Journal of Experimental Botany, Vol. 62, No. 15, pp. 5683–5698, 2011
doi:10.1093/jxb/err250 Advance Access publication 18 August, 2011
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

The *Arabidopsis sn*-1-specific mitochondrial acylhydrolase AtDLAH is positively correlated with seed viability

Young Sam Seo*, Eun Yu Kim* and Woo Taek Kim†

Department of Biology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Korea

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: wtkim@yonsei.ac.kr

Received 2 May 2011; Revised 5 July 2011; Accepted 25 July 2011

Abstract

Lipid-derived molecules produced by acylhydrolases play important roles in the regulation of diverse cellular functions in plants. In *Arabidopsis*, the DAD1-like phospholipase A1 family consists of 12 members, all of which possess a lipase 3 domain. In this study, the biochemical and cellular functions of AtDLAH, an *Arabidopsis thaliana* DAD1-like acylhydrolase, were examined. Bacterially expressed AtDLAH contained phospholipase A1 activity for catalysing the hydrolysis of phospholipids at the *sn*-1 position. However, AtDLAH displayed an even stronger preference for 1-lysophosphatidylcholine, 1-monodiacylglycerol, and phosphatidic acid, suggesting that AtDLAH is a *sn*-1-specific acylhydrolase. The *AtDLAH* gene was highly expressed in young seedlings, and its encoded protein was exclusively localized to the mitochondria. *AtDLAH*-overexpressing transgenic seeds (*35S:AtDLAH*) were markedly tolerant to accelerated-ageing treatment and thus had higher germination percentages than wild-type seeds. In contrast, the *atdlah* loss-of-function knockout mutant seeds were hypersusceptible to accelerated-ageing conditions. The *35S:AtDLAH* seeds, as opposed to the *atdlah* seeds, exhibited a dark red staining pattern following tetrazolium treatment under both normal and accelerated-ageing conditions, suggesting that AtDLAH expression is positively correlated with seed viability. The enhanced viability of *35S:AtDLAH* seeds was accompanied by more densely populated epidermal cells, lower levels of accumulated lipid hydroperoxides, and higher levels of polar lipids as compared with wild-type and *atdlah* mutant seeds. These results suggest that AtDLAH, a mitochondrial-localized *sn*-1-specific acylhydrolase, plays an important role in *Arabidopsis* seed viability.

Key words: DAD1-like acylhydrolase, lipid peroxidation, mitochondrial targeting, seed viability.

Introduction

Seed viability is important for reproduction and propagation in higher plant species. Seeds also play a significant role in food sources for animals and humans. Therefore, seed quality and longevity are critical factors for ecology, agriculture, and the economy (Chrispeels and Sadava, 2003; Walters *et al.*, 2005; Li and Pritchard, 2009). Seed quality is characterized by the seed's capability to germinate and maintain its storage contents (Coolbear, 1995). However, seeds are continuously exposed to harsh environments, such as high humidity, extreme temperatures, strong sun rays, and pathogen infections, during their development, harvest, and storage. Thus, most seeds suffer gradual deterioration, including decreased germination percentages, storability, and stress tolerance (Nakayama *et al.*, 1981; McDonald, 1999). A number of mechanisms of seed deterioration have been suggested, including disruption of nucleic acids, proteins, storage lipids, and membranes (Osborne, 1980; ReuZeau *et al.*, 1992; Bewley and Black, 1994; Sun and Leopold, 1995; Thapliyal and Connor, 1997; Pukacka, 1998). The disruption of membrane phospholipids and storage lipids by peroxidation is regarded as the primary reason for seed deterioration, because these lipids can be easily damaged by oxidative stress caused by unfavourable environmental conditions (Smirnoff, 1993).

Abbreviations: DGDG, digalactosyldiacylglycerol; LOOH, lipid hydroperoxide; 1-LPC, 1-lysophosphatidylcholine; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol.

© 2011 The Author(s).

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

However, the molecular and biochemical events underlying lipid peroxidation-mediated seed deterioration have not been well characterized (Clerkx *et al.*, 2004).

Several lines of evidence have suggested that seed deterioration is associated with lipid peroxidation caused by peroxyl radicals. Sattler *et al.* (2004) reported that tocopherol-deficient mutants had significantly reduced seed longevity and elevated levels of lipid hydroperoxides (LOOHs) during germination. Therefore, protection of membrane lipids and oils by tocopherols (lipid-soluble antioxidants) against various oxidative stresses is crucial for seed germination. Regeneration of ascorbate may play an important role in protecting storage reserves that serve as essential energy sources for seed germination (Eastmond, 2007). *Arabidopsis* mutants with defects in the peroxisomal membrane monodehydroascorbate reductase isoform, a protein that generates reduced ascorbate, exhibited elevated levels of H_2O_2, lipid peroxidation, and protein oxidation, resulting in impaired seedling establishment. This finding suggests that detoxifying H_2O_2 and preventing peroxisomal release of H_2O_2 are critical for protecting membrane lipids and storage oils. Phospholipase Dα1 (PLDα1), a membrane lipid-hydrolysing phospholipase, plays a role in *Arabidopsis* seed deterioration and ageing (Devaiah *et al.*, 2007). Therefore, knockout mutants of PLDα1 exhibited an increased tolerance to accelerated and natural ageing. PLDα1-deficient seeds lost fewer unsaturated fatty acids and accumulated fewer lipid peroxides than wild-type seeds after storage or exposure to adverse conditions. This result supports the theory that production of phosphatidic acid (PA) from phospholipids by PLD is an initial step of membrane degradation and seed deterioration, and that PA-derived lipid peroxyl radicals will subsequently attack phospholipids, resulting in a chain reaction of membrane lipid peroxidation (Thompson, 1988; Samama and Pearce, 1993).

