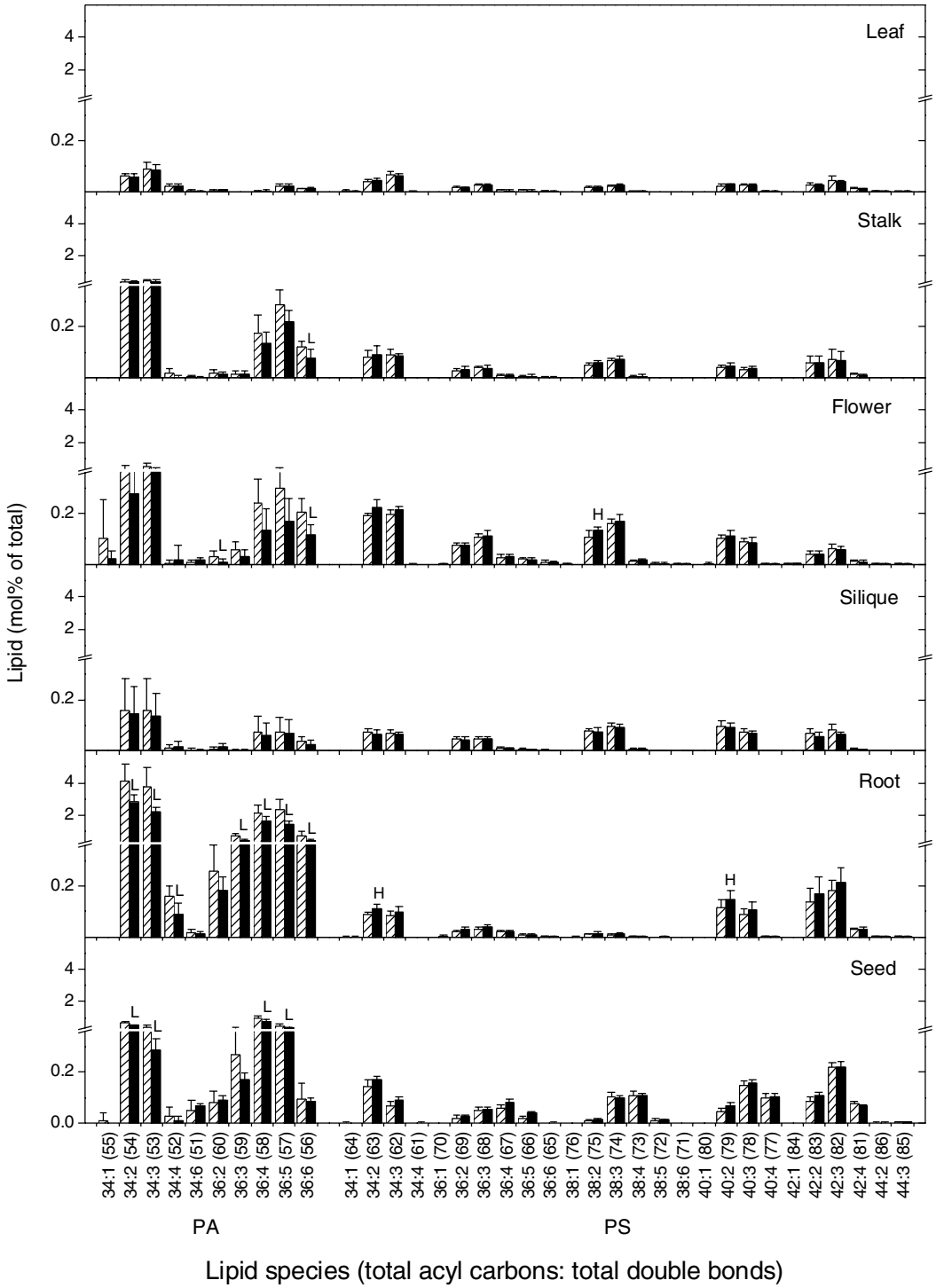

Fig. 5. Molecular species of PA and PS (mol% of total polar glycerolipids analyzed) in organs of wild-type (stippled bars) and PLDα1 knockout (solid bars) plants. Values are means ± SD ($n = 5$ for leaves and 10 for other organs). The data in tabular form can be found in the Supplementary data. [L]The value for the PLDα-deficient plant is lower than the value for wild-type plants; $P < 0.05$. [H]The value for the PLDα-deficient plant is higher than the value for wild-type plants; $P < 0.05$.

wild-type plants (Figs. 2 and 5, and Supplementary data). Although PLDα1 has been shown to account for about half of the hydrolysis that occurs in leaves during wounding and freezing stresses (Zien et al., 2001; Welti et al., 2002), the current data suggest that PLDα1 accounts for a smaller fraction of the basal PA. In fact, leaves and siliques showed insignificant differences between PA levels in *pldα1* plants as compared to wild-type plants, indicating that in these organs, the contribution of PLDα1 to basal PA levels was quite small under the growth conditions employed. Previous work indicated a small, but significant difference between PA levels in leaves from wild-type and

