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Enhanced seed viability and lipid compositional changes during natural aging by suppressing phospholipase D α in soybean seed

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Summary

Changes in phospholipid composition and consequent loss of membrane integrity are correlated with loss of seed viability. Furthermore, phospholipid compositional changes affect the composition of the triacylglycerols, i.e. the storage lipids. Phospholipase D (PLD) catalyzes the hydrolysis of phospholipids to phosphatidic acid, and PLD α is an abundant PLD isoform. Although wild type seeds stored for 33 months were non-viable, 30 to 50% of PLD α -knockdown (PLD-KD) soybean seeds stored for 33 months germinated. Wild type and PLD-KD seeds increased in lysophospholipid levels and in triacylglycerol fatty acid unsaturation during aging, but the levels of lysophospholipids increased more in wild type than in PLD-KD seeds. The loss of viability of wild type seeds was correlated with alterations in ultrastructure, including detachment of the plasma membrane from the cell wall complex and disorganization of oil bodies. The data demonstrate that, during natural aging, PLD α affects the soybean phospholipid profile and the triacylglycerol profile. Suppression of PLD activity in soybean seed has potential for improving seed quality during long-term storage.

Keywords

genetic transformation; phospholipase D; seed viability; phospholipid; triacylglycerol; seed oil

Introduction

The preservation of seed viability and quality in storage is an important trait both for food usage and for seed use (Bentsink *et al.*, 2000). Generally, viability and quality of seeds gradually deteriorate after harvest (Coolbear, 1995; McDonald, 1999), but the deterioration in long-term storage depends on environment, biochemical, biological, and genetic factors. Changes characterized during the aging process in seeds include alterations in membrane protein composition (Nowakowska & Rakowski, 2002), disruption of the nuclear envelope (Haithcock *et al.*, 2005), protein degradation (Kumar *et al.*, 1999), decreases in lipid content (Lin & Pearce, 1990), oxidative stress, and decreases in mRNA translation (Gidrol *et al.*,

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Supporting Information

Additional supporting information may be found in the online version of this article.

1990) and DNA replication capabilities (de Castro *et al.*, 1995). Reduced levels of antioxidant enzymes such as superoxide dismutase, catalase, and ascorbate peroxidase can also lead to oxidative damage (Bailly *et al.*, 1996; 1998).

Degradation of phospholipids and structural deterioration of biological membranes have been suggested to be particularly significant issues during aging in soybean seeds (Parrish & Leopold, 1978). When Ca^{2+} is added to young bean cotyledons, the action of phospholipase D (PLD) and phosphatidic acid phosphatase can induce lipid breakdown in smooth microsomal membranes (Duxbury *et al.*, 1991). Duxbury *et al.* suggested that degradation of the phospholipid bilayer during natural aging of soybean cotyledons, facilitated by lipolytic enzymes, stimulates loss of protein function and proteolysis. In natural aging of seeds, the loss of seed vigor and germination ability is also coincident with increased electrolyte leakage (Leinonen, 1998; Rakowski *et al.*, 1998). Moreover, changes in lipid composition, including oxidation and peroxidation can lead to loss of the economic and nutritional value of soybean seed through modifications in color, taste, odor, and viability during shipment and storage (Nakayama *et al.*, 1981; Samama & Pearce, 1993).

Lipid degrading enzymes may play important roles in the natural aging process in seeds (Devaiah *et al.*, 2007). PLD can catalyze degradation of membrane phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG), producing phosphatidic acid (PA) and a free head group. Accumulation of PA in the cell may lead to formation of hexagonal II-phase lipid particles with loss of cell membrane integrity (Kooijman *et al.*, 2003). PA produced by phospholipase D is also known to regulate other cellular activities (Wang *et al.*, 2006). It has been suggested that degradation of membrane lipids by PLDs also can lead to senescence and cell death (Fan *et al.*, 1997).

Recently, we investigated alterations in phospholipid and triacylglycerol profiles caused by suppression of PLD in transgenic soybean (Lee *et al.*, 2011). Knockdown of PLD induced increased unsaturation of phospholipids, mainly PC and PE, and decreased unsaturation of the triacylglycerol (TAG) fraction in fresh soybean seeds. These changes may have reflected a slowdown in formation of TAG from PC in the PLD-KD seeds compared to wild type seeds. Suppressing and ablating PLD α 1 in *Arabidopsis* seeds enhanced seed viability and lipid stability resulting from reduced loss of oil fatty acids during aging (Devaiah *et al.*, 2007). However, the effects of PLD α suppression on storage lipid profiles and particularly phospholipid membrane properties during seed aging are still not fully understood. Here, we investigate how knockdown of PLD α affects membrane phospholipids and storage lipids during aging and how these effects relate to stability of soybean seeds.

Results

Changes in the level of PLD α protein and enzyme activity in seeds of T₂, null-transgenic, and the background soybean cultivar

PLD α -suppressed (PLD-knockdown or PLD-KD) soybean lines were generated by transferring pSPLDi and pHG1 plasmids via particle inflow gun bombardment, resulting in knockdown of PLD α activity by RNA interference (RNAi) in the lines transformed with pSPLDi. *PLD* gene expression, physiological cost, and biochemical changes in PLD-KD transgenic soybean seeds were characterized in a previous report (Lee *et al.*, 2011). To confirm the suppression of PLD α in pSPLDi transgenic soybean seed, pSPLDi PCR, western blotting for PLD α , and a PLD α enzyme assay were performed using immature cotyledons of T₂ and T₅ SW lines. The presence of the transgene in T₂ SW1 and SW-derived T₅ line seeds was confirmed by PCR, specifically amplifying the pSPLDi construct (Figure 1a and c). Monitoring soybean PLD protein in the soybean extract using an *Arabidopsis thaliana* PLD α 1 (*AtPLD α 1*) antibody showed that there was decreased PLD α

in the immature cotyledons of transgenic SW1-3 and SW1-7 lines compared to wild type (Fayette) and the null-transgenic segregating seed, SW-N (Figure 1a). The size of the PLD α protein, 92 kDa in denaturing gels, was consistent with previous data (Ryu *et al.*, 1996). When the endogenous *PLD* gene expression was suppressed by the RNAi machinery, the immature cotyledons of T₂ and T₅ transgenic lines showed a 40 to 50 percent reduction in PLD α activity compared to wild type, whereas null-transgenic segregating seed did not show a PLD α activity reduction (Figure 1b and d).

Changes in PLD α -suppressed soybean seeds response to natural aging

To determine whether PLD α activity affects soybean seed viability, seed germination and electrolyte leakage of fresh and naturally aged PLD-KD and wild type seeds were evaluated. The germination percentage of fresh soybean seeds from each genotype was approximately 95 percent. After 33 months storage at room temperature, no aged wild type seeds had germinated after 10 days of imbibition, whereas the germination of aged PLD-KD seeds of the SW1-3 and SW1-7 lines was observed to be 50 and 30%, respectively (Figure 2a). Because T₂ seeds might be null, heterozygous, or homozygous for the transgene, we performed PCR analysis to determine the relationship between the presence of the transgene and seed viability in the SW1-3 progeny line (Figure 2b). PCR analysis showed that five out of 6 seedlings that germinated were transgenic, and only two out of 6 seeds that didn't germinate were transgenic. These results suggest that the viability of 33 month-old seeds was associated with suppression of PLD α .

Leakage of endogenous electrolyte is indicative of poor membrane integrity. Electrolyte leakage from aged null-transgenic and wild type seeds was significantly greater than from aged PLD-suppressed lines (Figure 2c). Ion leakage from SW1-3 and SW1-7 was around 14%, whereas leakage from wild type seeds and null-transgenic soybean seeds was about 20%. Thus, the improved viability of PLD-KD seeds compared to the wild type seeds under natural aging was correlated with increased membrane stability.