In higher plants, acylhydrolases play important roles in the regulation of diverse cellular metabolic functions, including seed germination, cell elongation, anther dehiscence, jasmonate-mediated defence signalling, and leaf senescence (Ishiguro *et al.*, 2001; He and Gan, 2002; Lee *et al.*, 2003; Eastmond, 2006; Hyun *et al.*, 2008). Based on their lipolytic specificities, acylhydrolases are classified into different types of lipases, such as galactolipases, triacylglycerol (TAG) lipases, and phospholipases (Brady *et al.*, 1990; Beisson *et al.*, 2003). The DEFECTIVE IN ANTHER DEHISCIENCE 1 (DAD1) (At2g44810) was originally identified as an *Arabidopsis* PLA1 that catalysed the initial step for jasmonic acid production in chloroplasts (Ishiguro *et al.*, 2001). The DAD1-like acylhydrolase family consists of 12 members, all of which contain *sn*-1-specific acylhydrolase activity, and is further divided into three subgroups based on predicted subcellular localizations (Beisson *et al.*, 2003; Ryu, 2004; Seo *et al.*, 2008, 2009; Kim *et al.*, 2011). Seven proteins, including DAD1, belong to class I, which is typified by a putative N-terminal chloroplast-targeting signal, while four cytosolic proteins belong to class II. The sole class III protein, At1g30370, was predicted to localize to the mitochondria with unknown function. The class II enzyme At2g42690 was suggested to be involved in the PR-1-mediated defensive response to ultraviolet-B (UV-B) irradiation (Lo *et al.*, 2004). The chloroplast-targeted DONGLE (DGL) (At1g05800) participates in wound-induced jasmonate formation and is functionally redundant with DAD1 in *Arabidopsis* (Ishiguro *et al.*, 2001; Hyun *et al.*, 2008; Ellinger *et al.*, 2010). In this study, the biochemical and cellular properties of the class III DAD1-like acylhydrolase isoform AtDLAH encoded by *At1g30370* was analysed. Transgenic *Arabidopsis* seeds that overexpress AtDLAH exhibited strongly enhanced resistance to lipid peroxidation and ageing treatments compared with wild-type and *atdlah* knockout mutant plants, suggesting that AtDLAH plays a significant role in *Arabidopsis* seed viability and longevity.

Materials and methods

Plant materials

Wild-type *Arabidopsis thaliana* (ecotype Columbia-0) and the T-DNA insertion *AtDLAH* (*At1g30370*) loss-of-function mutant line (WiscDsLox489-492N9) were obtained from the Ohio State University Arabidopsis Biological Resources Center (ABRC, Columbus, OH, USA). The *atdlah* mutant was confirmed by genotyping PCR using the T-DNA left-border primer and gene-specific primers (Supplementary Table S1 available at *JXB* online). Full-length *AtDLAH* cDNA was cloned into the binary vector pBI121 (ABRC stock number CD3-388), and the resulting plasmid was transformed into *Arabidopsis* as previously described (Seo *et al.*, 2008). *35S:AtDLAH* transgenic lines were selected due to their resistance to kanamycin (30 μg ml^{-1}). Expression levels of the *AtDLAH* gene in leaves and seeds of transgenic and mutant plants were examined by reverse transcription-PCR (RT-PCR) using gene-specific primers (Supplementary Table S1).

RNA extraction and cDNA synthesis

Total RNA was isolated from developing seeds (0, 12, and 21 d after pollination) and germinating seeds (0, 1, 2, 3, and 4 d after imbibition) as previously described (Ruuska and Ohlrogge, 2001). RNA samples were extracted using an RNAiso RNA purification kit according to the manufacturer's protocol (Takara, Shiga, Japan) and then treated with DNase I for 30 min. First-strand cDNA synthesis was performed as previously described (Kim *et al.*, 2010). RT-PCR was conducted using gene-specific primer sets (Supplementary Table S1) with the following conditions: 25 cycles were conducted, each consisting of 45 s at 95 °C, 1 min at 60 °C, and 90 s at 72 °C in an automatic thermal cycler (Applied Biosystems, Carlsbad, CA, USA).

Construction of the MBP–AtDLAH recombinant protein

AtDLAH cDNA lacking the N-terminal transit peptide sequence was amplified by PCR using gene-specific primers (Supplementary Table S1). The products were introduced into the pMal-c2X plasmid (New England BioLabs, Hertfordshire, UK). The fusion protein was expressed in the *Escherichia coli* BL21 (DE3) strain and purified by affinity chromatography using amylose resin (New England BioLabs) as previously described (Seo *et al.*, 2009).

In vitro lipase assay

The *in vitro* assay for measuring lipase activity was performed as previously described (Seo *et al.*, 2009). To examine phospholipase A1 activity using radiolabelled phosphatidylcholine (PC) with asymmetric fatty acids, a reaction mixture containing 15 pmol 1-palmitoyl-2-[^{14}C]palmitoyl-PC (2.22 GBq $mmol^{-1}$, GE Healthcare, Uppsala, Sweden) was incubated with 20 μg of recombinant

fusion proteins for 30 min at 30 °C in a final volume of 200 μl of 0.2% Triton X-100, 100 mM NaCl, and 50 mM sodium phosphate buffer (pH 6.8). Reaction products were separated by thin-layer chromatography (TLC) (Silica Gel 60; Merck, Whitehouse Station, NJ, USA) and developed with chloroform/methanol/CH_3COOH/water (85:15:12.5:3.5, v/v/v/v). The colorimetric assay for substrate specificity was conducted with a mixture containing various lipid substrates, including PC, phosphatidylethanolamine (PE), phosphatidic acid (PA), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), triolein (TAG), 1,2-diacylglycerol, 1,3-diacylglycerol, 1-monoacylglycerol, 2-monoacylglycerol, and 1-lysophosphatidylcholine (1-LPC). The recombinant fusion protein was incubated with the mixtures for 30 min at 30 °C, and then released free fatty acids were measured using NEFA-HR colorimetric kits (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's protocol.

Protoplast transient assay

A full-length *AtDLAH* cDNA clone and a synthetic nuclear localization signal (NLS; Woo *et al.*, 2010) were ligated into a soluble-modified green fluorescent protein (GFP) plasmid (psmGFP) (Cho *et al.*, 2008) to construct *35S:AtDLAH-GFP* and *35S:NLS-GFP*, respectively. The GFP fusion constructs were transformed into protoplasts prepared from wild-type and mt-yk CS16264 *Arabidopsis* rosette leaves by polyethylene glycol (PEG) treatment (Seo *et al.*, 2008). The mt-yk CS16264 plant was used as a mitochondria-localized marker (Nelson *et al.*, 2007). After 16 h of incubation, the expression of *35S:AtDLAH-GFP* and *35S:NLS-GFP* was monitored with a cooled CCD camera and a BX51 fluorescence microscope (Olympus, Tokyo, Japan) as previously described (Son *et al.*, 2009).