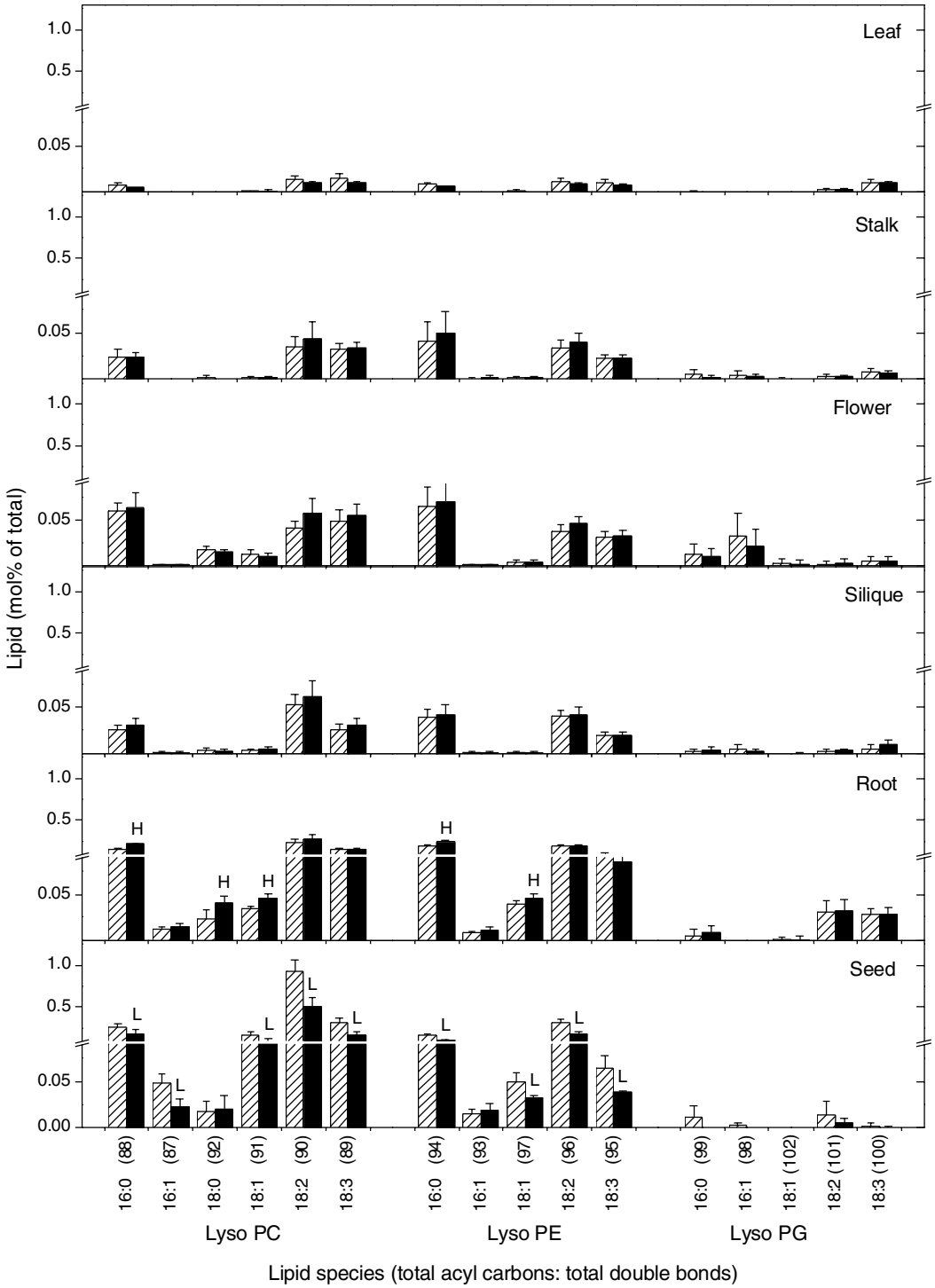

Fig. 6. Molecular species of lysolipids (mol% of total polar glycerolipids analyzed) in organs of wild-type (stippled bars) and PLDα1 knockout (solid bars) plants. Values are means ± SD ($n = 5$ for leaves and 10 for other organs). The data in tabular form can be found in the Supplementary data. [L]The value for the PLDα-deficient plant is lower than the value for wild-type plants; $P < 0.05$. [H]The value for the PLDα-deficient plant is higher than the value for wild-type plants; $P < 0.05$.

