The Plant Journal (2007) **50**, 950–957 doi: 10.1111/j.1365-313X.2007.03103.x

Enhancing seed quality and viability by suppressing phospholipase D in Arabidopsis

Shivakumar P. Devaiah[1,2], Xiangqing Pan[1,2], Yueyun Hong[1,2], Mary Roth[3], Ruth Welti[3] and Xuemin Wang[1,2,*]
[1]*Department of Biology, University of Missouri, St Louis, MO 63121, USA,*
[2]*Donald Danforth Plant Science Center, St Louis, MO 63132, USA, and*
[3]*Kansas Lipidomics Research Center, Division of Biology, Kansas State University, Manhattan, KS 66506, USA*

Received 10 October 2006; revised 29 January 2007; accepted: 6 February 2007.
*For correspondence (fax +1 314 516 6233; e-mail wangxue@umsl.edu).

Summary

Seed aging decreases the quality of seed and grain and results in agricultural and economic losses. Alterations that impair cellular structures and metabolism are implicated in seed deterioration, but the molecular and biochemical bases for seed aging are not well understood. Ablation of the gene for a membrane lipid-hydrolyzing phospholipase D (PLDα1) in Arabidopsis enhanced seed germination and oil stability after storage or exposure of seeds to adverse conditions. The PLDα1-deficient seeds exhibited a smaller loss of unsaturated fatty acids and lower accumulation of lipid peroxides than did wild-type seeds. However, *PLDα1*-knockdown seeds were more tolerant of aging than were *PLDα1*-knockout seeds. The results demonstrate the PLDα1 plays an important role in seed deterioration and aging in Arabidopsis. A high level of PLDα1 is detrimental to seed quality, and attenuation of *PLDα1* expression has the potential to improve oil stability, seed quality and seed longevity.

Keywords: seed deterioration, germination, seed oil content, polyunsaturated fatty acids, phospholipase D, lipid oxidation.

Introduction

High-quality seeds are of great socio-economic significance because seeds provide the majority of our food supply and are important sources of animal and industrial feedstock. Quality seeds are characterized by maintaining a high germination rate and stable content after storage. However, seeds gradually lose quality and viability after harvest (Coolbear, 1995; McDonald, 1999). In addition, environmental stresses in the field or during harvest can compromise seed quality and storability. High moisture, spontaneous heating and microbial infection can exacerbate the deterioration of seeds during storage, shipment and handling (Nakayama *et al.*, 1981; Robertson *et al.*, 1973). Besides decreasing germination, the undesirable consequences of seed deterioration include unpalatable food and inferior products. The loss of seed quality and viability is associated with biochemical changes, such as DNA lesions, decreases in lipid content and lowered protein and nucleic acid synthesis. Oxidative stress has been implicated as a major contributor to seed deterioration (Bailly *et al.*, 1996, 1998). However, the molecular and biochemical factors that leads to seed deterioration and loss of seed viability are not well understood (Clerkx *et al.*, 2004; McDonald, 1999).

One of the most characteristic features of aging and seed deterioration is a progressive loss of membrane phospholipids (Samama and Pearce, 1993; Thompson, 1988). Phospholipid-degrading enzymes are regarded as important contributors to membrane degradation and tissue deterioration. For example, increases in lipid degradation and oxidation are blamed for the undesirable taste, color, odor and instability of soybean seeds that deteriorated due to damage during harvest, shipment, or storage (List *et al.*, 1992; Nakayama *et al.*, 1981; Robertson *et al.*, 1973). The loss of phospholipids could result from the activities of different families of enzymes, such as phospholipases, acyl hydrolases and lipid-oxidizing enzymes. Of those, phospholipase D (PLD), which cleaves phospholipids to generate phosphatidic acid (PA), has been proposed to catalyze an early step in the process of membrane degradation and seed deterioration (List *et al.*, 1992; Samama and Pearce, 1993; Thompson, 1998). Support for PLD-initiated lipid

degradation came primarily from early studies that followed the time course of release of various lipolytic products (i.e. PA → free fatty acids → lipid peroxides) *in vitro* and *in vivo*. Increases in PA in seeds occur under various stress conditions (List *et al.*, 1992; Robertson *et al.*, 1973; Samama and Pearce, 1993; Thompson, 1998). However, it is unknown whether the increase in PLD activity leads to seed aging or is a consequence of seed damage. Thus, direct evidence is lacking for a role for PLD in seed deterioration. In addition, PLD is a multi-gene family in plants (Wang, 2005). The activities of different PLDs characterized in *Arabidopsis thaliana* are affected differentially by Ca^{2+}, polyphosphoinositides and free fatty acids. Phospholipase Ds are activated differently and have unique functions (Li *et al.*, 2004, 2006; Mishra *et al.*, 2006). These findings raise questions of whether and which PLDs are involved seed quality and viability, which is the focus of this study.

Results

To determine whether and which PLDs play a role in seed aging, the effect of genetic ablation of individual PLDs on *A. thaliana* seed germination after aging was investigated. Homozygous T-DNA insertion mutants were isolated for different PLDs. Seeds of PLD-knockout mutants and wild-type (WT) *A. thaliana* were subjected to a treatment of high temperature (43°C) and high humidity (100% relative humidity) that was used to accelerate seed aging (Byrd and Delouche, 1971). After the accelerated aging (AA) treatment, the germination rate for WT seeds decreased by 70% (Figure 1a). Seeds of most PLD-knockout mutants exhibited a germination rate similar to WT, whereas the germination rate of PLDδ-deficient seeds was lower than that of WT seeds. However, ablation of PLDα1 rendered seeds more resistant to the aging treatment than WT seeds (Figure 1a). The data indicate that PLDα1 is likely to be involved in lipid degradation and seed aging. Further study was focused on the role of PLDα1 in seed viability and oil stability.