Purification of chloroplasts and mitochondria

Chloroplasts and mitochondria were isolated from light-grown 2-week-old leaves from wild-type and *35S:AtDLAH-HA* T_4 transgenic plants as previously described (Tanaka *et al.*, 2004) with some modifications. The collected leaves were homogenized in an isolation solution containing 50 mM HEPES-KOH (pH 7.4), 0.33 M sorbitol, 1 mM $MnCl_2$, 2 mM EDTA, and 0.2% bovine serum albumin (BSA). The homogenate was filtered through four layers of nylon mesh.

To fractionate chloroplasts, extracts were centrifuged at 350 *g* for 5 min at 4 °C and the resulting supernatant was layered on to an uncontinuous gradient consisting of 30% and 60% (v/v) Percoll in isolation solution. The gradients were centrifuged at 8000 *g* for 15 min at 4 °C. The intact chloroplasts distributed around the 30/60% Percoll interface were isolated and diluted with the isolation solution. After samples were centrifuged at 4000 *g* for 10 min at 4 °C to remove Percoll, pellets were re-suspended in isolation solution.

To separate mitochondria, the filtered extracts were centrifuged at 3000 *g* for 5 min at 4 °C and supernatants were re-centrifuged at 22 000 *g* for 15 min at 4 °C. Pellets were resuspended with isolation solution and centrifuged at 3000 *g* for 15 min at 4 °C. To obtain mitochondrial pellets, supernatants were centrifuged at 22 000 *g* for 15 min at 4 °C and pellets were resuspended with isolation solution. The quality of the purified chloroplasts and mitochondria was assessed by an immunoblot analysis using a specific antibody for the mitochondrial protein VDAC1 (voltage-dependent anion-selective channel protein 1) (Clausen *et al.*, 2004). The signals were detected with an ECL Western Detection kit (Millipore, Billerica, MA, USA).

Measurement of mitochondrial lipase enzyme activities in wild-type, 35S:AtDLAH *transgenic, and* atdlah *mutant plants*

Total proteins were isolated from purified mitochondrial fractions from wild-type, *35S:AtDLAH* transgenic, and *atdlah* knock-out mutant seedlings as previously described by Seo *et al.* (2008). Cell extracts were used for colorimetric assays (40 μg total protein) as previously described by Seo *et al.* (2008).

Accelerated-ageing treatment and germination tests

For normal growth conditions, freshly harvested seeds were surface-sterilized and imbibed at 4 °C for 5 d. These seeds were plated on 0.5× MS medium (Duchefa, Haarlem, The Netherlands) and 0.7% phytoagar (Duchefa), pH 5.7, and then incubated in a growth chamber. For the accelerated-ageing treatment, seeds were incubated for 48 h at 43 °C and 100% relative humidity in a closed bottle before the cold imbibition (Sattler *et al.*, 2004). In order to validate comparisons, all seeds are grown under the same conditions and harvested at the same time. Each day for 5 d, germination was scored by radicle emergence from the seeds.

Scanning electron microscopy

Dry seeds were coated with platinum–palladium in a sputter-coater as previously described by Ryu *et al.* (2009). The surface structure was subjected to high-resolution scanning electron microscopy (model S-800, FESEM, Hitachi, Tokyo, Japan) at an accelerating voltage of 3 kV under high vacuum conditions (Penfield *et al.*, 2001; Atia *et al.*, 2009).

FOX assay

To determine the level of LOOHs, total lipids were extracted and assayed as previously described (Griffiths *et al.*, 2000; Zhu *et al.*, 2009) with minor modifications. Briefly, the lipid extracts were incubated with FOX solution [90% methanol (v/v), 25 mM H_2SO_4, 4 mM butylated hydroxytoluene (BHT), 250 μM ferrous ammonium sulphate hexahydrate, and 100 μM xylenol orange] for 30 min at 25 °C. Absorbancies were immediately measured at 560 nm. Because the reactivity of 18:2-derived hydroperoxides with the FOX reagent was reported to be nearly identical to that of hydrogen peroxide (DeLong *et al.*, 2002), serial concentrations (0, 0.05, 0.10, 0.20, 0.25, 0.30, and 0.50 mM) of hydrogen peroxide were used to make a standard curve, and the levels of LOOHs in the seeds were calculated using the standard curve.

TLC analysis of total polar lipid contents

Total lipids were extracted from 30 mg of dry seeds as previously described (Welti *et al.*, 2002; Devaiah *et al.*, 2006). To inhibit phospholipase activities, seeds were homogenized in 1 ml of isopropanol with 0.01% BHT at 75 °C. For lipid extraction, the ground samples were extracted several times with chloroform. The lipid extracts were separated on TLC plates and stained with iodine vapour. Stained bands were quantified using Multi Gauge v.3.1 (Fuji Film, Tokyo, Japan).

Results

Characterization of AtDLAH

At1g30370 is classified as the only member of the class III PLA1 family because it contains a putative N-terminal transit peptide for localizing to the mitochondria (Ryu, 2004). The *At1g30370* gene is comprised of a single 1,590 bp exon, and encodes a 530 amino acid protein containing a lipase 3 domain (Fig. 1A). The predicted molecular mass of the At1g30370 protein was determined to be 60.8 kDa and the calculated pI was 9.9. Sequences common to lipase active sites, such as the GXSXG motif and a catalytic triad (serine, aspartate, and histidine residues), were conserved in the lipase