PLDα-deficient plants (Welti et al., 2002). In flower stalks, the difference between wild-type and PLDα1 knockout plants could mean that PLDα1 contributed about 20% of the basal level of PA, while in seeds, PLDα1 contributed 30%, in roots, 35–40%, and in flowers, 40–55%, with the values within the ranges depending on whether mole percentage or absolute amounts, normalized to dry weight, are compared. Thus, in general, as might be expected, PLDα1 tends to contribute most to the basal levels of PA in the organs in which PLDα1 is most active. However, PLDα1 does contribute to the basal PA level in flower stalks, even though the protein levels and measured activity

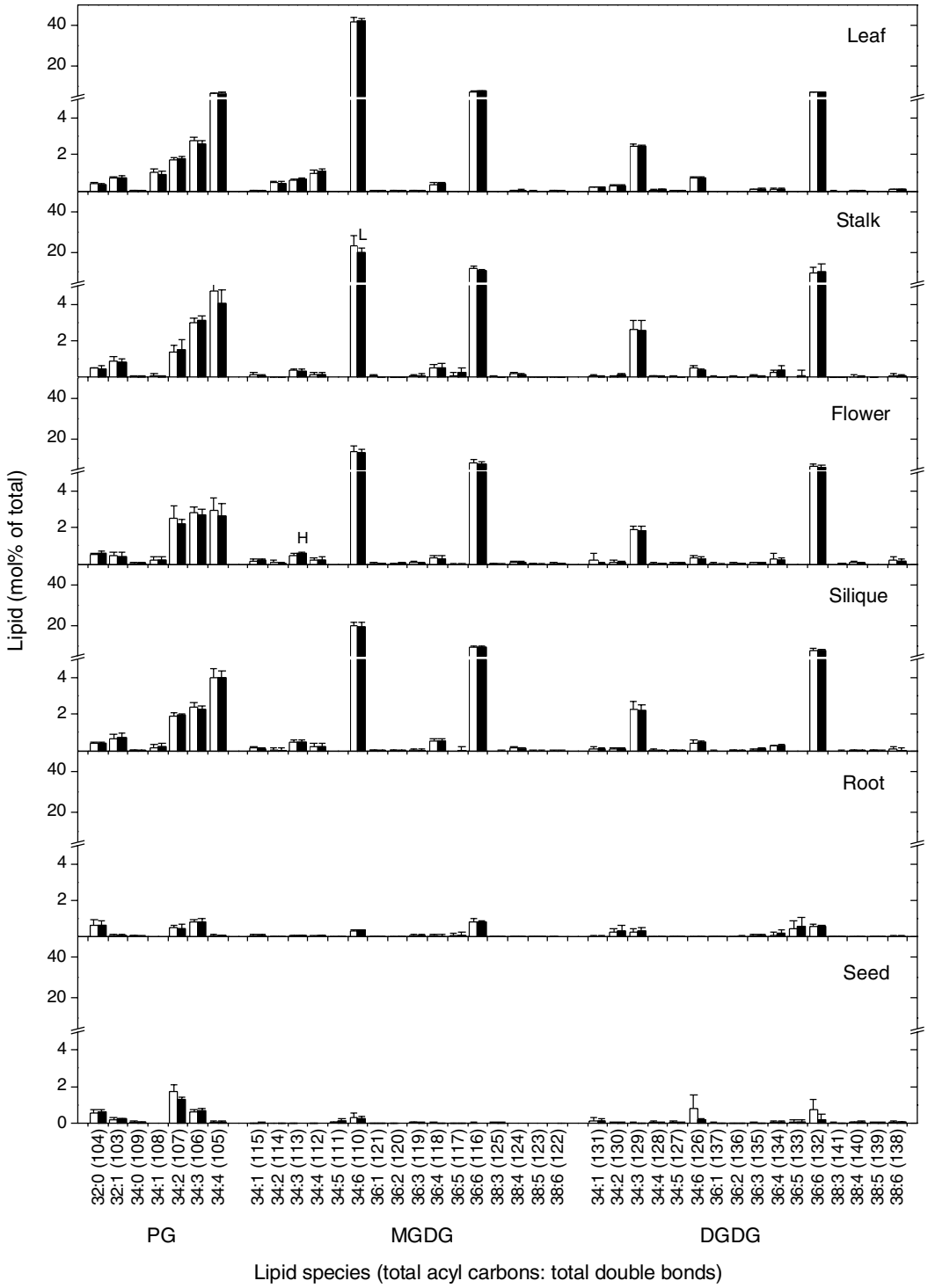

Fig. 7. Molecular species of PG, MGDG, and DGDG (mol% of total polar glycerolipids analyzed) in organs of wild-type (stippled bars) and PLDα1 knockout (solid bars) plants. Values are means ± SD ($n = 5$ for leaves and 10 for other organs). The data in tabular form can be found in the Supplementary data. [L]The value for the PLDα-deficient plant is lower than the value for wild-type plants; $P < 0.05$. [H]The value for the PLDα-deficient plant is higher than the value for wild-type plants; $P < 0.05$.

levels are quite low. It should also be noted that this simple comparison between wild-type and *pldα1* organs may underestimate the actual contribution of PLDα1 because other PLDs may compensate for the loss of PLDα1 and reduce the impact of the PLD knockout.