Phospholipase Dα1 is the most abundant form of PLD in *A. thaliana* and its activity is characterized by an *in vitro* requirement for millimolar concentrations of Ca^{2+} for activity. Phospholipase Dα1 protein was present in dry seeds and its level increased during seed germination and seedling growth (Figure 1b). The *PLDα1* knockout (KO) mutant, designated *pldα1-1*, had no detectable PLDα1 protein or PLD activity requiring millimolar Ca^{2+} concentrations (Figure 1c). The null mutation resulted from a T-DNA insertion at the third exon, or 936 nucleotides downstream of the initiation codon (Zhang *et al.*, 2004). The mutant contained a single T-DNA in the genome and introducing a wild-type *PLDα1* into the *pldα1-1* plants genetically complemented the expression and function of *PLDα1* (Mishra *et al.*, 2006; Zhang *et al.*, 2004). To verify the role of *PLDα1* in seed deterioration, a *PLDα1*-knockdown mutant (*pldα1-AS*), in

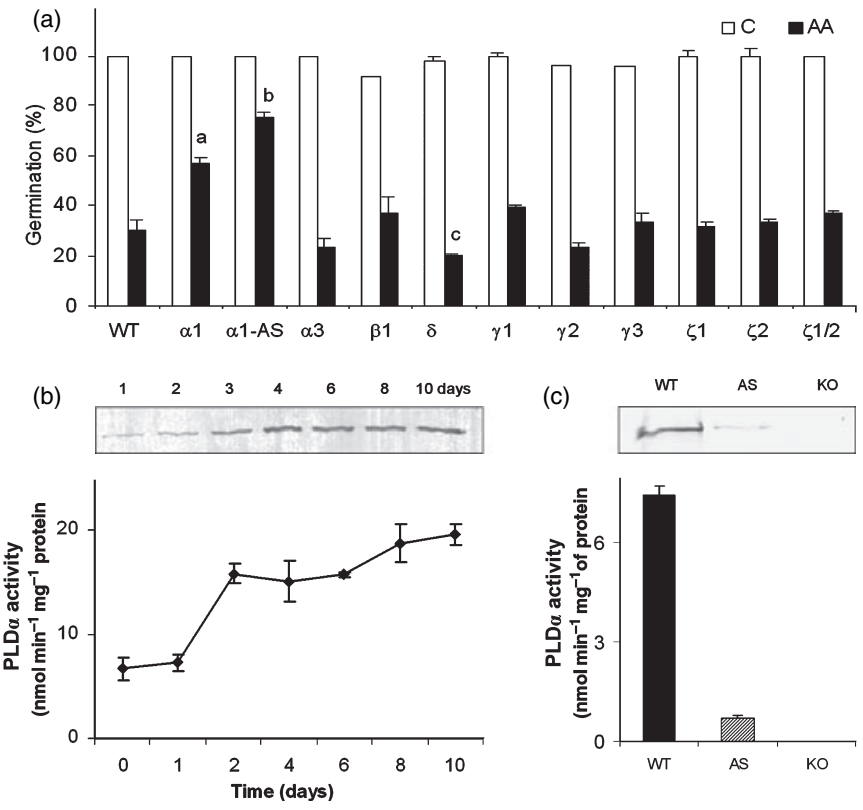

Figure 1. Phospholipase D mutations and their effect on seed germination after aging.

(a) Germination rate of WT and PLD mutant seeds without (C = control seeds) and with aging (AA = accelerated aging) treatment. Accelerated aging was performed by placing the seeds at 43°C in a tightly closed box with 100% relative humidity for 48 h. Values are means + SD (standard deviation; $n = 3$; each time 50 seeds were scored). Label 'a' above the bar indicates that the value of the mutant seeds is significantly different from that of WT seeds at $P < 0.05$. Labels 'b' or 'c' above the bar indicate that the mutant values are significantly different from WT and also one another at $P < 0.05$.

(b) Changes in PLDα1 protein (upper panel) and activity (lower panel) during seed germination and seedling growth in WT. Proteins (20 μg per lane) were separated on an 8% SDS-PAGE gel and blotted with PLDα1 antibody; PLDα was made visible by staining with alkaline phosphatase.

(c) Immunoblotting of PLDα1 protein (upper panel) and PLDα1 activity (lower panel) in seeds of WT, *pldα1-1* (KO) and *pldα1-AS* (AS).

which the expression of *PLDα1* was decreased by expressing a *PLDα1* antisense fragment, was utilized (Fan *et al.*, 1997). In dry *pldα1-AS* seeds, the level of PLDα1 protein and activity was approximately 15% of that in WT (Figure 1c). Under normal laboratory growth conditions, *pldα1-1* and *pldα1-AS* mutant plants displayed no significant difference from WT in growth, development, seed yield or seed weight (Fan *et al.*, 1997).