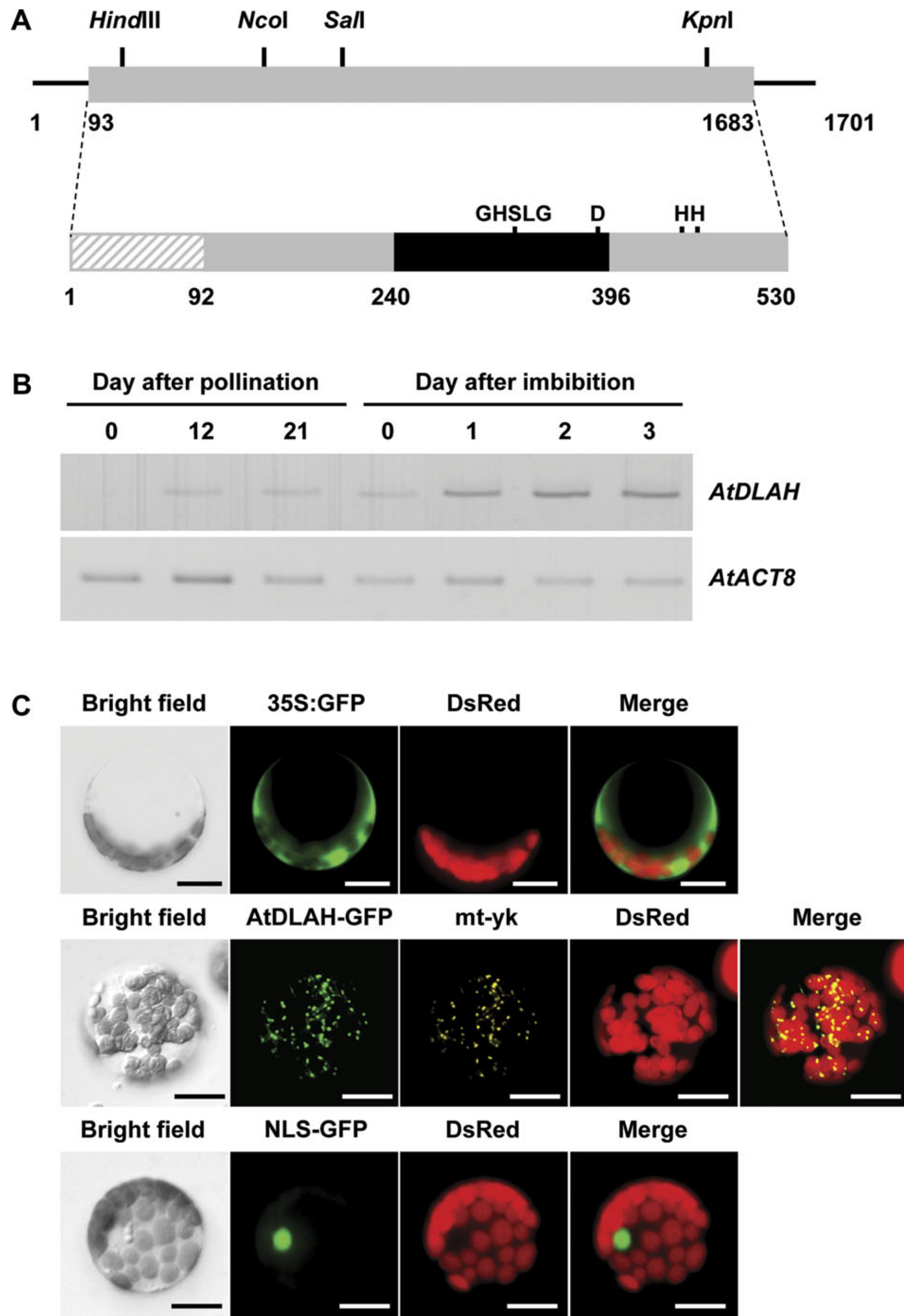

Fig. 1. Structure, expression, and subcellular localization of *Arabidopsis* AtDLAH. (A) Schematic representation of *AtDLAH* (At1g30370) cDNA and its deduced protein. Solid lines depict the 5'- and 3'-untranslated regions. The coding region (grey box) with restriction enzyme sites, the lipase 3 domain (black box), and the putative N-terminal transit peptide (hatched box) are represented. The lipase consensus sequence (GHSLG) and the catalytic triad (serine, aspartate, and two candidate histidine residues) are indicated. (B) *AtDLAH* expression in developing and early germinating seeds was analysed by RT-PCR. *AtACT8* was used as a loading control. (C) Subcellular localization of AtDLAH in *Arabidopsis* protoplasts. The *35S:AtDLAH-GFP* fusion gene was introduced into protoplasts using a PEG-mediated method. The *35S:GFP* and *35S:NLS-GFP* constructs were used as controls for cytosolic and nuclear proteins, respectively. The mt-yk plant was used as a mitochondria-localized marker. Scale bars=10 μm.

3 domain, indicating that At1g30370 has typical lipase features. Therefore, At1g30370 was termed AtDLAH (*Arabidopsis thaliana* DAD1-like acylhydrolase). A database search revealed that AtDLAH was most closely related to a poplar protein (*Populus trichocarpa*, XP_002314049.1) and a castor bean triacylglycerol lipase (*Ricinus communis*, XP_002531054.1) with 68% and 63% identities, respectively (Supplementary Fig. S1 at *JXB* online). AtDLAH also shared relatively high sequence identity with a grape protein (*Vitis vinifera*, XP_002272780.1; 59% identity) and a rape chloroplast lipase (*Brassica napus*, ACJ76846.1; 45% identity). The cellular functions of these putative plant lipases are currently

unknown. Protein sorting signal prediction programs, such as PSORT (http://psort.ims.u-tokyo.ac.jp/form.html) and TargetP (http://www.cbs.dtu.dk/services/TargetP), predicted that AtDLAH has a 92 amino acid N-terminal transit peptide for targeting to the mitochondria (TargetP score: 0.513). These results strongly suggest that AtDLAH is a mitochondria-localized DAD1-like acylhydrolase.

Expression and subcellular localization of AtDLAH

Using semi-quantitative RT-PCR with gene-specific primers, the temporal expression patterns of *AtDLAH* in developing and early germinating *Arabidopsis* seeds were examined. Figure 1B shows that *AtDLAH* transcript was slightly detected during seed development stages, while its level was elevated in germinating seeds after imbibition. Since the mRNA for *AtDLAH* was gradually expressed in germinating seeds, *AtDLAH* probably plays a role in early *Arabidopsis* seedling development.