Changes in ultrastructure of cotyledon tissues in response to natural aging

Transmission electron microscopy was used to investigate ultrastructural changes in plasma membrane and the cell wall complex of fresh wild type, aged wild type, and aged PLD-KD cotyledons (Figure 3). Obvious changes in cell structure were detected by light microscopy with 100 \times magnification (Figure S1). The plasma membrane in aged wild type cotyledon tissue was partially plasmolyzed and detached from the cell wall complex (Figure 3b). Plasma membrane detachment was observed in both aged PLD-KD and wild type cotyledon tissues; however, the plasma membrane in wild type cotyledons was irregularly piled, resulting from cell dehydration (Figure 3b and e). The presence of voids between the plasma membrane and secondary cell wall in aged wild type cotyledons indicated that a structural alteration of the plasma membrane occurred during the aging process. The greater structural changes that occurred in wild type seeds, in comparison to PLD-KD seeds, were coincident with the greater loss of seed viability and higher electrolyte leakage.

Changes in phospholipid and triacylglycerol profiles after natural aging of wild type and PLD-KD soybean seeds

To determine the metabolic basis for the changes in membrane structure and electrolyte leakage during aging and to identify differences in lipid metabolism between wild type and PLD-KD seeds, the lipid compositions of the soybean seeds were analyzed by ESI-MS/MS. Total lysophosphatidylcholine (lysoPC), lysophosphatidylethanolamine (lysoPE), and lysophosphatidylglycerol (lysoPG) were dramatically increased in both wild type and PLD-KD seeds by natural aging, but increases were higher for lysoPC and lysoPG in wild type

seeds compared to PLD-KD seeds (Table 1). PA was not significantly increased in aged seeds, compared to fresh seeds.

Analysis of lipids of wild type seeds at the level of molecular species showed that the phospholipids of WT seeds exhibited more significant changes during aging than did the phospholipids of PLD-KD seeds (Figure 4). The phospholipids of wild type seeds increased in unsaturation during natural aging, while the molecular species of PLD-KD phospholipids did not change as much during aging. In particular, diacyl 34-C phospholipid species with two or more double bonds and diacyl 36-C phospholipid species with four or more double bonds in the two acyl chains tended to increase during aging of wild type seeds, while those with fewer double bonds tended to decrease. For example, the lipid profiling data showed that 34:2-PG and 36:4-PC were dramatically increased in wild type seeds after natural aging, while 36:2- and 36:3- species of PE, PC, and PA were decreased.

Table 2 delineates the individual fatty acyl composition of PCs, PEs, and PIs, with diacyl species designated 36:4, 36:3, and 36:2. Phospholipids with 36:4 species are primarily di18:2 combinations, and this combination becomes more predominant during aging in wild type seeds, while species containing 18:1 with 18:3 decline during aging. Phospholipids with 36:3, which tend to decrease during aging of wild type seeds, are primarily 18:1 with 18:2. PC and PE with 36:2, which decline during aging of wild type seeds, are a mixture of di18:1 and 18:0 with 18:2; during aging, di18:1 species exhibit a large decline in both genotypes. Overall, phospholipid data, shown in Figure 4 and Table 2, and summarized in Supplementary Figure S2, show that phospholipids containing 18:1 decline during aging in wild type seeds.

Figure 5 shows the molecular species of lysolipids formed during aging. As seen here and in Table 2, PLD-KD seeds produced fewer of these lipolytic products during natural aging than wild type seeds. Nearly every molecular species of lysoPC, lysoPE, and lysoPG levels increased during seed aging. The significantly greater gains during aging in lysoPC and lysoPG formation in wild-type compared to PLD-KD seeds (Table 1) were due mainly to increases in 18:2-lysoPLs.

Triacylglycerols containing particular fatty acyl species were measured by ESI-MS/MS (as normalized mass spectral signal; Table 1 and Figure 6). This technique reveals overlapping subsets of TAG molecular species with one acyl chain specified and the other two determined together (Lee et al., 2011). During aging, the total amount of triacylglycerol signal in wild type seeds tended to decrease, albeit not quite significantly ($p = 0.069$) (Table 1). In addition TAG species in both genotypes became more unsaturated (Figures 6 and 7). Unsaturation index (UI), the average number of fatty acyl double bonds per fatty acid present, provides a simple measure of overall unsaturation (Figures 7 and S3).

There were significant decreases in 18:1-containing TAG molecular species in both genotypes (Table 1 and Figure 6), and in most TAG molecular species containing 36:2- and 36:3-diacyl components (Figure 6). 36:3 and 36:2 molecular species were demonstrated to contain 18:1 fatty acyl chains in PC (Table 2); because TAG diacyl groups are derived from PC (Bates et al., 2009), it is likely that 36:3 and 36:2 TAG molecular species also contain 18:1. Thus, these data, which are summarized in Supplementary Figure S3, are consistent with a tendency toward an overall decrease in 18:1 in TAG species in seeds; this trend was also detected directly by fatty acid compositional analysis (Supplementary Table 1).

Discussion

Post-harvest degradation of phospholipids reduces seed viability and oil quality during long-term storage of soybean seed (Wilson & McDonald, 1986; Danielle *et al.*, 1993; Bailly *et al.*,

1996). PLDs can be involved in membrane phospholipid degradation and loss of seed viability by natural and accelerated aging (Devaiah *et al.*, 2007). We demonstrated that suppression of PLD α preserves soybean seed viability and quality during long-term storage. Immature seeds of PLD-suppressed T₂ and T₅ transgenic lines showed lower levels of PLD α protein and PLD α activity than null-transgenic and wild type cultivars (Figure 1). Agricultural traits of PLD-suppressed soybean were described in our previous report (Lee *et al.*, 2011).

During storage, temperature, moisture, and time are critical factors affecting physical, physiological and biochemical changes that impact seed properties. These changes include loss of viability, grain color changes, alterations in moisture content, decomposition of lipids, degradation of protein, and damage to plasma and organelle membranes (Bailly *et al.*, 1996; 1998; Kumar *et al.*, 1999). After storage for 33 months, wild type seeds failed to germinate, whereas aged PLD-KD transgenic seeds maintained 30 to 50% germination (Figure 2a). The greater viability of PLD-KD seeds compared to wild type after aging was associated with (1) decreased electrolyte leakage (Figure 2) (2) fewer ultrastructural changes (Figure 3), and (3) fewer lipid compositional changes. In particular, PLD-KD seeds produced lower levels of lysoPLs than wild type seeds, and TAG levels in PLD-KD seeds were maintained during aging, while TAG levels in wild type seeds tended to decrease during aging.

Loss of viability has been shown to be positively correlated with an increase in electrolyte leakage in sunflower seeds during natural aging (Corbinau *et al.*, 2002). Electrolyte leakage could be caused by membrane phospholipid deterioration. Sphingolipids, ubiquitous and particularly abundant integral components of the plasma membrane in eukaryotic cells, are involved in various cellular functions (Hait *et al.*, 2006). Phospholipase D and phosphatidic acid have been shown to affect activity of plant sphingolipid-metabolizing enzymes and sphingolipid profiles (Guo *et al.*, 2011). Recently, altered sphingolipid profiles have been shown to change the leaf ionome in *Arabidopsis* (Chao *et al.*, 2011). Thus, we cannot rule out the notion that differences in membrane integrity between non-transgenic and transgenic seeds are caused by differences in sphingolipid metabolism, secondary to the phospholipase D differences, during natural aging. Additionally, the storage lipids, mainly TAG, in soybean seeds are coated with a phospholipid monolayer to form a lipid body in the cytosol. Lipolysis of phospholipids at the oil body surface may destabilize the oil bodies, contributing to their degradation, eventually causing deterioration of seed quality. PLD α is located in the cytosol and can act on lipids in the plasma membrane or in intracellular (microsomal) membranes (Xu *et al.*, 1996).