Fresh control seeds (C) of WT, *pldα1-AS* and *pldα1-1* all showed nearly 100% germination, but the rates of seed germination decreased greatly after the seeds were subjected to an aging treatment. After 2 days of incubation at 43°C and 100% humidity, the germination rates were 30% for WT seeds and 58% and 75% for *pldα1-1* and *pldα1-AS*, respectively (Figure 1a). To examine whether the improved resistance to aging also occurred in naturally aged seeds, the germination abilities of *pldα1-1*, *pldα1-AS* and WT seeds stored for 3 years at room temperature were compared. The seeds were harvested from plants grown under the same conditions. After 3 years of natural aging (NA), only 34% of WT seeds germinated, whereas the germination rates for *pldα1-1* and *pldα1-AS* seeds were 62% and 72%, respectively (Figure 2a). After 6 years' storage, the germination rate of *pldα1-AS* seeds was approximately three-fold greater than that of WT seeds (23% versus 8%; Figure 2b). Seeds of *pldα1-1* were not included in the 6-year aging analysis because the mutant seeds were not available at the onset of the experiment.

In addition, the seedlings from *pldα1-1* and *pldα1-AS* seeds grew faster and more strongly, as indicated by longer roots and bigger cotyledon leaves (Figure 2c,d). After 3 years' aging, the seedling vigor, as indicated by root length and shoot length, of *pldα1-1* and *pldα1-AS* seeds was stronger than that of WT seeds (Figure 2d). Without aging, the vigor was similar among the three types of seedlings. The results from the naturally aged seeds are consistent with those for the seeds with accelerated aging, and both results show that suppression of *PLDα1* improves seed viability.

To determine the metabolic effect of the suppression of *PLDα1*, the fatty acid composition and oil content of WT and mutant seeds after aging were analyzed. Under both natural and accelerated aging conditions, WT seeds lost more fatty acids than did *pldα1-AS* or *pldα1-1* seeds. After 3 years' storage, the content of all fatty acids in WT seeds decreased significantly, and the loss of individual fatty acids ranged from 25% to 60% (Figure 3a,b). In comparison, *pldα1-1* and *pldα1-AS* seeds exhibited a significantly smaller decrease in all fatty acids, except for palmitic acid (Figure 3a,b). Similarly, the decreases in fatty acids in *pldα1-1* and *pldα1-AS* seeds were also smaller than in WT seeds after the 2-day accelerated aging treatment (Figure 3a,c). There are some differences in the extent of individual fatty acid decreases between the 3-year storage and accelerated aging treatments and between *pldα1-1* and *pldα1-AS* seeds. While *pldα1-1* seeds suffered little loss of highly unsaturated fatty acids, such as 18:3, 20:2 and 20:3, after 3 years' storage, in

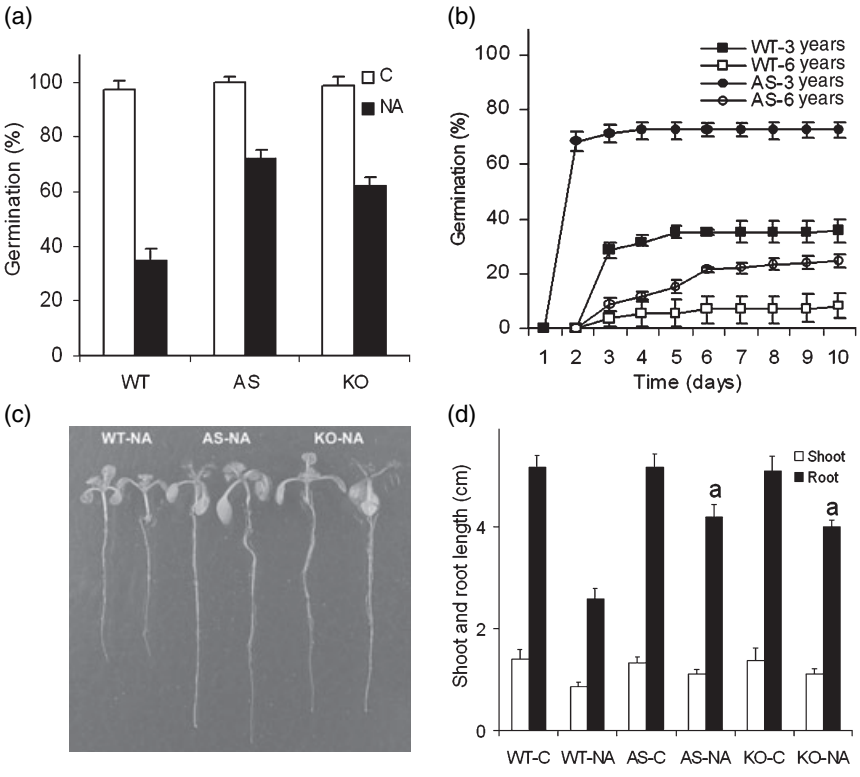

Figure 2. Effect of PLDα1 deficiency on seed germination and seedling growth.
(a) Germination rate of WT, *pldα1-AS* (AS) and *pldα1-1* (KO) seeds after storage for 3 years (NA = natural aging; C = control seeds). Germination was scored on seeds plated on 0.5 MS medium after 10 days. Values are means + SD (n = 10).
(b) Time course of WT and *pldα1-AS* germination after storage for 3 and 6 years at room temperature.
(c) Seedling phenotype of WT, *pldα1-AS* and *pldα1-1* after growth on 0.5 MS medium for 10 days.
(d) Shoot and root length of 6-day-old seedlings of NA and C seeds of WT, *pldα1-AS* and *pldα1-1*. Values are means + SD (n = 10). Label 'a' indicates that the value of NA mutant seeds is significantly different from that of NA WT seeds at $P < 0.05$.