To determine if AtDLAH was localized to the mitochondria as predicted, a protoplast transient assay using AtDLAH-fused GFP as a fluorescent marker was performed. Figure 1C shows that control GFP was uniformly distributed throughout the cytosolic fractions of protoplasts, and GFP fused to the synthetic NLS sequence (NLS–GFP) was exclusively localized to the nuclei. In contrast, the AtDLAH–GFP fusion protein displayed a spotted pattern. Because similar spotted signals were previously detected for mitochondria-localized proteins (Kabeya and Sato, 2005; Sheahan *et al.*, 2005), there is a possibility that the AtDLAH–GFP fusion protein is also localized to the mitochondria. To test this possibility, the AtDLAH–GFP fusion protein was expressed in protoplasts of organelle-specific marker plants (mt-yk CS16264), which contain a mitochondrial protein fused with yellow fluorescence protein (YFP) (Nelson *et al.*, 2007). The GFP signals were overlaid with the yellow fluorescence signal of the mitochondrial marker. These results indicate that the AtDLAH–GFP fusion protein is primarily localized to the mitochondria of *Arabidopsis* leaf protoplasts.

In vitro *enzyme activity and substrate specificity of AtDLAH*

Ishiguro *et al.* (2001) previously reported that DAD1 exhibits PLA1 activity. Because AtDLAH has a highly conserved lipase 3 domain similar to that in DAD1, AtDLAH is also considered to be a member of the PLA1 family (Ryu, 2004). To test whether the AtDLAH protein exhibits PLA1 activity, AtDLAH was expressed without its transit peptide in *E. coli* as a fusion with maltose-binding protein (MBP). The cleavage site of the transit peptide for the protein was determined using the TargetP program (probability score: 0.513) and analysis of previous reports (Ishiguro *et al.*, 2001; Padham *et al.*, 2007; Hyun *et al.*, 2008). The purified MBP–AtDLAH protein produced 1-LPC after incubation with 1-palmitoyl-2-[^{14}C]linoleoyl-PC as a substrate (Fig. 2A). These results indicate that AtDLAH can catalyse the hydrolysis of PC at the *sn*-1 position *in vitro*.

Although AtDLAH has PLA1 activity *in vitro*, it could possess activities for other lipid substrates as reported previously (Padham *et al.*, 2007; Hyun *et al.*, 2008; Seo *et al.*, 2009). To examine this possibility, various lipid substrates, including PC, PE, PA, MGDG, DGDG, triolein, 1,2-diacylglycerol, 1,3-diacylglycerol, 1-monodiacylglycerol, 2-monodiacylglycerol, and 1-LPC, were used for *in vitro* enzyme assays. Under the experimental conditions used in this study, MBP–AtDLAH displayed a strong preference for 1-LPC, 1-monodiacylglycerol, and PA, and, to a lesser extent, MBP–AtDLAH possessed phopholipase activity toward PC and PE (Fig. 2B). Therefore, AtDLAH contains lipase activity toward a broad range of lipid substrates with preferential specificities for lipids with an acyl chain on their *sn*-1 position. Additionally, AtDLAH functions optimally at a pH of 6.6 with 1-LPC as a substrate (Fig. 2C).

Generation and characterization of AtDLAH-*overexpressing transgenic and* atdlah *loss-of-function mutant plants*

To address the cellular function of AtDLAH, overexpression and reverse-genetic approaches were used. Transgenic *Arabidopsis* plants (*35S:AtDLAH*) that ectopically expressed *AtDLAH* under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter were developed. Overexpression of *AtDLAH* in independent T_4 transgenic lines was confirmed by RT-PCR (Fig. 3A). The loss-of-function T-DNA knockout mutant (WiscDsLox489_492N9; *atdlah*) for *AtDLAH* was also identified by genotyping PCR and RT-PCR (Fig. 3B, C). In normal and accelerated-ageing-treated seeds, transcript levels of *AtDLAH* in wild-type, *35S:AtDLAH*, and mutant plants were highly similar to those in leaves (Fig. 3D). These plants were subsequently used for phenotypic analysis.

To ensure further the ectopic expression of AtDLAH and its mitochondrial localization at the protein level, chloroplast and mitochondrial fractions were isolated from light-grown 2-week-old leaves of wild-type and *35S:AtDLAH-HA* T_4 transgenic leaves. Protein extracts were prepared from each fraction and subsequently analysed by protein gel blotting using an anti-haemagglutinin (HA) antibody. As shown in Fig. 4A, the 65.9 kDa band specific to AtDLAH-HA was predominantly present in the mitochondrial fraction, confirming its mitochondrial localization. VDAC1, a mitochondria-specific marker protein, was exclusively detected in the corresponding fraction.

AtDLAH lipase activity was measured using mitochondrial protein extracts from wild-type, *35S:AtDLAH-HA*, and *atdlah* leaves. The results in Fig. 4B demonstrate that the level of mitochondrial lipase activity in *35S:AtDLAH-HA* (lines #2 and #3) was 1.3 times greater than that of the wild-type leaves. These results indicate that ectopic expression of *AtDLAH* caused a small but specific increase in mitochondrial lipase activity. However, the predicted decrease in mitochondrial lipase activity in the *atdlah* mutant as compared with that of the wild-type leaves was not identified.