It has been suggested that high levels of PLD α 1 activity cause formation of PA, resulting in membrane and storage lipid degradation in seeds (List *et al.*, 1992). In this study, PA levels in seeds at 33 months were not significantly increased in either genotype (Table 1 and Figure 4). Devaiah *et al.* (2007) reported that levels of PA increased 30% after accelerated aging treatment in wild type *Arabidopsis*. Despite the lack of a measurable change, PLD α may be acting locally to create PA involved in signaling responses (Wang *et al.*, 2006). Such responses may lead to local alterations in enzyme activities that result in compromised membrane integrity. For example, the lower level of lysophospholipids observed in PLD-KD seed, compared to wild type seed, may reflect lower levels of lipolytic activity by phospholipase As during natural aging. It has been reported that endogenously accumulated PA formed by PLD action can enhance PLA₂ activity in mammalian cells (Sato *et al.*, 1993; Kinkald *et al.*, 1998), and it's possible that PLD or its PA may also stimulate phospholipase A activity in soybean seeds. LysoPLs may increase membrane permeability during natural aging, and the somewhat higher levels of these lipolytic products in the wild type seeds

compared to the PLD-KD seeds may contribute to the higher electrolyte leakage observed in the wild type seeds.

Significant differences in the unsaturation of the PC of wild type and PLD-KD seeds were observed (Figures 4 and 7; Lee *et al.*, 2011). Our previous report suggested that knockdown of PLD may retard the conversion of PC to DAG to TAG in developing soybean seed, causing fatty acids retained in PC, which is the substrate for desaturases, to become more unsaturated in fresh seeds (Lee *et al.*, 2011). Here, we show that the overall levels of TAG unsaturation in aged wild type and PLD-KD seeds are similar, but aged seeds of both wild type and PLD-KD lines contain more unsaturated TAG species than fresh seeds. The data suggest the possibility that lipid metabolism, including acyl desaturation, occurs during aging of mature seeds. Further work is needed to test this notion. Stable isotope labeling would be an attractive approach, but labeling intact seeds and analyzing over an extended time is logistically challenging.

In the current study, RNAi silencing with a partial *PLD α* sequence and seed specific β -conglycinin α' subunit promoter showed around 40% reduction in PLD α activity compared to wild type seed. Devaiah *et al.* (2007) showed that, in *Arabidopsis*, seed vigor and germination of PLD α -knockout *Arabidopsis* was better than that of the PLD α -ablated *plda1-1* after natural aging, and they proposed that PLD α activity is needed for optimal germination and seedling growth. PLD α -attenuation did affect some agronomic traits of soybean, such as seed quality, plant height, and seed weight (Lee *et al.*, 2011). Considering the data described herein, we propose that suppression of PLD α in soybean seeds improves seed oil storability and long-term viability of seeds after harvest.

Experimental Procedures

Plant material, seed storage, germination test, and electrolyte leakage

The soybean cultivar, Fayette (background cultivar or “wild type” in this study) (Bernard *et al.*, 1988), was co-transformed with pSPLDi harboring RNAi structure (800 bp sense and 1300 bp antisense soybean *PLD* fragments) and pHG1 (CaMV35S:HPH) via particle inflow gun bombardment and regenerated (Lee *et al.*, 2011). T₁ soybean lines SW1-3, SW1-7, SW1-5-N (null transgenic line) and wild type were grown in a greenhouse under 25 ± 2 °C day and 20 ± 2 °C night temperatures and a 16 h light/8 h dark photoperiod. Immature pods were analyzed at approximately 26 days after fertilization (DAF) by PCR, enzyme assay, and western blotting for PLD α . T₂ seeds were harvested from each T₁ plant, and dried to 13% moisture. T₂ transgenic and wild type seeds were stored in paper envelopes at 25 °C and 17% relative humidity for 33 months, and T₅ seeds derived from SW1-3 and SW1-7 lines were harvested and stored under the same conditions for two months. The viability of transgenic, SW1-3 and SW1-7, and wild type seeds stored for 2 and 33 months was evaluated using a standard germination test. Three replicates of ten seeds from each genotype were wrapped in a 25 cm-long roll of paper and drenched in water. One end of the paper was submerged. The water level was maintained 5 cm below the seeds in order to supply the same amount of water to each seed. The number of soybean sprouts was counted after 10 days.

Electrolyte leakage from seeds stored 2 and 33 months was measured by a modification of the method of Fan *et al.* (1997). Briefly, three soybean seeds were agitated in 3 ml of 0.4 M mannose for 6 h to induce ion leakage. Conductivity of the solution was measured with a YSI model 32 conductance meter (Yellow Springs Instrument Co., Inc.) after vortexing the samples for 30 s. The total electrolyte concentration was determined after boiling samples for 15 min and cooling to 25 °C. Electrolyte leakage was expressed as a percentage of the

initial electrolytic concentration divided by total the electrolyte concentration in the solution. Three replicates with three seeds per replication were evaluated for each soybean line.

PCR analyses

Genomic DNA was extracted from young soybean leaves and immature seeds using a simplified CTAB (cetyl trimethylammonium bromide) DNA extraction method by Saghai-Marooof et al. (1984). Two hundred ng of genomic DNA were used as a template in a 50 μ l PCR reaction containing 20 pmol each of forward and reverse primer, 0.2 mM of deoxynucleotide triphosphate (dNTPs), 1.25 U Taq DNA polymerase, and 1.5 mM MgCl₂ in PCR buffer (New England BioLabs, MA, USA). Specific primer sequences of the transgene left arm (forward: 5'-TATTCAACACCCGTCA-3' and reverse: 5'-CCACCATGACATGCGGAAACC-3'), the transgene right arm (forward: 5'-TTTCCGCATGTTCATGTGGTA-3' and reverse: TAGCCCGATACTTTCCT-3'), and hygromycin phosphotransferase (forward: 5'-GCACAATCCCACTATCCTTCGCAA-3' and reverse: 5'-CTTCTACACAGCCATCGGTCCAG-3'), designed by primer3 software (<http://frodo.wi.mit.edu>), were used for transgene PCR using a Hybaid PCR Express Thermocycler (Hybaid, UK) and a Multigene Thermocycler (Labnet International Inc., NJ, USA). The left arm and right arm of the pSPLDi construct were amplified using one cycle at 94 °C for 5 min; 30 cycles at 94 °C for 30 sec, 52 °C for 60 sec, and 72 °C for 90 sec; and a final cycle at 72 °C for 7 min with forward β CONGF (5'-taa ttc aac acc cgt ca-3') and reverse SQSP1 (5'-cca caa tga cat gcg gaa acc-3'). PCR products were run on 0.8% agarose gel with 1X TAE buffer at 50 V. The gel was stained in ethidium bromide solution for 10 min, and the stained gel image was acquired with Kodak 1D™ image analysis software (Eastman Kodak Company, New Haven, CT, USA) on a UV transilluminator.

Western blot analysis and PLD enzyme assay

Total protein from a T₂ immature cotyledon was extracted using a chilled pestle in a 1.5 mL microcentrifuge tube with liquid N₂ and a homogenization buffer (50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 0.5 mM phenyl methylsulfonyl fluoride, and 2 mM DTT) at 4 °C (Fan *et al.*, 1997). The concentration of total native protein was measured with a Nanodrop ND1000 spectrophotometer (Nanodrop, DE, USA). For western blot analysis, 20 μ g of total native proteins were incubated at 95 °C for 15 min with SDS-PAGE loading buffer (100 μ l of 50 mM Tris-HCl, pH 6.8, 10 mM DTT, 2% SDS, 0.01% bromophenol blue, and 10% glycerol). Denatured protein was separated in 10% SDS-PAGE gel at 100 V until the loading dye reached the bottom of the gel. Gels were transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) using electrophoretic transfer with a Bio-Rad criterion blotter (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The transferred membrane was blotted with *Arabidopsis* PLD α antibodies as described by Zhang *et al.* (2003). Briefly, non-fat milk was used for membrane blocking for 1 h with agitation at 4 °C, and membranes were soaked in *At*PLD α antibody (1:1000 in TBST containing 10 mM Tris, 140 mM NaCl and Tween 20, pH 7.4) overnight. The blots were washed 3 times in 1X TBST for 5 min per wash. The blots were incubated in a secondary antibody (1:1000 diluted Goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (Bio-Rad, CA, USA) in TBST buffer) for 1 h and then washed two times in 1X TBST followed by one wash in TBS (10 mM Tris, 140 mM NaCl, pH 7.5). The protein bands binding to antibodies were visualized with a HRP color development reagent (Bio-Rad, CA, USA).