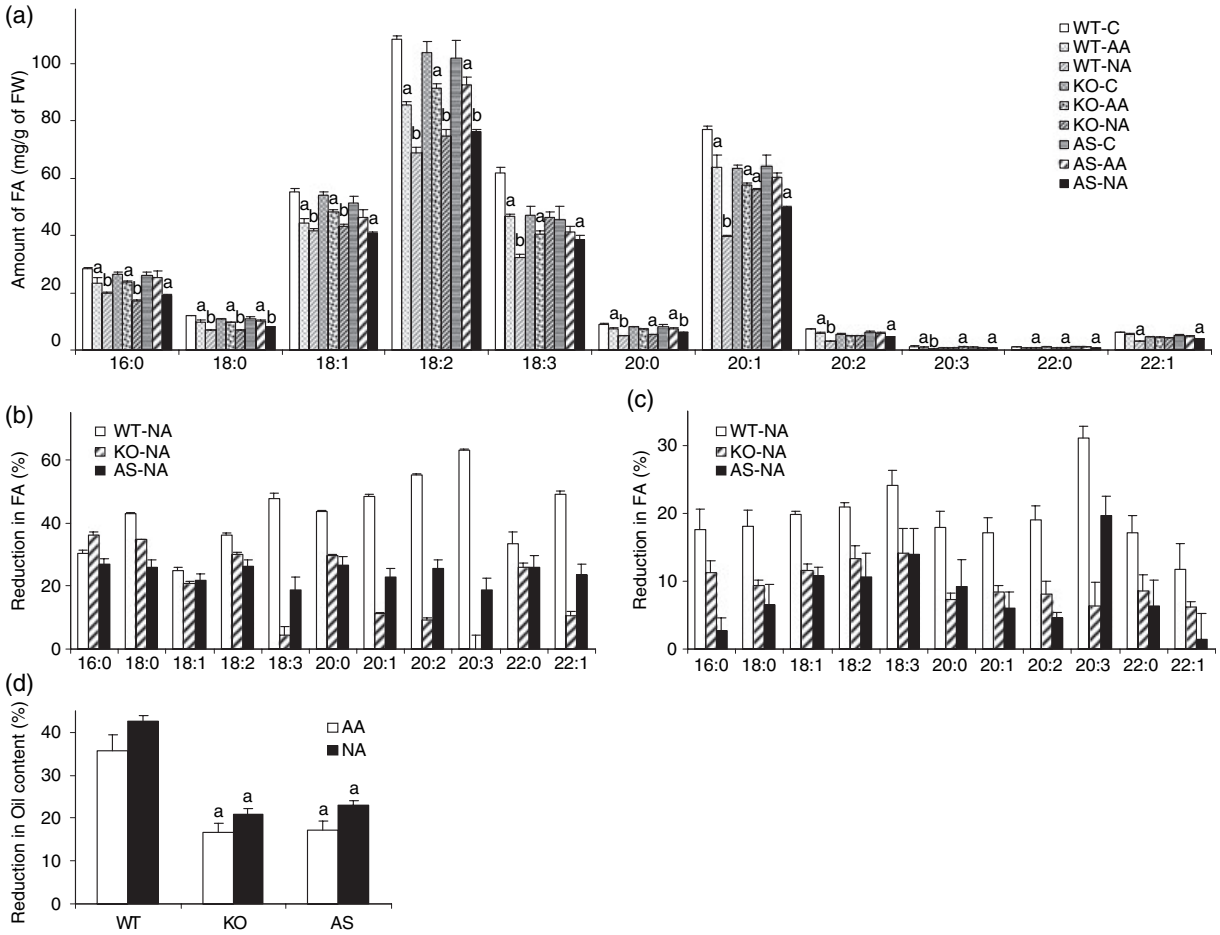

Figure 3. Effect of *PLDα1* deficiency on seed fatty acid composition and oil content after aging.
(a) Amounts of fatty acids in WT, *pldα1-KO* and *pldα1-AS* seeds in fresh, control seeds (C), after 3 years' storage at room temperature (NA) and after 2 days' accelerated aging (AA).
(b) Decreases in fatty acids between control and WT, *pldα1-AS* and *pldα1-KO* seeds aged for 3 years at room temperature.
(c) Decreases in fatty acids between control and AA seeds of WT, *pldα1-AS* and *pldα1-KO*. Seeds were aged by treating at 43°C and 100% relative humidity for 48 h.
(d) Decrease (%) in seed oil content after accelerated aging and after 3 years' storage, calculated as oil content of aged seeds divided by oil content of control seeds × 100. Values are means + SD ($n = 6$). Label 'a' indicates that values of the AA-treated or NA-treated seeds are significantly lower than those of control at $P < 0.05$. Label 'b' indicates that values of NA-treated seeds are significantly lower than those of WT seeds and also than those of AA-treated seeds at $P < 0.05$.