The pattern of PA molecular species is similar to that of PC and PE, and this could represent a hydrolytic connection or similarity generated through biosynthesis. However, the molecular species composition of the PA in leaves hints at a different source for a fraction of the PA

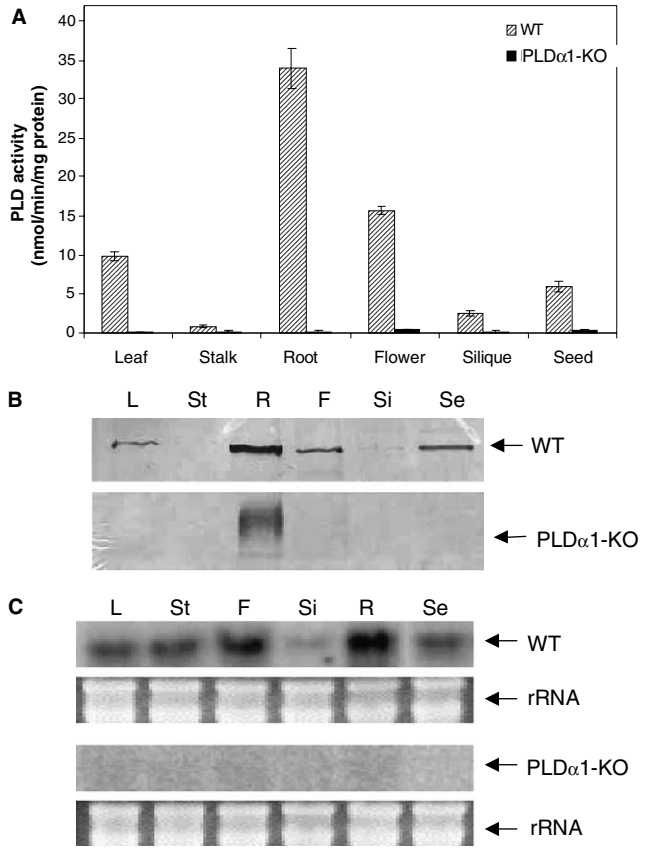

Fig. 8. PLDα1 activity, protein, and mRNA in Arabidopsis organs. (A) PLDα1 enzyme activity in organs of wild-type and *pldα1* plants. (B) Immunoblot of proteins from organs of wild-type and *pldα1* plants with an antibody raised against a PLDα1 peptide. Proteins (20 μg/lane) were separated by SDS–PAGE using 8% acrylamide and transferred to a polyvinylidene difluoride membrane. The membrane was blotted with a PLDα-specific antibody, followed by incubation with a second antibody conjugated to alkaline phosphatase. L, leaves; St, flower stalks; R, roots; F, flowers; Si, siliques; and Se, seeds. (C) RNA blot of *PLDα1* mRNA from different organs of wild-type and *pldα1* plants. Equal amounts of total RNA (10 μg/lane) were separated on denaturing gels. The arrow indicates the position of the *PLDα1* mRNA band. Ethidum bromide-stained rRNA (*lower panels*) is shown to demonstrate equal loading of total RNA in each lane.

in that organ. Leaf PA contains a larger fraction of 34:4 species (**52**) (10% of the total PA) and 34:6 species (**51**) (2% of the total PA) than any other organ. Indeed, no more than 2% of the PA of the other organs was the 34:4 (**52**) species. The basal levels of 34:4 (**52**) and 34:6 PA (**51**) were the same in leaves of wild-type and PLDα1 knockout plants, suggesting that these molecular species arise by a PLDα1-independent mechanism. The 34:6 species are found only in trace amounts in diacyl phospholipids other than PA, whereas 34:6, consisting of 18:3–16:3, is the major species of MGDG. The 34:4 species, consisting of an 18:3–16:1 combination, is considerably more prominent in PG than in other phospholipid classes. 34:6 PA (**51**) was increased substantially during freezing stress in a PLDα-independent manner (Welti et al., 2002). These data suggest that a fraction of the basal PA in leaves arises from polar chloroplast lipids by a PLDα1-independent mechanism. If 34:4 and 34:6 are representative of PG and MGDG species converted to basal PA, then the fraction of the PA derived from polar chloroplast lipids in leaves would be 20–25%, since 34:4 PG (**105**) represents about half of the PG and 34:6 MGDG (**110**) represents about 80% of total MGDG.