Aliquots of 20 μ g of native protein were used for the PLD enzyme assay as described previously (Fan *et al.*, 1997). Briefly, the reaction mixture contained 100 mM MES, pH 6.5, 25 mM CaCl₂, 0.5 mM SDS, 1% (v/v) ethanol, and 2 mM PC (egg yolk) containing dipalmitoyl glycerol-3-phosphate-(methyl-³H)-choline in a final volume of 200 μ l. Substrate preparation, reaction conditions, and product separation were based on a previously

described procedure (Wang *et al.*, 1993). The release of ^3H -labeled choline into the aqueous phase was quantified by liquid scintillation counting (Beckman-Coulter, CA, USA).

Transmission electron microscopy (TEM)

Soybean tissues were prepared for TEM and the image captured using a protocol modified from that of Boyle and Takemoto (1998). Briefly, soybean cotyledon tissues were immersed and fixed in 2% paraformaldehyde (Ladd, Burlington, VT, USA), 2% glutaraldehyde, and 10% sucrose in 0.1 M sodium cacodylate buffer pH 6.8 for 16 h at room temperature with constant rotation. Samples were post-fixed with 2% osmium tetroxide and 10% sucrose in 0.1 M sodium cacodylate buffer pH 6.8. Fixed tissues were dehydrated in an ascending series of acetone with 50% – 100% at room temperature, embedded in Spurr's embedding media in gelatin capsules, and polymerized at 65 °C for 24 h. Embedded soybean tissues were cut with glass knives on a Reichert Ultracut S microtome (Leica, Austria) and held on 100 nickel slotted grids. All images were captured using an Advanced Microscopy Techniques digital image capturing system (ATM, Chazy, NY, USA).

Polar lipid, triacylglycerol, and major fatty acid analyses

Lipids were extracted from soybean seed and analyzed by ESI-MS/MS as described by Lee et al. (2011). Fatty acid composition was determined by gas-liquid chromatography on a Supelco 30-m Omegawax 250 capillary column (Sigma-Aldrich, St. Louis, MO, USA) after derivatization of the fatty acids to methyl esters in 1.5 M methanolic HCl, followed by addition of water, and extraction with pentane.

Unsaturation index and percentage of MUFA and PUFA

The unsaturation index (UI) was calculated as the average number of double bonds per fatty acid present (the sum of the percentage of each lipid \times number of double bonds)/(number of fatty acid species per lipid, i.e. 2 for PLs and 3 for TAGs) with molecular species composition of TAG and PLs determined by ESI-MS/MS. UI data are presented as mean \pm SD and Tukey's studentized range test with five determinations. Percentage of monounsaturated fatty acid (% MUFA) and percentage of polyunsaturated fatty acid (% PUFA) were calculated from molecular species composition of each PL class and total TAG, and then % unsaturated fatty acid was calculated from each molecular species composition (sum of MUFA and PUFA divided by total molecular composition \times 100). The molecular species compositions used for the calculations (for phospholipids and TAGs) were: 36:6, di18:3; 36:5, 18:3/18:2; 36:4, 36:3, and 36:2, as indicated in Table 2; 34:3, 16:0/18:3; 34:2, 16:0/18:2; 34:1, 16:0/18:1; 32:0, di16:0.

Statistical analysis

Data collected from the fatty acid composition, electrolyte leakage analysis, PLD enzyme assays, and UI were examined by analysis of variance using SAS (SAS Institute Inc., NC, USA) PROC general linear model (GLM) procedure. Means were separated using the LSD or Tukey test at the $p < 0.05$ probability level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Bailly C, Benamar A, Corbineau F, Come D. Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seeds as related to deterioration during accelerated aging. *Physiol Plant*. 1996; 97:104–110.
- Bailly C, Benamar A, Corbineau F, Come D. Free radical scavenging as affected by accelerated ageing and subsequent priming in sunflower seeds. *Physiol Plant*. 1998; 104:646–652.
- Bates PD, Durrett TP, Ohlrogge JB, Pollard M. Analysis of acyl fluxes through multiple pathways of triacylglycerol synthesis in developing soybean embryos. *Plant Physiol*. 2009; 150:55–72. [PubMed: 19329563]
- Bentsink L, Alonso-Blanco C, Vreugdenhil D, Tesnier K, Groot SPC, Koornneef M. Genetic analysis of seed-soluble oligosaccharides in relation to seed storability of *Arabidopsis*. *Plant Physiol*. 2000; 124:1595–1604. [PubMed: 11115877]
- Bernard RL, Noel GR, Anand SC, Shannon JG. Registration of Fayette soybean. *Crop Sci*. 1988; 28:1028–1029.
- Boyle DL, Takemoto LJ. Finger-like projections of plasma membrane in the most senescent fiber cells of human lenses. *Current Eye Res*. 1998; 17:1118–1123.
- Chao DY, Gable K, Chen M, Baxter I, Dietrich CR, Cahoon EB, Guerinot ML, Lahner B, Lü S, Markham JE, Morrissey J, Han G, Gupta SD, Harmon JM, Jaworski JG, Dunn TM, Salt DE. Sphingolipids in the root play an important role in regulating the leaf ionome in *Arabidopsis thaliana*. *Plant Cell*. 2011; 23:1061–1081. [PubMed: 21421810]
- Coolbear, P. Mechanism of seed deterioration. In: Basra, AS., editor. *Seed Quality: Basic Mechanisms and Agricultural Implications*. New York: Food Product Press; 1995. p. 223-277.
- Corbineau F, Gay-Mathieu C, Vinel D, Côme D. Decrease in sunflower (*Helianthus annuus*) seed viability caused by high temperature as related to energy metabolism, membrane damage and lipid composition. *Physiol Plant*. 2002; 116:489–496.
- de Castro RD, Zheng X, Bergervoet JHW, De Vos CHR, Bino RJ. β -tubulin accumulation and DNA replication in imbibing tomato seeds. *Plant Physiol*. 1995; 109:499–504. [PubMed: 12228608]
- Devaiah SP, Pan X, Hong Y, Roth M, Welti R, Wang X. Enhancing seed quality and viability by suppressing phospholipase D in *Arabidopsis*. *Plant J*. 2007; 50:950–957. [PubMed: 17565616]
- Duxbury CL, Legge RL, Thompson JE. Lipid breakdown in smooth microsomal membranes from bean cotyledons alters membrane proteins and induces proteolysis. *J Exp Bio*. 1991; 42:103–112.
- Fan L, Zheng S, Wang X. Antisense suppression of phospholipase D α retards abscisic acid- and ethylene-promoted senescence of postharvest *Arabidopsis* leaves. *Plant Cell*. 1997; 9:2183–2196. [PubMed: 9437863]
- Gidrol S, Noubhani A, Pradet A. Biochemical changes induced by accelerated aging in sunflower seeds. II RNA populations and protein synthesis. *Physiol Plant*. 1990; 80:598–604.
- Guo L, Mishra G, Taylor K, Wang X. Phosphatidic acid binds and stimulates Arabidopsis sphingosine kinases. *J Biol Chem*. 2011; 286:13336–13345. [PubMed: 21330371]
- Hait NC, Oskeritzian CA, Paugh SW, Milstien S, Spiegel S. Sphingosine kinases, sphingosine 1-phosphate, apoptosis and disease. *Biochim Biophys Acta*. 2006; 1758:2016–2026. [PubMed: 16996023]
- Haitcock E, Dayani Y, Neufeld E, Zahand AJ, Feinstein N, Mattout A, Gruenbaum Y, Liu J. Age-related changes of nuclear architecture in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA*. 2005; 102:16690–16695. [PubMed: 16269543]