WT seeds these same fatty acids were the ones that were lost to the greatest extent (Figure 3b). The extent of decreases in 18:3, 20:2 and 20:3 in *pldα1-AS* seeds was greater than in *pldα1-1*, but smaller than in WT seeds. After the accelerated aging treatment, decreases in polyunsaturated fatty acids were also the greatest, but both *pldα1-1* and *pldα1-AS* seeds exhibited similar decreases in 18:3 (Figure 3c). The decreases in individual fatty acids were reflected in the aging-associated loss of total oil content. After 3 years' storage the decrease in oil content was approximately 42% in WT seeds, but 22% in *pldα1-AS* or *pldα1-1* seeds. After the 2-day accelerated aging process, WT seeds lost about 36% of their oil content whereas *pldα1-AS* and *pldα1-1* seeds lost approximately 16% over fresh seeds of the same genotype (Figure 3d).

It has been proposed that the loss of fatty acids and oil during aging results from lipid peroxidation, and that oxidative stress is regarded as a major contributor to seed deterioration (Bailly *et al.*, 1996; 1998). The extent of lipid peroxidation was first determined using the TBARS (thio-barbituric acid-reactive-substances) assay, which measures malondialdehyde (MDA), a secondary end-product of the oxidation of polyunsaturated fatty acids. In fresh seeds no difference occurred in MDA content between WT, *pldα1-AS* and *pldα1-1* seeds (Figure 4a). After 2 days' accelerated aging, MDA content increased significantly in WT but no apparent increase was detected in *pldα1-1* or *pldα1-AS* seeds. Malondialdehyde can be formed only from unsaturated fatty acids with three or more double bonds and, thus, the TBARS assay can underestimate lipid peroxidation

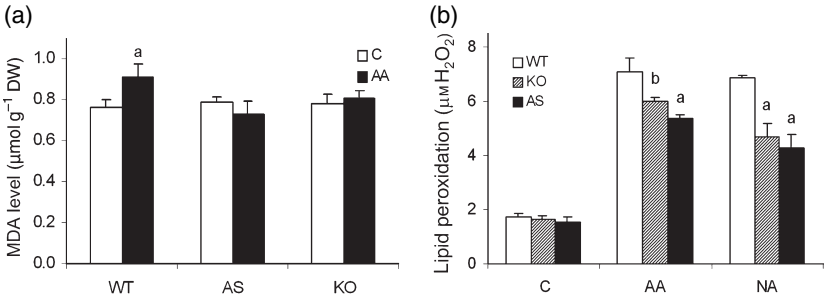

Figure 4. Effect of seed aging on lipid peroxidation.
(a) Malondialdehyde (MDA) content of WT, *pldα1-AS* and *pldα1-1* seeds after 2-days' aging at 43°C and 100% relative humidity.
(b) Increases in lipid hydroperoxide content in WT, *pldα1-AS* and *pldα1-1* seeds after 2 days' aging and 3 -years' storage. Values are means + SD (n = 3). Label 'a' indicates that values of AA- or NA-treated seeds are significantly different from those of control seeds at $P < 0.05$. Label 'b' indicates that values of *pldα1-AS* seeds are significantly different from those of WT and *pldα1-1* seeds at $P < 0.05$.

because oxidation of fatty acids with fewer double bonds is not determined. The ferrous oxidation-xylenol orange (FOX) assay was also used to assay lipid peroxidation (DeLong *et al.*, 2002). The content of lipid hydroperoxides increased three-fold after aging treatments, and the amount of peroxidation in WT seeds was significantly higher than that in *pldα1-AS* and *pldα1-1* seeds after storage and accelerated aging (Figure 4b).

The lipid product of the enzymatic activity of PLD is PA, so the level of PA might be expected to be altered by ablation of *PLDα1* in seeds. The PA level increased after aging treatment of WT seeds. Aged WT seeds contained about 30% more PA than fresh WT seeds (Figure 5a). The increase in PA came mostly from PA species with acyl groups of 34:1, 34:2, 36:2, 36:3 and 36:4 (total acyl carbons:total acyl double bonds). However, the levels of PA did not increase in aged *pldα1-AS* and *pldα1-1* seeds (Figure 5b,c). The lack of increase of PA in PLDα1-deficient seeds indicates that PLDα1 is responsible for aging-promoted production of PA.

Discussion

The above data indicate that PLD has an important role in seed quality and that the roles of individual PLDs in seed deterioration are varied. Distinguishable functions have been identified for specific PLDs in plant stress responses (Li *et al.*, 2004, 2006; Mishra *et al.*, 2006). The present data show that the ablation of most PLDs did not improve seed oil stability or seed viability, suggesting that these PLDs are not directly involved in seed aging or that other PLDs compensate for the missing function. On the other hand, ablation of *PLDδ* rendered seeds even less tolerant to aging. This effect of PLDδ-knockout implies that PLDδ plays a positive role in ameliorating the effects of seed aging, and this role is