Besides differences in PA levels between wild-type and *pldα1* plants, the major difference is in the seeds, where lyso PC and lyso PE levels were significantly lower in the *pldα1* plants (Fig. 6). Lysophospholipids are products of phospholipase As. The lower levels of the catabolic products, PA, lyso PC, and lyso PE in *pldα1* seeds could mean that the activity of PLDα1 may promote membrane lipid degradation and adversely affect seed quality.

In contrast to the observations of changes in levels of lipid hydrolytic products, comparative profiling of the lipid molecular species of the wild-type and *pldα1* plants revealed no major differences in the plastidically produced lipid species (Fig. 7). Likewise, although some molecular species of the extra-plastidically produced diacyl lipids (PC, PE, PI, and PS) appear to be subtly altered in the PLDα1 knockout plants as compared to the wild-type plants, some of these small changes can be explained by the presentation of the data in mol% of species analyzed, because the PA species are significantly lower in the PLDα1 knockout plants, particularly in roots and seeds (Figs. 4 and 5, and Supplementary data). In the PC and PE of seeds, however, there is a slight, but significant tendency toward relatively higher levels of species with >36 carbons in the *pldα1* plants as compared to the wild-type plants (Fig. 4 and Supplementary data). This trend is notable in both data expressed as mol% or in absolute amounts, and could mean that PLDα1 affects metabolism of very-long-chain fatty acyl species, although no genotype-specific differences were observed in the PS of wild-type as compared to *pldα1* plants.

3. Concluding remarks

This study shows that ESI-MS/MS-based lipid profiling is capable of generating quantitative data for most common-occurring, polar glycerolipid species. This analysis has measured 140 glycerolipid species in six organs and has revealed significant differences in the amounts and types of lipid molecular species among the organs. This provides, to date, the most complete analysis of molecular species of polar lipids in Arabidopsis. The comparative profiling of lipids of wild-type and *pldα1* plants provide clues about the roles of *PLDα1* and its enzyme product in various Arabidopsis organs. *PLDα1* contributes significantly to the accumulation of PA in seeds, roots, flower stalks, and flowers, but not to the basal level of PA in leaves or siliques. In addition, the loss of *PLDα1* decreases the accumulation of lysophospholipids in seeds, pointing to a role for PLDα1 in membrane lipid degradation in seeds.

4. Experimental

4.1. Plant materials and growth

Seeds of *Arabidopsis thaliana* wild-type (Columbia ecotype) and the PLDα1-knockout line *pldα1* were sown in Scotts Metromix 360 soil. The pots were kept at 4 °C for 2 days and then moved to a growth chamber at 23 °C (day) and 18 °C (night) with a 12-h day length, daytime fluorescent lighting at 120 μmol m^{-2} s^{-2}, and 58% relative humidity. The *pldα1* mutant of *A. thaliana* was identified from SALK_053785. T-DNA left border primer, 5′-GCG TGG ACC GCT TGC TGC AACT-3′, and two pairs of gene specific primers for PLDα1: forward 5′-CTT CTT CTG ACC ACC GAA CGA TTG AGT TT-3′ and reverse 5′-TCA CAA AGC TAC ATT CTC TCA CCA CGT C-3′ were used for mutant screening. The presence and location of a T-DNA insertion was confirmed by PCR and sequencing to be in the third exon, 936 nucleotides downstream of the initiation codon. PLDα1 deficiency was confirmed by assaying PLDα1 activity and immunoblotting with a PLDα specific antibody, following the procedure described previously (Zhang et al., 2004).

4.2. Sampling and lipid extraction

Organs (leaves, flower stalks, flowers, siliques, seeds and roots) were harvested for lipid extraction and profiling. Wild-type and mutant plants were grown at the same time and in the same growth chamber. All organs of the two genotypes were sampled at the same time and at the same stage of growth. Flower samples include all parts of fully open flowers. Seeds were harvested from the plants grown at the same conditions, stored at room temperature for eight weeks, resulting in dried seeds, which were extracted. Sample preparation and lipid extraction were carried out as described previously (Welti et al., 2002). After harvesting each organ, it was immediately transferred to *i*-PrOH (3 ml) with 0.01% butylated hydroxytoluene (BHT) at 75 °C and incubated for 15 min. At this time, seeds were crushed with a glass rod, while other organs were not disrupted, and $CHCl_3$ (1.5 ml) and H_2O (0.6 ml) were added. The tubes were shaken for 1 h, followed by removal of the extract. The organs were re-extracted with $CHCl_3$:MeOH (2:1) with 0.01% BHT 5 times with 30 min agitation each time. The remaining plant organ was heated overnight at 105 °C and weighed. The weights of these dried, extracted organs are the "dry weights". Dry weights of each sample ranged from 8 to 13 mg for leaves, 6–11 mg for flower stalks, 0.5–0.9 mg for flower stalks, 3–4 mg for siliques, 4–8 mg for roots, and 4–9 mg for seeds. The dry weights for individual samples are provided with the mass spectrometry data in the Supplementary data. The combined extracts were washed once with 1 ml of 1 M KCl and once with 2 ml water. The solvent was evaporated under nitrogen, and the lipid extract was dissolved in $CHCl_3$ (1 ml).