- Kinkald AR, Othman R, Voysey J, Wilton DC. Phospholipase D and phosphatidic acid enhance the hydrolysis of phospholipids in vesicles and in cell membranes by human secreted phospholipase A₂. *Biochim Biophys Acta*. 1998; 1390:173–185. [PubMed: 9507109]
- Kooijman EE, Chupin V, de Kruijff B, Burger KN. Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. *Traffic*. 2003; 4:162–174. [PubMed: 12656989]
- Kumar GNM, Houtz LR, Knowles NR. Age-induced protein modifications and increased proteolysis in potato seed-tubers. *Plant Physiol*. 1999; 119:89–100. [PubMed: 9880350]
- Lee J, Welti R, Schapaugh WT, Trick HN. Phospholipid and triacylglycerol profiles modified by PLD suppression in soybean seed. *Plant Biotech J*. 2011; 9:359–372.
- Leinonen K. Effect of storage conditions on dormancy and vigor of *Picea abies* seeds. *New Forest*. 1998; 16:231–249.
- Lin SS, Pearce RS. Changes in lipids of bean seed (*Phaseolus vulgaris*) and corn caryopses (*Zea mays*) aged in contrasting environments. *Ann Botany*. 1990; 65:451–456.
- List GR, Mounts TL, Lanser AC. Factors promoting the formation of nonhydratable soybean phosphatides. *JAOCS*. 1992; 69:443–446.
- McDonald MB. Seed deterioration: physiology, repair and assessment. *Seed Sci Technol*. 1999; 27:177–237.
- Nakayama Y, Sayo K, Kito M. Decomposition of phospholipids in soybean during storage. *Cereal Chem*. 1981; 58:260–264.
- Nowakowska J, Rakowski K. Accelerated and natural ageing processes change the properties of plasma membrane in Norway spruce (*Picea abies* L. Karst) seeds during storage. *Dendrobiology*. 2002; 47:79–82.
- Parrish DJ, Leopold AC. On the mechanism of aging in soybean seeds. *Plant Physiol*. 1978; 61:365–368. [PubMed: 16660294]
- Rakowski K, Behzadipour M, Ratajczak R, Kluge M. Effect of seeds freezing and storage conditions on plasma membrane properties of Norway spruce (*Picea abies* Karst) seedlings. *Botanica Acta*. 1998; 111:236–240.
- Ryu SB, Zheng L, Wang X. Changes in phospholipase D expression in soybeans during seed development and germination. *JAOCS*. 1996; 73:1171–1176.
- Saghai-Marooof MA, Soliman KM, Jorgensen RA, Allard RW. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc Natl Acad Sci USA*. 1984; 81:8014–8018. [PubMed: 6096873]
- Samama AM, Pearce RS. Ageing of cucumber and onion seeds-phospholipase-D, lipoxygenase activity and changes in phospholipid content. *J Exp Bot*. 1993; 44:1253–1265.
- Sang Y, Cui D, Wang X. Phospholipase D and phosphatidic acid-mediated generation of superoxide in *Arabidopsis*. *Plant Physiol*. 2001; 126:1449–1458. [PubMed: 11500544]
- Sato T, Ishimoto T, Akiba S, Fujii T. Enhancement of phospholipase A₂ activation by phosphatidic acid endogenously formed through phospholipase D action in rat peritoneal mast cell. *FEBS Lett*. 1993; 24:23–26. [PubMed: 8495740]
- Wang X, Dyer JH, Zheng L. Purification and immunological analysis of phospholipase D from Cater Bean Endosperm. *Arch Biochem Biophys*. 1993; 306:486–494. [PubMed: 8215453]
- Wang X, Devaiah SP, Zhang W, Welti R. Signaling functions of phosphatidic acid. *Biochim Biophys Acta*. 2006; 45:250–278.
- Wilson DO Jr, McDonald MB Jr. The lipid peroxidation model of seed ageing. *Seed Science and Technology Zürich*. 1986; 14:269–300.
- Xu L, Paulsen AQ, Ryu SB, Wang X. Intracellular localization of phospholipase D in leaves and seedling tissues of castor bean. *Plant Physiol*. 1996; 111:101–107. [PubMed: 12226277]
- Zhang W, Wang C, Qin C, Wood T, Olafsdottir G, Welti R, Wang X. The oleate-stimulated phospholipase D, PLD δ and phosphatidic acid decrease H₂O₂-induced cell death in *Arabidopsis*. *Plant Cell*. 2003; 15:2285–2295. [PubMed: 14508007]

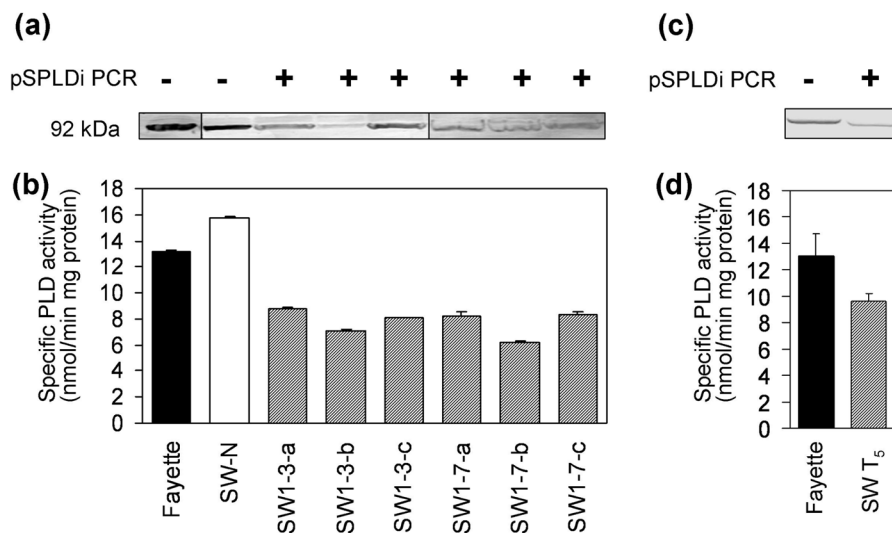


Figure 1. pSPLDi PCR, western blotting for PLD α , and a PLD α enzyme activity in immature cotyledons from, (a) and (b), heterozygous T₂ SW1 and, (c) and (d), a T₅ SW line. (a) and (c) The presence of the pSPLDi transgene was confirmed with GOI PCR. + indicates PCR-positive for pSPLDi, and - indicates PCR-negative. Immunoblot analysis of PLD α was performed with *Arabidopsis* PLD α 1 antibody, and PLD α proteins were detected at the 92 kDa position, based on protein markers. (b) and (d) PLD α enzyme assay of T₂ SW1 and T₅ SW seeds. PLD α enzyme activity was expressed as nmol of PC hydrolyzed min⁻¹ mg⁻¹total protein. Error bars indicate \pm SE from three independent measurements .