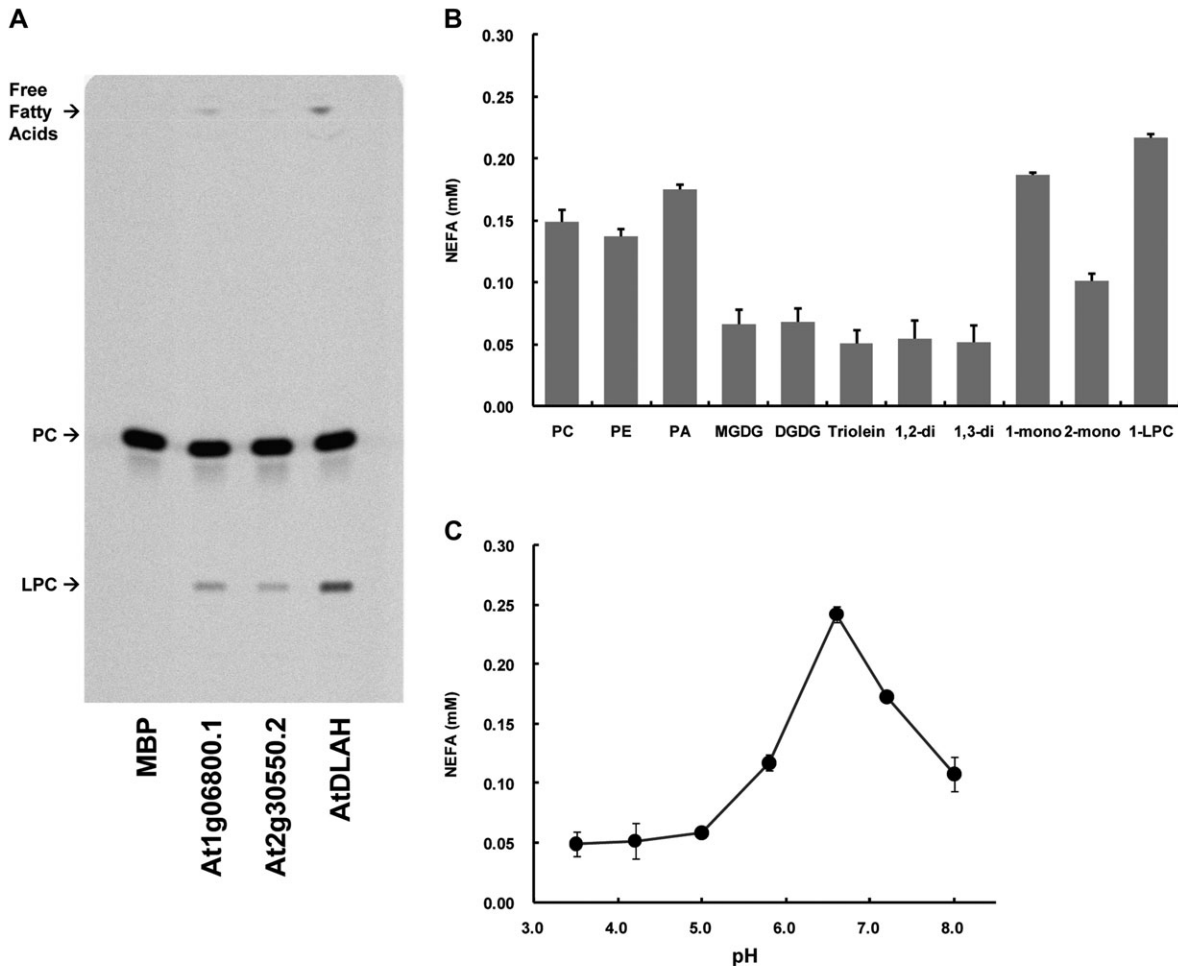

Fig. 2. AtDLAH enzyme assays. (A) AtDLAH catalyses the hydrolysis of PC at the *sn*-1 position. PLA1 activity was measured by the production of radiolabelled lysophosphatidylcholine (LPC) after incubation with 1-palmitoyl-2-[^{14}C]palmitoyl-PC. The resultant ^{14}C-labelled LPC was detected by TLC. MBP was used as a negative control, and At1g06800.1 and At2g30550.2, which have PLA1 activity (Seo *et al.*, 2009), were used as positive controls. (B) Lipolytic enzyme assays to determine substrate specificity. Lipolytic activities were determined using an NEFA-HR kit with PC, PE, PA, MGDG, DGDG, triolein, 1,2-diacylglycerol, 1,3-diacylglycerol, 1-monodiacylglycerol, 2-monodiacylglycerol, and 1-LPC as substrates. Results are expressed as the means ±SD from four independent experiments.
(C) Optimal pH for AtDLAH activity. Lipase activity of AtDLAH was determined by quantifying the release of free fatty acids from 1-LPC in phosphate buffers with different pHs at 30 °C for 30 min. Results are expressed as the means ±SD from four independent experiments.

AtDLAH-*overexpressing transgenic seeds had higher germination percentages after ageing treatment as compared with wild-type and* atdlah *mutant seeds*

The morphological comparison of light-grown wild-type, *35S:AtDLAH* (lines #2 and #3), and *atdlah* mutant seedlings at an early stage of development is presented in Fig. 5A. The *AtDLAH*-overexpressing transgenic plants displayed significantly longer roots than control seedlings 5 d after germination, while the *atdlah* mutant plants exhibited shorter roots than did the wild-type plants (Fig. 5A). To investigate whether the longer roots in *35S:AtDLAH* seedlings were due to enhanced cell elongation and/or cell division, expression patterns of cell elongation- and division-associated genes were monitored. The expression levels of several cell elongation/division marker genes, including *AtCYCD3*, *AtCDC2b*, *AtEXP5*, and *AtPCNA* (Seo *et al.*, 2008), were indistinguishable between the three types of seedlings (Supplementary Fig. S2 at *JXB* online), suggesting that the longer roots in the *35S:AtDLAH* seedlings may not be a consequence of enhanced cell elongation or cell cycle progression.

The germination percentages of wild-type, *35S:AtDLAH* transgenic, and *atdlah* mutant seeds were examined. Under normal germination conditions, *35S:AtDLAH* transgenic seedlings (lines #2 and #3) displayed higher (~15%) germination percentages 1 d after germination than the wild-type and mutant seedlings (left panels in Fig. 5B, C). Thereafter, the germination percentages were very similar among the three different types of plants, though the germination percentage of the mutant plant was slightly lower (~3%). The higher germination percentage of the transgenic seeds probably resulted in the slightly larger cotyledons in the *AtDLAH*-overexpressing plants 5 d after germination (Fig. 5A, B). Therefore, the longer roots of the *35S:AtDLAH* seedlings were also probably not due to increased cell elongation/