Every effort was made to ensure that sampling for lipid analysis occurred as rapidly as possible and that lipolytic activity was immediately stopped by submersion of the organs in isopropanol at 75 °C for 15 min. This precaution is important to minimize injury during sampling, which might increase lipolytic activities (Lee et al., 1997; Zien et al., 2001). Lipids from five replicates of leaf samples and 10 replicates of all other samples of both wild-type and PLDα1-knockout plants were extracted and analyzed.

4.3. ESI tandem mass spectrometry and lipid profiling

An automated electrospray ionization-tandem mass spectrometry approach was used and data acquisition and analysis and acyl group identification were carried out as described previously (Welti et al., 2002; Wanjie et al., 2005) with minor modifications. The samples were dissolved in $CHCl_3$ (1 ml). Two aliquots were taken for mass spectrometry analysis, one sample for phospholipid analysis and one for galactolipid analysis. The volumes of each aliquot are provided with the mass spectrometry data in the Supplementary data. For phospholipid analysis, Arabidopsis lipid extract in $CHCl_3$ was combined with solvents and internal standards, such that the ratio of $CHCl_3$/MeOH/300 mM ammonium acetate in water was 300/665/35 and the final volume was 1 ml. Internal standards, obtained and quantified as previously described (Welti et al., 2002), were 0.66 nmol di14:0-PC, 0.66 nmol di24:1-PC, 0.66 nmol 13:0-lyso PC, 0.66 nmol 19:0-lyso PC, 0.36 nmol di14:0-PE, 0.36 nmol di24:1-PE, 0.36 nmol 14:0-lyso PE, 0.36 nmol 18:0-lyso PE, 0.36 nmol di14:0-PG, 0.36 nmol di24:1-PG, 0.36 nmol 14:0-lyso PG, 0.36 nmol 18:0-lyso PG, 0.36 nmol di14:0-PA, 0.36 nmol di20:0(phytanoyl)-PA, 0.24 nmol di14:0-PS, 0.24 nmol di20:0(phytanoyl)-PS, 0.20 nmol 16:0–18:0-PI and 0.16 nmol di18:0-PI. For galactolipid analysis, plant lipid extract in $CHCl_3$ was combined with solvent and internal standards, such that the ratio of $CHCl_3$/MeOH/50 mM sodium acetate in water was 300/665/35, the final volume was 1 ml, and the sample contained 2.01 nmol 16:0–18:0-MGDG, 0.39 nmol di18:0-MGDG, 0.49 nmol 16:0–18:0-DGDG, and 0.71 nmol di18:0-DGDG.

Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems, Foster City, CA). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 μl/min. The collision gas pressure was set at 2 (arbitrary units) for phospholipids and 1 for galactolipids. The collision energies, with nitrogen in the collision cell, were 28 V for PE, 40 V for PC, −58 V for PI, −57 V for PA and PG, −34 V for PS, 50 V for MGDG, and 84 V for DGDG. Declustering potentials were 100 V for PE and PC, −100 V for PA and PG, PI, and PS, 150 V for MGDG, and 215 V for DGDG. Entrance potentials were 15 V for PE, 14 V for PC, −10 V for PI,

PA and PG, and PS, 14 V for MGDG, and 15 V for DGDG. Exit potentials were 11 V for PE, 14 V for PC, −15 V for PI, −14 V for PA and PG, −13 V for PS, and 17 V for MGDG and DGDG. The mass analyzers were adjusted to a resolution of 0.7 amu full width at half height. For each spectrum, 9–150 continuum scans were averaged in multiple channel analyzer (MCA) mode. The source temperature (heated nebulizer) was 100 °C, the interface heater was on, +5.5 kV or −4.5 kV were applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units).

Lipid species were detected using the scans previously described (Brügger et al., 1997; Kim et al., 1999; Welti et al., 2002). Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. The background of each spectrum was subtracted, the data were smoothed, and peak areas were integrated using a custom script and Applied Biosystems Analyst software. The lipids in each class were quantified in comparison to the two internal standards of that class using correction curves determined for the API 4000 mass spectrometer.

4.4. Acyl group identification

The acyl groups of the major phospholipid species in a lipid extract from wild-type plants were identified as acyl anions from the appropriate negative ion precursors. The collision energies were 20–55 V. The solvent was $CHCl_3$/MeOH/300 mM ammonium acetate in water (300/665/35). PG, PI, PE, PS, lyso PG, lyso PE, SQDG, and PA were analyzed as $[M-H]^-$ ions, MGDG and DGDG were analyzed as $[M-H]^-$ and $[M+OAc]^-$, and PC and lyso PC were analyzed as $[M+OAc]^-$.