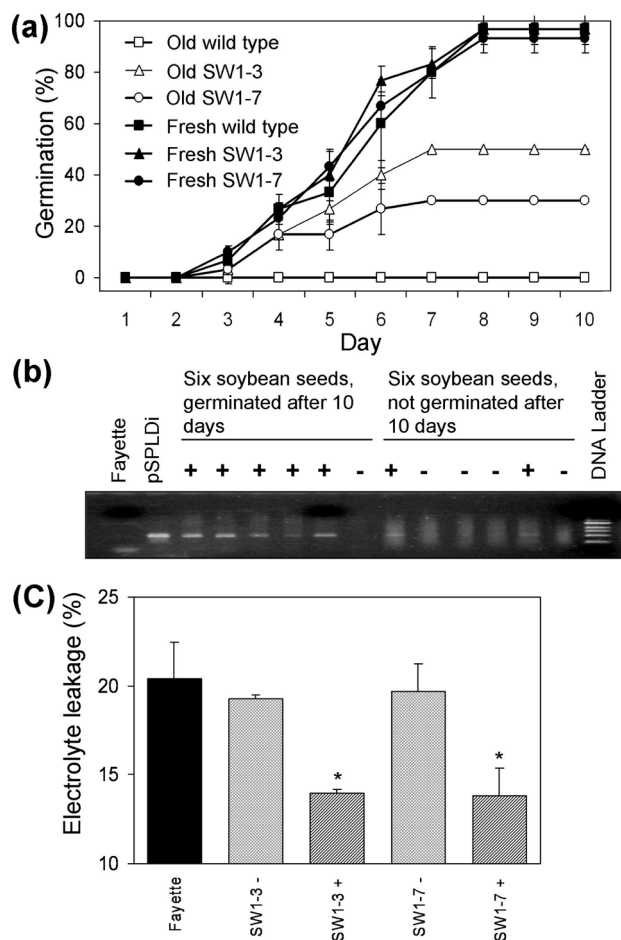


Figure 2. Effect of PLD α suppression on seed germination of soybean seeds stored for 33 months. T₂ SW1-3 and SW1-7 seeds were tested for germination, the presence of the pSPLDi transgene, and electrolyte leakage. (a) Fresh and aged soybean seeds were placed on a wet paper roll for 10 days, and soybean sprouts were counted to calculate germination (%), number of germinated seeds/total number of seeds \times 100). Values are mean \pm SD (n = 30 in three replications). (b) PCR analysis for the presence of pSPLDi transgene in soybean sprout from 33 month-old soybean seeds. (c) Effect of PLD α suppression on electrolyte leakage of 33 month-old soybean seeds. Wild type (Fayette), SW1-3 transgene PCR negative (-), SW1-3 transgene PCR positive (+), SW1-7 transgene PCR negative (-), and SW1-7 transgene PCR positive (+) seeds were tested for ion leakage by conductivity measurements. * indicates a significant difference at the $p < 0.05$ level in transgenic (PCR positive) or null-transgenic (PCR negative) compared to the wild type genotype (n = 3).

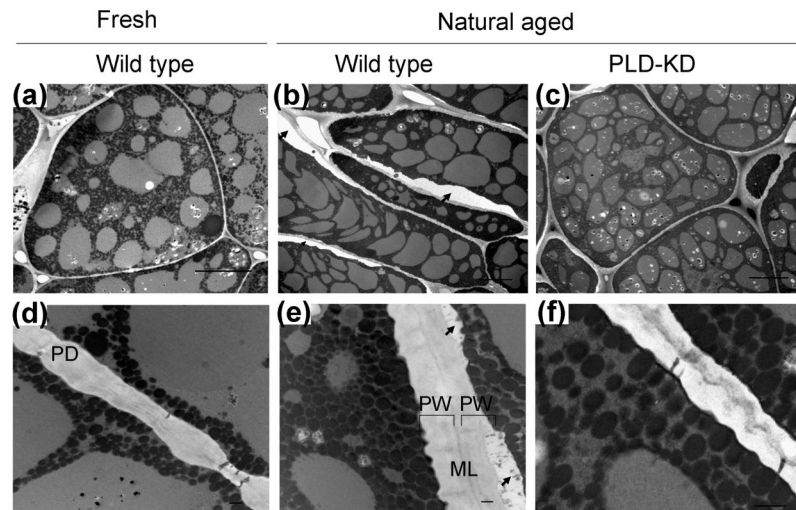


Figure 3. Ultrastructure of cotyledon tissues and cell wall complex from fresh wild type [(a) and (d)], aged wild type [(b) and (e)], and aged PLD-KD [(c) and (f)] cotyledon tissues. In (b) and (e), black arrows indicate signs of partial plasmolysis. Bars in a, b, and e = 10 μm . Bars in d, e, and f = 0.5 μm . ML, middle lamella; PW, primary walls; PD, plasmodesmata.

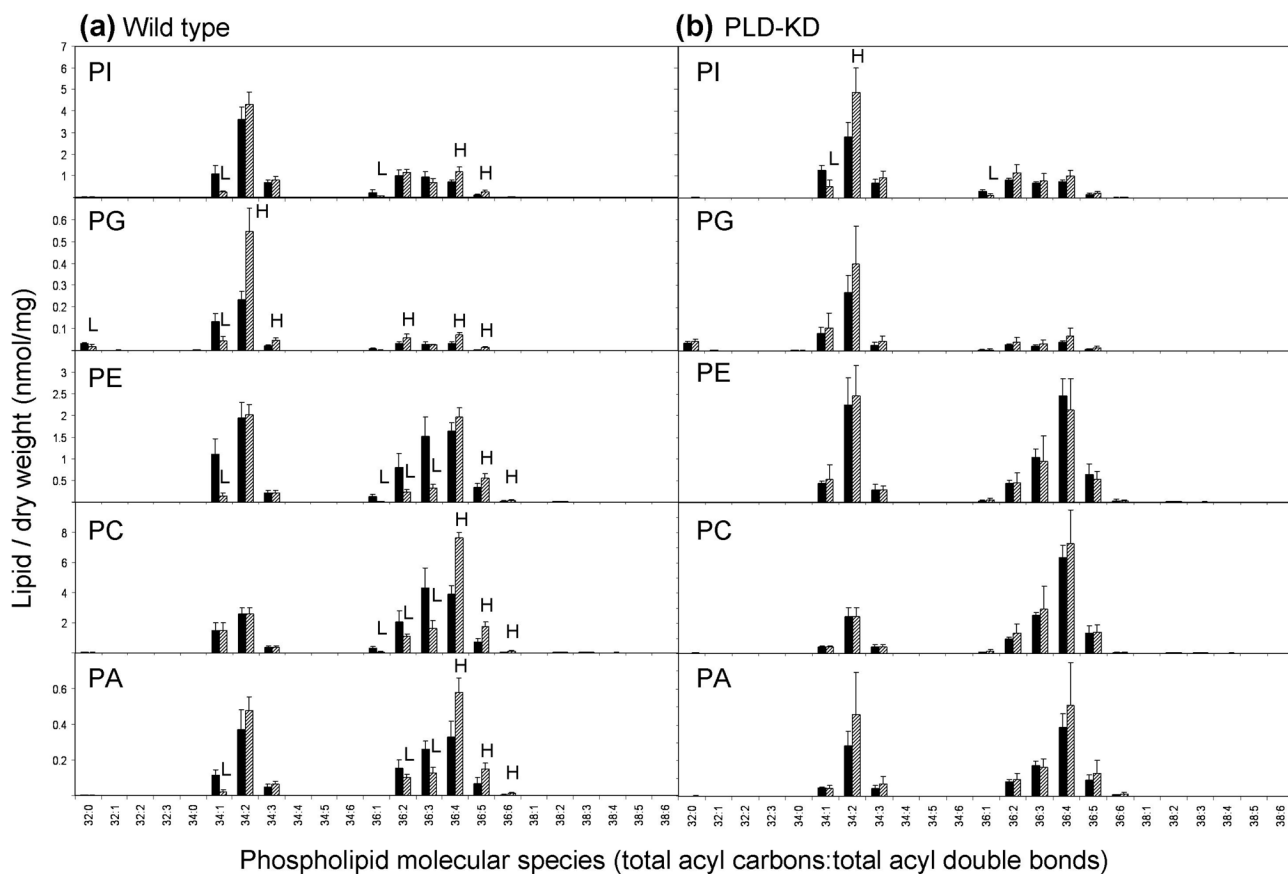


Figure 4.

Lipid molecular species of wild type and PLD-KD fresh and aged seeds. (a) The black bars and the hatched bars represent wild type fresh and aged seeds, respectively. (b) The black bars and the hatched bars represent PLD-KD fresh and aged seeds, respectively. The values are the mean \pm SD ($n = 5$). An H or L in the hatched bars indicates that the value is significantly higher or lower than that of fresh seeds at $p < 0.05$, respectively.