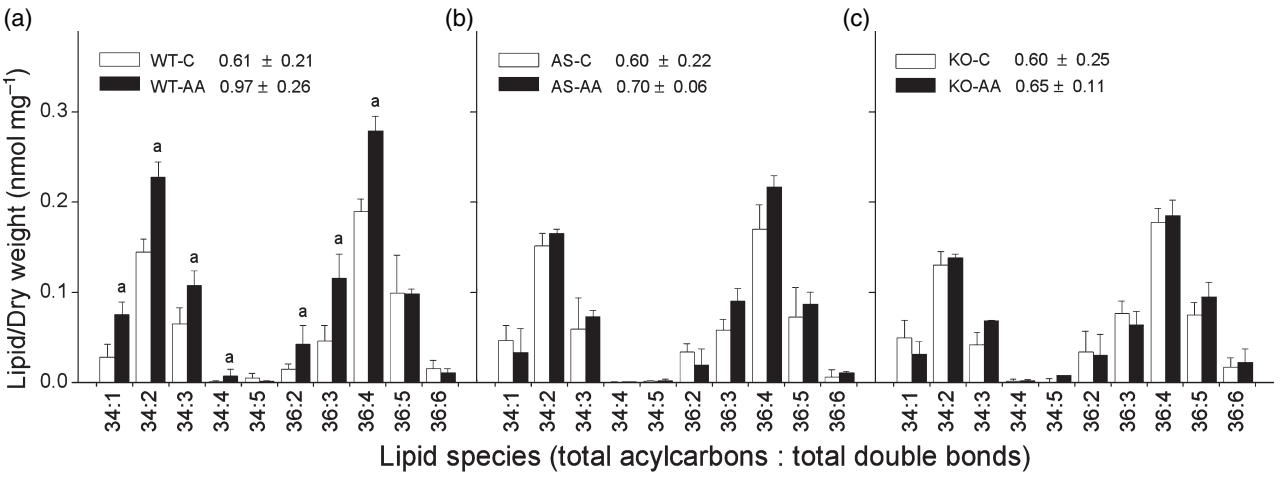

Figure 5. Levels of PA and PA molecular species in fresh (C) and aged (AA) seeds of WT, *pldα1-AS* and *pldα1-1* (KO). Seed aging was accelerated by placing seeds in a tightly closed box at 43°C with 100% relative humidity for 48 h. Lipid profiling was performed by electrospray ionization tandem mass spectroscopy (ESI ms/ms) on five independent extracts. The PA molecular species were denoted as total acyl carbon: total double bonds. The number following each treatment in the panel shows the total PA. Values are means + SD (n = 5). Label 'a' indicates that values of aged seeds are significantly different from those of control seeds at $P < 0.05$.

consistent with previous findings that PLDδ protects Arabidopsis from stress damage, such as hydrogen peroxide-induced cell death and freezing injuries (Li *et al.*, 2004; Zhang *et al.*, 2003). Of the different PLD mutants examined, only ablation of *PLDα1* significantly improved the resistance of seeds to deterioration during storage or accelerated aging.

The results suggest that high levels of PLDα1 in seeds are detrimental, promoting seed aging and deterioration. The formation of PA by PLD is suggested to initiate a chain of reactions that damage cell membranes and storage lipids in seeds (List *et al.*, 1992). The aging-induced increase in PA in WT, but not in *PLDα1*-deficient seeds, indicates that PLDα1 is active and responsible for the aging-induced formation of PA. The PLDα1-mediated hydrolysis of membrane lipids could destabilize oil bodies that consist of triacylglycerol, coated by a phospholipid monolayer containing proteins. Such loss of oil body integrity would expose polyunsaturated fatty acids to peroxidation. Another effect of high PLDα1 activity is to promote the production of reactive oxygen species. Phosphatidic acid activates NADPH oxidase activity to produce superoxide, a reactive oxygen species that can be quickly converted to H_2O_2. The role of *PLDα1* and PA in increasing the formation of reactive oxygen species has been reported in *A. thaliana* leaves (Park *et al.*, 2004; Sang *et al.*, 2001). The decrease in membrane integrity and increased ROS production promotes lipid peroxidation. Lipid peroxidation and free radical propagation cause oxidative damage that is considered to be a major contributor to seed deterioration (Bailly *et al.*, 1996; 1998; McDonald, 1999). The present results show that ablation of *PLDα1* abolished aging-induced increases in PA and decreased the levels of lipid peroxidation, which may underlie the basis for increased resistance to aging in the *PLDα1*-deficient seeds.

In addition, the study found that *pldα1-AS* seeds consistently germinated better than *pldα1-1* seeds after aging. Although the *pldα1-1* mutation completely disrupted *PLDα1* expression, *pldα1-AS* seeds still have a detectable amount of PLDα1 protein and activity. The result indicates that seeds of the *PLDα1*-knockdown are more tolerant to aging than those of the *PLDα1*-knockout. Thus, some PLDα1 activity is needed for optimal germination and seedling establishment. It is worth noting that *PLDα1* has multi-faceted roles, and besides its catabolic function, PLDα1 mediates plant hormonal and stress responses (Mishra *et al.*, 2006; Sang *et al.*, 2001; Wang, 2005). Thus, to improve seed storability and viability in plants, the manipulation of *PLDα1* should be limited to seeds. Based on the present results and previous studies (List *et al.*, 1992; Nakayama *et al.*, 1981; Robertson *et al.*, 1973; Samama and Pearce, 1993), we propose that high levels of PLDα1 increase membrane lipid degradation, oxidative stress and seed deterioration, and that suppression of *PLDα1* in seeds has the potential to decrease the loss of unsaturated fatty acids and oxidative stress, and to enhance seed quality and longevity.