4.5. Protein extraction and PLDα activity assay

Wild-type and *pldα1* plants that were grown at the same time and in the same growth chamber were used for protein and RNA analyses. Total protein from organs of wild-type and *pldα1* plants was extracted by grinding in an ice-chilled mortar and pestle with buffer A as described previously (Fan et al., 1997). Equal amounts of protein were separated on an 8% gel and transferred onto polyvinylidene difluoride filters. The membranes were blotted with a PLDα1-specific antibody, followed by incubation with a secondary antibody conjugated to alkaline phosphatase. PLDα activity was determined using egg yolk (PC) mixed with dipalmitoylglycerol-3-phospho-(methyl-[^{3}H]) choline as described in Fan et al. (1997).

4.6. RNA gel blot analysis

Total RNA was isolated from organs of *Arabidopsis* wild-type and *pldα* plants, using a cetyltrimethylammonium bromide extraction method (Wang et al., 1994). RNA (20 μg/lane) was subjected to denaturing 1% formaldehyde-agarose gel electrophoresis and transferred onto a nylon membrane. The *PLDα1*-specific probe was labeled with [α-^{32}P]dATP by random priming. The probe made from this region detected only sense *PLDα1* RNA. The membrane was prehybridized and hybridized at 65 °C overnight and then washed as described previously (Fan et al., 1997). The blots were exposed to X-ray film.

4.7. Data analysis

Differences between genotypes (wild-type and *pldα1*) per organs, lipid classes and lipid species were assessed by *t*-test using a significance level of 0.05. PCA was used to separate and group organs of the two genotypes based on their overall composition in polar lipid species as well as to identify lipid species important for the observed groupings. For PCA, the dataset was composed of 110 samples (including the replicates per organ and per genotype) and 140 lipid molecular species. Lipids, expressed in mol%, were used and the data were log-transformed and standardized to the same scale. PCA was performed with the software package SYSTAT, version 10 (Systat Software Inc., Richmond, CA, USA).

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Appendix A. Supplementary data

Two supplementary data files are included in Excel format. "Supplementary data 1" contains the data shown in Figs. 2, 4–6, and 7 in tabular format. "Supplementary data 2" contains all the mass spectrometry data used in the lipid profiling, the dry weights of each sample, and the sample aliquot volumes for the mass spectrometry measurements. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.06.005.

References

Andersson, M.X., Larsson, K.E., Tjellström, H., Liljenberg, C., Sandelius, A.E., 2005. Phosphate-limited oat: the plasma membrane and the tonoplast as major targets for phospholipid-to-glycolipid replacement and stimulation of phospholipases in the plasma membrane. J. Biol. Chem. 280, 27578–27586.

Bohn, M., Heinz, E., Lüthje, S., 2001. Lipid composition of fluidity of plasma membranes isolated from corn (*Zea mays* L.) roots. Arch. Biochem. Biophys. 387, 35–40.

Brügger, B., Erben, G., Sandhoff, R., Wieland, F.T., Lehmann, W.D., 1997. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. Proc. Natl. Acad. Sci. USA 94, 2339–2344.

Fan, L., Zheng, S., Wang, X., 1997. Antisense suppression of phospholipase Dα retards abscisic acid and ethylene-promoted senescence of post-harvest Arabidopsis leaves. Plant Cell 9, 2183–2196.

Forrester, J.S., Milne, S.B., Ivanova, P.T., Brown, H.A., 2004. Computational lipidomics: a multiplexed analysis of dynamic changes in membrane lipid composition during signal transduction. Mol. Pharmacol. 65, 813–821.

Han, X., Gross, R.W., 2005. Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. Mass Spectrom. Rev. 24, 367–412.

Katavic, V., Reed, D.W., Taylor, D.C., Giblin, E.M., Barton, D.L., Zou, J., Mackenzie, S.L., Covello, P.S., Kunst, L., 1995. Alteration of seed fatty acid composition by an ethyl methanesulfonate-induced mutation in *Arabidopsis thaliana* affecting diacylglycerol acyltransferase activity. Plant Physiol. 108, 399–409.

Kim, Y.H., Choi, J.S., Yoo, J.S., Park, Y.M., Kim, M.S., 1999. Structural identification of glycerolipid molecular species isolated from cyanobacterium *Synechocystis* sp. PCC 6803 using fast atom bombardment tandem mass spectrometry. Anal. Biochem. 267, 260–270.

Lee, S., Suh, S., Kim, S., Crain, R.C., Kwak, J.M., Nam, H.-G., Lee, Y., 1997. Systemic elevation of phosphatidic acid and lysophospholipid levels in wounded plants. Plant J. 12, 547–556.