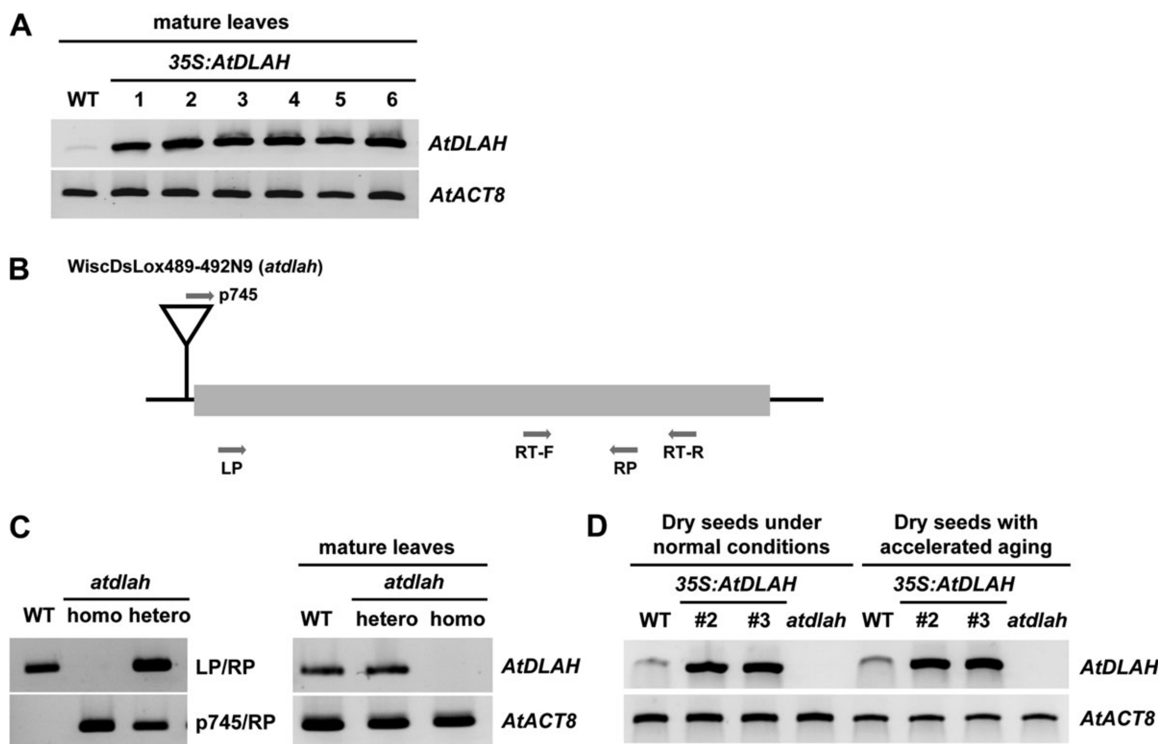

Fig. 3. Molecular characterization of wild-type, *AtDLAH*-overexpressing T_4 transgenic, and *atdlah* mutant plants. (A) RT-PCR analysis of wild-type and six independent *AtDLAH*-overexpressing T_4 transgenic plants (lines #1, #2, #3, #4, #5, and #6) in leaves. The *AtACT8* gene was used as a loading control. (B) Schematic representation of the *atdlah* mutant. The T-DNA insertion is shown as an inverted triangle. The shaded bar indicates the coding region, and gene-specific (LP and RP) and T-DNA-specific (p745) primers used for genotyping and RT-PCR are indicated with arrows. (C) PCR analysis of the *atdlah* mutant in leaves. Genomic PCR analysis of the *atdlah* loss-of-function mutant using gene-specific and T-DNA-specific primers (left panel). RT-PCR analysis of the *atdlah* mutant (right panel). The *AtACT8* gene was used as a loading control. DNA sequences of primers used in this study are shown in Supplementary Table S1 at *JXB* online. (D) RT-PCR analysis of wild-type, *35S:AtDLAH* (transgenic lines #2 and #3), and *atdlah* mutant seeds without or with accelerated-ageing treatment. The *AtACT8* gene was used as a loading control.

division, but rather due to quicker germination. The slightly quicker germination in overexpressors and later germination in the mutant were also supported by the finding that there were apparent differences in root length even in the earlier germination stage (1–3 d after imbibition) (the inset graph of the right panel in Fig. 5A). In contrast, the root elongation rates remained constant in wild-type, *35S:AtDLAH*, and *atdlah* seedlings for 10 d after germination (right panel in Fig. 5A), further supporting the role of *AtDLAH* in germination.

The germination of wild-type, *35S:AtDLAH*, and *atdlah* mutant seeds was further analysed following treatment with accelerated-ageing conditions. Seeds were incubated with high temperature (43 °C) and high humidity (100% relative humidity) for 2 d, conditions known to accelerate seed ageing (Tesnier *et al.*, 2002; Devaiah *et al.*, 2007; Oge *et al.*, 2008; Rajjou *et al.*, 2008). Aged seeds were imbibed at 4 °C for 5 d to break dormancy, and then placed at 22 °C to germinate while root radicle emergence was monitored (Sattler *et al.*, 2004). In response to the accelerated-ageing treatment, the germination percentage of wild-type seeds was reduced to the level of 16–27% at 1–5 d after germination (right panels in Fig. 5B, C). In contrast, aged *35S:AtDLAH* seeds (lines #2 and #3) displayed markedly higher germination percentages (61–83%) than aged wild-type seeds, whereas aged mutant seeds rarely germinated (1–5%).

Abscisic acid (ABA) is a plant hormone that inhibits seed germination (Leung and Giraudat, 1998). To test whether the higher germination percentages of *35S:AtDLAH* seeds under normal and accelerated-ageing treatments were due to reduced sensitivity to ABA, seed germination percentages of wild-type, *35S:AtDLAH*, and *atdlah* plants in the presence of various ABA concentrations (0.1, 0.5, or 1 μM) were examined. All three types of plants displayed highly similar sensitivities to the various concentrations of exogenously applied ABA (Supplementary Fig. S3 at *JXB* online). Thus, the greater germination percentage of the *35S:AtDLAH* seeds was probably due to increased seed viability, not decreased sensitivity to ABA. Taken together, the results in Fig. 5 suggest that AtDLAH activity is positively associated with germination ability, and this effect was more evident after accelerated-ageing treatments.