Experimental procedures

Plant materials

Phospholipase D mutants were isolated from *A. thaliana* Columbia-0 (WT) ecotype. The PLD knockout line was obtained from the Salk T-DNA lines through the analysis of the SiGnAL database and seeds were obtained from the Ohio State University Arabidopsis Biological Resources Center (ABRC). Homozygous mutant plants were isolated using the T-DNA left-border primer and gene-specific primers (Zhang *et al.*, 2004). The isolation of homozygous T-DNA-insertional mutants for *pldα1-1*, *pldζ1-1*, *pldζ2-1* and *pldζ1/ζ2* double knockouts was described previously (Li *et al.*, 2006; Zhang *et al.*, 2004). The generation of *pldα1-AS* plants was previously reported (Fan *et al.*, 1997). The deficiency in PLDα1 was confirmed by assaying PLDα1 activity and immunoblotting with a PLDα-specific antibody, following the procedure described previously (Fan *et al.*, 1997). The SALK identification numbers for the knockout lines for other PLDs were SALK_130690 for *pldα3-1*, SALK_092469 for *pldδ-1*, SALK_079133 for *pldβ1-1*, SALK_066687 for *pldγ1-1*, SALK_078226 for *pldγ2-1* and SALK_126694 for *pldγ3-1*. The disruption of respective PLD genes was verified by real-time PCR and/or immunoblotting with antibodies specific to individual PLDs.

Seed aging and germination

Accelerated aging was carried out by placing the seeds at 43°C in tightly closed boxes with 100% relative humidity for different periods of time according to Byrd and Delouche (1971). Naturally aged seeds were seeds stored at 23 ± 2°C and 50 ± 10% relative humidity for 3 and 6 years, and the seeds of *pldα1-1*, *pldα1-AS* and WT were collected from plants grown under the same conditions. Plants were grown in a growth chamber with cool white fluorescent light of 100 μmol m^{-2} sec^{-1} under 14-h light/10-h dark and 23°C/18°C cycles. Seeds were surface-sterilized and plated on 0.5 MS medium. Germination was scored when radicles emerged from seeds; counts were made daily for 10 days.

Protein extraction and PLDα activity assay

Seeds and seedlings were harvested at different days of germination, frozen in liquid nitrogen and stored at −80°C. Total protein from the samples was extracted by grinding in an ice-chilled mortar and pestle with a buffer as described previously (Fan *et al.*, 1997). Equal amounts of protein were separated on an 8% gel and transferred to polyvinylidine difluoride filters. The membranes were blotted with a PLDα-specific antibody, followed by incubation with a secondary antibody conjugated to alkaline phosphatase (Fan *et al.*, 1997). The PLDα activity was determined using egg yolk phosphatidylcholine (PC) mixed with dipalmitoylglycerol-3-phospho [methyl-^{3}H] choline as described (Fan *et al.*, 1997).

Fatty acid analysis

Total lipids from Arabidopsis seeds were extracted and methyl esters were formed in Teflon-lined screw-capped glass tubes, using 1.5 ml of methanol containing 1.5% H_2SO_4 and 0.01% butylated hydroxytoluene (BHT). Twenty-five microliters of 5.4 mM 17:0 triacylglycerol (TAG) was added to each sample as an internal standard. The tubes were incubated at 90°C for 1 h, and after incubation 1 ml of water and 1 ml of hexane were

added. After vortexing, the upper solvent phase was separated, treated with Na_2SO_4 and used for analysis. Fatty acid methyl esters were analyzed by gas chromatography (Shimadzu GC-17A) on a silica capillary column (DB-5MS, 30 m × 0.25 mm, 0.25 μm; Agilent Technologies, http://www.chem.agilent.com) using splitless injection with helium as a carrier gas at 25 ml min^{-1}; the injection volume was 2 μl. The temperatures of the injector, column and flame ionization detector (FID) were 220, 170 and 220°C respectively. The oil content was calculated based on the amount of fatty acid methyl esters relative to the internal standard 17:0 from TAG and expressed as percentage of the weight of TAG over the amount of seed dry weight.

Phosphatidic acid analysis

Lipid extraction and PA quantification were performed as described (Welti *et al.*, 2002). Briefly, seeds were immersed into 3 ml of hot isopropanol with 0.01% butylated hydroxytoluene at 75°C to inhibit lipolytic activities. The tissues were extracted with chloroform/methanol five times with 30-min agitation each time. The remaining plant tissues were dried in an oven at 105°C overnight and then weighed. The weights of these dried, extracted tissues are the 'dry weights' of the samples. Lipid samples were analyzed on an electrospray ionization triple quadrupole mass spectrometer (API 4000, Applied Biosystems, http://www.appliedbiosystems.com/). The molecular species of PA were quantified in comparison to two internal standards using a standard curve as previously described (Devaiah *et al.*, 2006; Welti *et al.*, 2002). Five replicates of each treatment for each phenotype were processed and analyzed. The *Q*-test for discordant data was performed on the replicates of the total lipid. Paired values were subjected to Student's *t*-test to determine the statistical significance.

Malondialdehyde and lipid hydroperoxide assays

Seeds were ground in 0.5 ml of 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl buffer (pH 8.0) containing 175 mM NaCl and homogenized with an equal volume of 0.5% (w/v) 2-thiobarbituric acid in 20% (w/v) trichloroacetic acid. The homogenate was incubated at 95°C for 30 min and then centrifuged at 16 000 ***g*** for 30 min. To determine malondialdehyde, the absorbance of the supernatant at 540 and 600 nm was measured, and malondialdehyde content was expressed as μmol g^{-1} dry weight (Bailly *et al.*, 1996). For the FOX assay, total lipids were extracted and assayed as described previously (DeLong *et al.*, 2002). Peroxide values were expressed as H_2O_2 equivalents using a standard curve from 0–20 μM H_2O_2. The reactivity of linoleic acid-derived LOOHs with the FOX reagent was reported to be nearly identical to H_2O_2 (DeLong *et al.*, 2002).