Li, W., Li, M., Zhang, W., Welti, R., Wang, X., 2004. The plasma membrane-bound phospholipase Dδ enhances freezing tolerance in *Arabidopsis thaliana*. Nature Biotechnol. 22, 427–433.

Meijer, H.J., Munnik, T., 2003. Phospholipid-based signaling in plants. Ann. Rev. Plant Biol. 54, 256–306.

Miquel, M., Cassagne, C., Browse, J., 1998. A new class of *Arabidopsis* mutants with reduced hexadecatrienoic acid level. Plant Physiol. 117, 923–930.

Mishra, G., Zhang, W., Deng, F., Zhao, J., Wang, X., 2006. A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis. Science 312, 264–266.

Murata, N., Sato, N., Takahashi, N., 1984. Very-long-chain fatty acids in phosphatidylserine from higher plant organs. Biochim. Biophys. Acta 795, 147–150.

Schneiter, R., Brügger, B., Amann, C.M., Prestwich, G.D., Epand, R.F., Zellnig, G., Wieland, F.T., Epand, R.M., 2004. Identification and biophysical characterization of a very-long-chain-fatty-acid-substituted phosphatidylinositol in yeast subcellular membranes. Biochem. J. 381, 941–949.

Somerville, C., Browse, J., Jaworski, J.G., Ohlrogge, J.B., 2000. In: Buchanan, B.B., Gruissem, W., Jones, R.L. (Eds.), ''Lipids'' in Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD, pp. 456–527.

Testerink, C., Munnik, T., 2005. Phosphatidic acid: a multifunctional stress signaling lipid in plants. Trends Plant Sci. 10, 368–375.

Vincent, P., Maneta-Peyret, L., Sturbois-Balcerzak, B., Duvert, M., Cassagne, C., Moreau, P., 1999. One of the origins of plasma membrane phosphatidylserine in plant cells is a local synthesis by a serine exchange activity. FEBS Lett. 464, 80–84.

Vincent, P., Maneta-Peyret, L., Cassagne, C., Moreau, P., 2001. Phosphatidylserine delivery to endoplasmic reticulum-derived vesicles of plant cells depends on two biosynthetic pathways. FEBS Lett. 498, 32–36.

Wang, C., Zien, C.A., Afitlhile, M., Welti, R., Hildebrand, D.F., Wang, X., 2000. Involvement of phospholipase D in wound-induced accumulation of jasmonic acid in Arabidopsis. Plant Cell 12, 2237–2246.

Wang, X., 2002. Phospholipase D in hormonal and stress signaling. Curr. Opin. Plant Biol. 5, 408–414.

Wang, X., 2004. Lipid signaling. Curr. Opin. Plant Biol. 7, 329–336.

Wang, X., Xu, L., Zheng, L., 1994. Cloning and expression of phosphatidylcholine-hydrolyzing phospholipase D from *Ricinus communis* L. J. Biol. Chem. 269, 20312–20317.

Wang, X., Devaiah, S.P., Zhang, W., Welti, R., 2006. Signaling functions of phosphatidic acid. Prog. Lipid Res. 45, 250–278.

Wanjie, S.W., Welti, R., Moreau, R.A., Chapman, K.D., 2005. Identification and quantification of glycerolipids in cotton fibers: reconciliation with metabolic pathway predictions from DNA databases. Lipids 40, 773–785.

Welti, R., Wang, X., 2004. Lipid species profiling: a high-throughput approach to identify lipid compositional changes and determine the function of genes involved in lipid metabolism and signaling. Curr. Opin. Plant Biol. 7, 337–344.

Welti, R., Li, W., Li, M., Sang, Y., Biesiada, H., Zhou, H.-E., Rajashekar, C.B., Williams, T.D., Wang, X., 2002. Profiling of membrane lipids in plant stress responses. J. Biol. Chem. 277, 31994–32002.

Welti, R., Shah, J., LeVine, S., Esch, S.W., Williams, T.D., Wang, X., 2005. High throughput lipid profiling to identify and characterize genes involved in lipid metabolism, signaling, and stress response. In: Feng, L., Prestwich, G.D. (Eds.), Functional Lipidomics. Marcel Dekker, New York, pp. 307–322, Chapter 14.

Wenk, M.R., 2005. The emerging field of lipidomics. Nat. Rev. Drug Discov. 4, 594–610.

Zhang, W., Qin, C., Zhao, J., Wang, X., 2004. Phospholipase Dα1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc. Natl. Acad. Sci. USA 101, 9508–9513.

Zien, C.A., Wang, C., Wang, X., Welti, R., 2001. In vivo substrates and the contribution of the common phospholipase D, PLDα, to wound-induced metabolism of lipids in Arabidopsis. Biochim. Biophys. Acta 1530, 236–248.