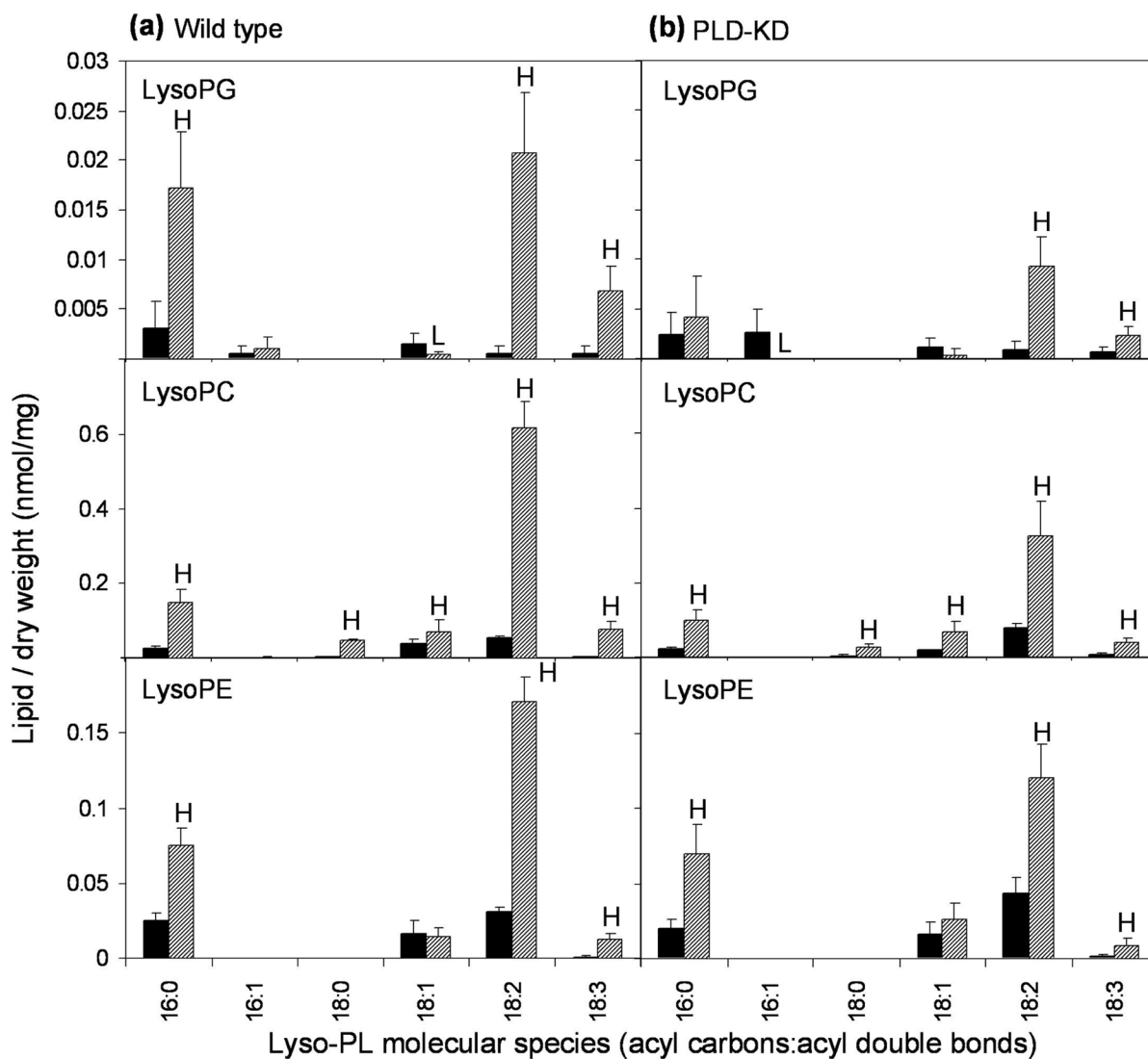


Figure 5. Changes in molecular species of lysoPC, lysoPE and lysoPG caused by natural aging of (a) wild type and (b) PLD-KD seeds, as revealed by ESI-MS/MS. The black bars represent fresh seeds and the hatched bars represent old seeds. The values are the mean \pm SD ($n = 5$). An H or L indicates that the value is significantly higher or lower than that of fresh seeds at $p < 0.05$.

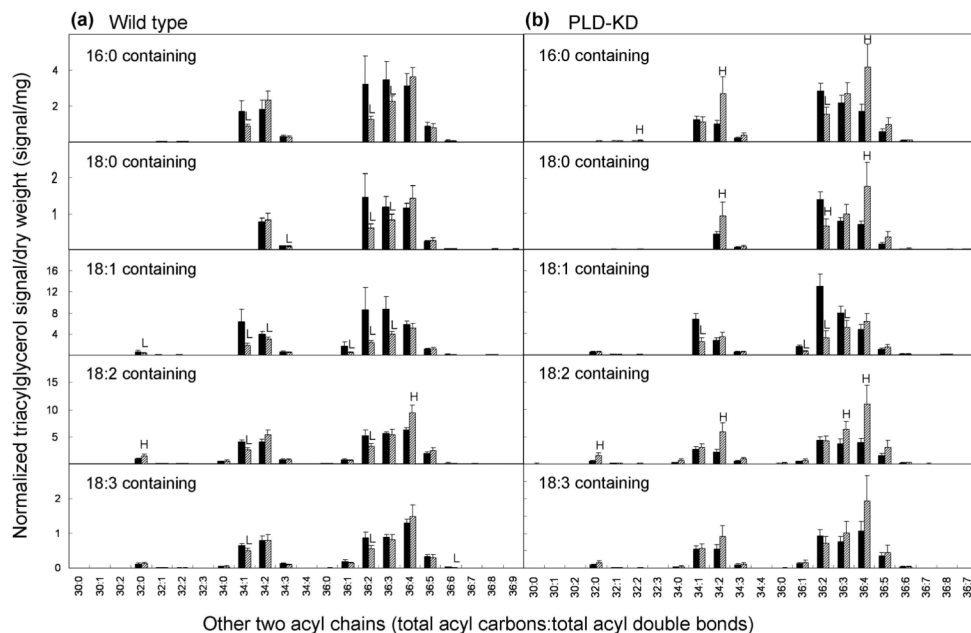


Figure 6. Changes in triacylglycerol molecular species resulted by natural aging in (a) wild type and (b) PLD-KD seeds measured by ESI-MS/MS with standards. The black bars and the hatched bars represent fresh and aged respectively. The value are the mean \pm SD ($n = 5$). An H or L in the aged seed indicates that the value is significantly higher or lower than that of fresh seed of each cultivar at $p < 0.05$.

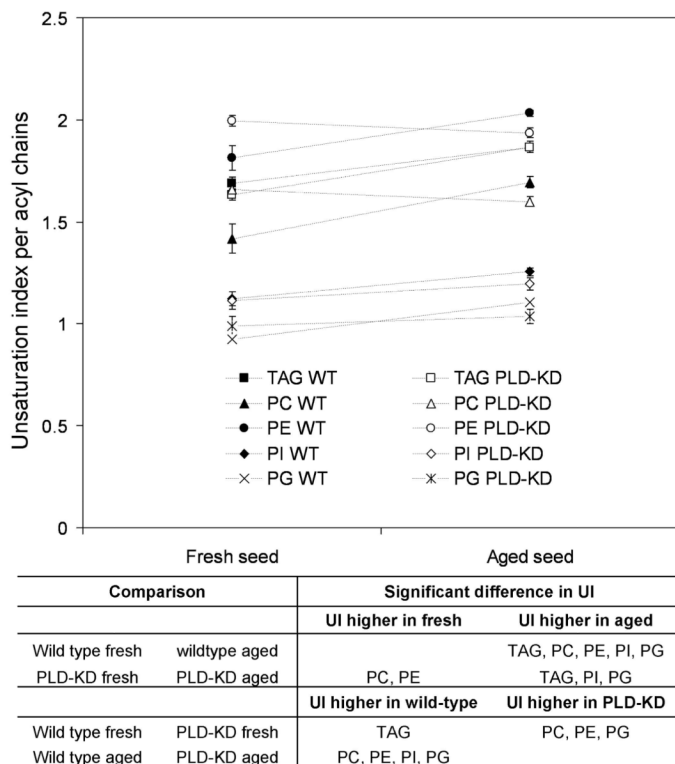


Figure 7. Indexes of PL and TAG species in wild type and PLD-KD soybean seeds during natural aging. Unsaturation index of PL and TAG from fresh wild type (WT) seeds, fresh PLD-KD seeds, aged wild type seeds, and aged PLD-KD seeds. All values are mean \pm SD of five biological replicates, and the significance data is shown below at $p < 0.05$ level.