Acknowledgements

This work was supported by grants from the US Department of Agriculture (2005-35818-15253) and the National Science Foundation (IOB-0454866). The Kansas Lipidomics Research Center's research was supported by grants from NSF (MCB 0455318, DBI 0521587, and Kansas EPSCoR's award, EPS-0236913), with support from the State of Kansas through Kansas Technology Enterprise Corporation, Kansas State University, and from US Public Health Service grant P20 RR016475 from the INBRE program of the National Center for Research Resources.

References

Bailly, C., Benamar, A., Corbineau, F. and Come, D. (1996) 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.* **97**, 104–110.

Bailly, C., Benamar, A., Corbineau, F. and Come, D. (1998) Free radical scavenging as affected by accelerated ageing and subsequent priming in sunflower seeds. *Physiol. Plant.* **104**, 646–652.

Byrd, H.W. and Delouche, J.C. (1971) Deterioration of soybean seed in storage. *Proc. Assoc. Offic. Seed Analysts* **61**, 41–57.

Clerkx, E.J., Blankestijn-De Vries, H., Ruys, G.J., Groot, S.P. and Koornneef, M. (2004) Genetic differences in seed longevity of various Arabidopsis mutants. *Physiol. Plant.* **121**, 448–461.

Coolbear, P. (1995) Mechanism of seed deterioration. In *Seed Quality: Basic Mechanisms and Agricultural Implications* (Basra, A.S., eds). New York: Food Product Press, pp. 223–277.

DeLong, J.M., Prange, R.K., Hodges, D.M., Forney, C.F., Bishop, M.C. and Quilliam, M. (2002) Using a modified ferrous oxidation-xylenol orange (FOX) assay for detection of lipid hydroperoxides in plant tissue. *J. Agric. Food Chem.* **50**, 248–254.

Devaiah, S.P., Roth, M.R., Baughman, E., Li, M., Tamura, P., Jeannotte, R., Welti, R. and Wang, X. (2006) Quantitative profiling of polar glycerolipid species and the role of phospholipase Dα1 in defining the lipid species in Arabidopsis tissues. *Phytochemistry* **67**, 1907–1924.

Fan, L., Zheng, S. and Wang, X. (1997) Antisense suppression of phospholipases Dα retards abscisic acid- and ethylene-promoted senescence of post harvest Arabidopsis leaves. *Plant Cell*, **9**, 2916–2919.

Li, W., Li, M., Zhang, W., Welti, R. and Wang, X. (2004) The plasma membrane-bound phospholipase Dδ enhances freezing tolerance in *Arabidopsis*. *Nat. Biotech.* **22**, 427–433.

Li, M., Qin, C., Welti, R. and Wang, X. (2006) Double knockouts of phospholipase Dζ1 and ζ2 in Arabidopsis affect root elongation during phosphate-limited growth, but do not affect root hair patterning. *Plant Physiol.* **140**, 761–770.

List, G.R., Mounts, T.L. and Lanser, A.C. (1992) Factors promoting the formation of nonhydratable soybean phosphatides. *J. Am. Oil Chem. Soc.* **69**, 443–446.

McDonald, M.B. (1999) Seed deterioration: physiology, repair and assessment. *Seed Sci. Technol.* **27**, 177–237.

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

Nakayama, Y., Sayo, K. and Kito, M. (1981) Decomposition of phospholipids in soybean during storage. *Cereal Chem.* **58**, 260–264.

Park, J., Gu, Y., Lee, Y., Yang, Z. and Lee, Y. (2004) Phosphatidic acid induces leaf cell death in Arabidopsis by activating the Rho-related small G protein GTPase-mediated pathway of reactive oxygen species generation. *Plant Physiol.* **134**, 129–136.

Robertson, J.A., Morrison, W.H. III and Burdick, D. (1973) Chemical evaluation of oil from field- and storage-damaged soybeans. *J. Am. Oil Chem. Soc.* **50**, 443–446.

Samama, A.M. and Pearce, R.S. (1993) Ageing of cucumber and onion seeds-phospholipase-D, lipoxygenase activity and changes in phospholipid content. *J. Exp. Bot.* **44**, 1253–1265.

Sang, Y., Cui, D. and Wang, X. (2001) Phospholipase D- and phosphatidic acid-mediated generation of superoxide in Arabidopsis. *Plant Physiol.* **126**, 1449–1458.

Thompson, J.E. (1988) The molecular basis for membrane deterioration during senescence. In *Senescence and Aging in Plants*

(Nooden, L.D. and Leopold, A.C., eds). New York: Academic Press, pp. 52–84.

Wang, X. (2005) Regulatory functions of phospholipase D and phosphatidic acid in plant growth development, and stress responses. *Plant Physiol.* **139**, 566–573.

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

Zhang, W., Wang, C., Qin, C., Wood, T., Olafsdottir, G. and Wang, X. (2003) Phospholipase Dδ and phosphatidic acid decrease H_2O_2-induced cell death in Arabidopsis. *Plant Cell*, **15**, 2285–2295.

Zhang, W., Qin, C., Zhao, J. and 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.