Table 1

Total amount of lipid in each head group class in fresh and aged seeds of wild type and PLD-KD soybean. All PL classes and TAG were represented as mean \pm SD ($n = 5$), and the same lower case letter indicates no significant difference at LSD ($\alpha < 0.05$) level among fresh wild type seed, aged wild type seed, fresh PLD-KD, and aged PLD-KD seed.

Polar lipid	Wild type		PLD-KD	
	Fresh seed	Aged seed	Fresh seed	Aged seed
Lipid per dry weight (nmol/mg)				
PC	15.90 \pm 3.09 ^a	16.59 \pm 2.00 ^a	14.92 \pm 1.79 ^a	18.43 \pm 6.15 ^a
PE	7.75 \pm 1.27 ^a	5.56 \pm 0.62 ^a	7.67 \pm 1.18 ^a	7.47 \pm 2.82 ^a
PG	0.52 \pm 0.08 ^a	0.83 \pm 0.15 ^a	0.52 \pm 0.09 ^a	0.75 \pm 0.35 ^a
PI	8.38 \pm 1.52 ^a	8.75 \pm 1.27 ^a	7.54 \pm 0.80 ^a	9.63 \pm 2.58 ^a
PS	0.26 \pm 0.04 ^a	0.22 \pm 0.05 ^a	0.23 \pm 0.03 ^a	0.24 \pm 0.07 ^a
PA	1.35 \pm 0.24 ^a	1.53 \pm 0.24 ^a	1.11 \pm 0.20 ^a	1.48 \pm 0.67 ^a
LysoPC	0.13 \pm 0.02 ^c	0.96 \pm 0.16 ^a	0.14 \pm 0.02 ^c	0.56 \pm 0.16 ^b
LysoPE	0.07 \pm 0.02 ^b	0.27 \pm 0.03 ^a	0.08 \pm 0.01 ^b	0.23 \pm 0.05 ^a
LysoPG	0.01 \pm 0.00 ^b	0.05 \pm 0.01 ^a	0.01 \pm 0.00 ^b	0.02 \pm 0.01 ^b
MGDG	0.04 \pm 0.01 ^a	0.03 \pm 0.02 ^a	0.04 \pm 0.01 ^a	0.03 \pm 0.01 ^a
DGDG	0.34 \pm 0.11 ^{ab}	0.47 \pm 0.13 ^a	0.23 \pm 0.06 ^b	0.45 \pm 0.12 ^a
Total polar lipids	34.75 \pm 6.40 ^a	35.27 \pm 4.11 ^a	32.49 \pm 4.10 ^a	39.29 \pm 11.75 ^a
Triacylglycerol				
Triacylglycerol	Wild type		PLD-KD	
	Fresh seed	Aged seed	Fresh seed	Aged seed
Lipid per dry weight (normalized mass spectral signal/mg)				
16:0 containing	14.75 \pm 4.46 ^a	11.95 \pm 1.72 ^a	9.73 \pm 1.63 ^a	13.88 \pm 3.83 ^a
18:0 containing	4.99 \pm 1.25 ^a	4.17 \pm 0.90 ^a	3.60 \pm 0.33 ^a	4.90 \pm 1.64 ^a
18:1 containing	38.72 \pm 11.57 ^a	19.80 \pm 2.65 ^b	40.03 \pm 5.58 ^a	24.54 \pm 4.97 ^b
18:2 containing	31.02 \pm 1.52 ^{ab}	32.96 \pm 5.64 ^{ab}	20.89 \pm 3.98 ^b	38.65 \pm 10.14 ^a
18:3 containing	5.41 \pm 0.36 ^a	4.97 \pm 1.00 ^a	4.65 \pm 1.04 ^a	6.20 \pm 2.11 ^a
Total TAG signals	94.89 \pm 19.16 ^a	73.96 \pm 11.92 ^a	78.90 \pm 12.56 ^a	88.16 \pm 22.68 ^a

Table 2

Individual fatty acyl groups of the major molecular species of PC, PE and PI of soybean seeds. Data were obtained by ESI-MS/MS in negative product ion scanning mode. Each PL species was determined to be a combination of two 18:n fatty acyl chains. PL species levels are indicated as mean mole % of that diacyl combination \pm SD ($n = 5$) and differentiated at $p < 0.05$ among fresh wild type seed, aged wild type seed, fresh PLD-KD, and aged PLD-KD seed.

	36:4-PC		36:3-PC		36:2-PC	
	D18:2	18:1+18:3	18:0+18:3	18:1+18:2	D18:1	18:0+18:2
Fresh wild type	92.8 \pm 1.9 ^b	7.2 \pm 1.9 ^a	0.3 \pm 0.2 ^b	99.7 \pm 0.2 ^a	77.1 \pm 9.2 ^a	22.9 \pm 9.2 ^c
Aged wild type	97.6 \pm 1.4 ^a	2.4 \pm 1.4 ^b	2.1 \pm 1.7 ^a	97.9 \pm 1.7 ^b	17.4 \pm 6.5 ^c	82.6 \pm 6.5 ^a
Fresh PLD-KD	96.5 \pm 1.1 ^a	3.5 \pm 1.1 ^b	0.3 \pm 0.2 ^b	99.7 \pm 0.2 ^a	49.9 \pm 11.4 ^b	50.1 \pm 11.4 ^b
Aged PLD-KD	98.1 \pm 1.0 ^a	1.9 \pm 1.0 ^b	0.6 \pm 0.7 ^b	99.4 \pm 0.7 ^a	37.4 \pm 13.0 ^b	62.6 \pm 13.0 ^b
36:4-PE						
		36:3-PE		36:2-PE		
D18:2						
Fresh wild type	94.1 \pm 1.5 ^b	5.9 \pm 1.5 ^a	0.5 \pm 0.3 ^a	99.5 \pm 0.3 ^a	64.0 \pm 14.0 ^b	36.0 \pm 14.0 ^c
Aged wild type	98.3 \pm 0.8 ^a	1.7 \pm 0.8 ^b	1.5 \pm 2.1 ^a	98.5 \pm 2.1 ^a	7.2 \pm 3.3 ^c	92.8 \pm 3.3 ^a
Fresh PLD-KD	95.5 \pm 0.8 ^b	4.5 \pm 0.8 ^a	1.0 \pm 1.0 ^a	99.0 \pm 1.0 ^a	47.5 \pm 16.8 ^{ab}	52.5 \pm 16.8 ^{bc}
Aged PLD-KD	97.8 \pm 0.8 ^a	2.2 \pm 0.8 ^b	0.8 \pm 0.7 ^a	99.2 \pm 0.7 ^a	29.6 \pm 18.1 ^b	70.4 \pm 18.1 ^b
36:4-PI						
		36:3-PI		36:2-PI		
D18:2						
Fresh wild type	89.1 \pm 4.9 ^b	10.9 \pm 4.9 ^a	8.6 \pm 3.4 ^{ab}	91.4 \pm 3.4 ^{ab}	27.4 \pm 6.5 ^a	72.6 \pm 6.5 ^b
Aged wild type	95.8 \pm 3.4 ^a	4.2 \pm 3.4 ^b	14.4 \pm 7.4 ^{ab}	85.6 \pm 7.4 ^{ab}	1.6 \pm 0.5 ^b	98.4 \pm 0.5 ^a
Fresh PLD-KD	94.6 \pm 4.6 ^{ab}	5.4 \pm 4.6 ^{ab}	7.8 \pm 6.3 ^b	92.2 \pm 6.3 ^a	27.7 \pm 7.6 ^a	72.3 \pm 7.6 ^b
Aged PLD-KD	95.4 \pm 4.6 ^a	4.6 \pm 4.6 ^b	17.9 \pm 9.7 ^a	82.1 \pm 9.7 ^b	5.0 \pm 6.8 ^b	95.0 \pm 6.8 ^a