AtDLAH activity was positively correlated with seed viability and longevity

Seed coats play an important role in seed viability and longevity since they provide the primary line of defence

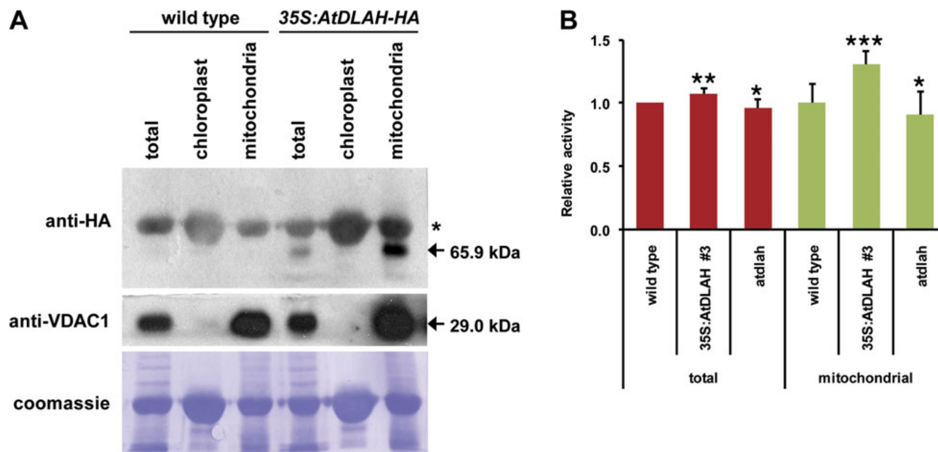

Fig. 4. Mitochondrial localization of AtDLAH-HA and mitochondrial lipase activities in wild-type, *AtDLAH*-overexpressing T_4 transgenic, and *atdlah* mutant plants. (A) Cellular fractionation analysis of the AtDLAH-HA fusion protein. Extracts of total, chloroplast, and mitochondrial fractions were prepared from wild-type and *35S:AtDLAH-HA* T_4 transgenic plants. Total proteins from each fraction were analysed with anti-HA and anti-VDAC1 antibodies (control for mitochondrial proteins). Loaded proteins were visualized by Coomassie staining. Arrows indicate AtDLAH-HA (65.9 kDa) and VDAC1 (29.0 kDa) proteins. An asterisk indicates non-specific binding to a 70 kDa protein by the anti-HA antibody. (B) Mitochondrial lipase enzyme assays. Total and mitochondrial proteins were prepared from wild-type, *AtDLAH*-overexpressing transgenic (line #3), and *atdlah* mutant plants and incubated with 1-palmitoyl-2-[^{14}C]palmitoyl-PC as the substrate at 30 °C for 30 min. Lipase activities were determined by quantifying the release of ^{14}C-labelled lyso-PC as described in Fig. 2A. Results are expressed as the means ±SD from three independent experiments. The data were analysed by Student's *t*-test. The statistical significance was determined at ****P* <0.01, ***P* <0.05, and **P* <0.1, respectively.

against unfavourable environmental conditions (Mohamed-Yasseen *et al.*, 1994). Development of the epidermal layer in the *Arabidopsis* seed coat is a complex process, involving cell growth, biosynthesis and secretion of pectinaceous mucilage, and production of a secondary cell wall (Beeckman *et al.*, 2000; Western *et al.*, 2000; Windsor *et al.*, 2000). Because of the water-holding capacity of the mucilage layer in *Arabidopsis* seeds, it is difficult to monitor seed viability (Debeaujon and Koornneef, 2000). Therefore, the tetrazolium uptake assay was employed to assess seed viability. Upon entry of the tetrazolium solution into seeds, the aleurone layer of live embryos stain red, whereas seeds with low viability stain a whitish colour (Rossetto *et al.*, 2004; Oge *et al.*, 2008). The *35S:AtDLAH* (lines #2 and #3) seeds had a dark-red staining pattern after tetrazolium treatment under both normal and accelerated-ageing conditions (Supplementary Fig. S4A at *JXB* online). However, the *atdlah* mutant seeds clearly had less of a red-stained pattern as compared with the *AtDLAH* overexpressors. As expected, the wild-type seeds had an intermediate degree of tetrazolium staining (Supplementary Fig. S4A). Therefore, the level of *AtDLAH* expression was positively correlated with seed viability.

The structural aspects of the seed coat of wild-type, *35S:AtDLAH* transgenic, and *atdlah* mutant seeds was examined using a scanning electron microscope. In general, the epidermal layers of *Arabidopsis* seed coats have hexagonal morphologies, a volcano-shaped structure known as the columella, and mucilage. The epidermal cells of wild-type and mutant seed coats displayed these typical properties (Fig. 6). However, the *AtDLAH*-overexpressing transgenic seed coats exhibited unusual epidermal cells that were more populous and had amorphous shapes (Fig. 6). To determine if this structural abnormality affects the extrusion of pectinaceous mucilage, wild-type, *35S:AtDLAH*, and *atdlah* mutant seeds were subjected to ruthenium red staining that selectively stains pectinaceous mucilage layers (Arsovski *et al.*, 2009). The mucilage layers extruded from seed coats were equally stained with ruthenium red, regardless of the plant type (Supplementary Fig. S4B at *JXB* online), indicating that the unusual morphology of the cell layers of the *35S:AtDLAH* seed coat did not alter the mucilage layers. The overexpression of *AtDLAH* resulted in dense and amorphous epidermal cells in seed coats, which correlated with the enhanced seed longevity of *35S:AtDLAH* transgenic plants.

Analysis of lipid peroxidation and total polar lipid contents after ageing treatments

To examine further why *35S:AtDLAH* seeds have a higher germination percentage, qualitative and quantitative changes in total seed lipid content were evaluated. Total lipids from wild-type, *35S:AtDLAH*, and *atdlah* mutant seeds with or without accelerated-ageing treatment were extracted, separated on TLC plates, and visualized with iodine vapour. Under normal growth conditions, the total polar lipid content was highly similar among these seeds. More detailed inspection, however, indicates that there were detectable differences in intensities of 2–3 polar lipid bands on TLC plates. These lipid bands are indicated by an asterisk in Fig. 7A. Furthermore, after accelerated-ageing treatments, the quantities of polar lipids in wild-type and *atdlah* mutant seeds were reduced by ~50% as compared with seeds under normal conditions (Fig. 7A), a finding consistent with previous results (Pearce and Abdel-Samad, 1980; Pukacka

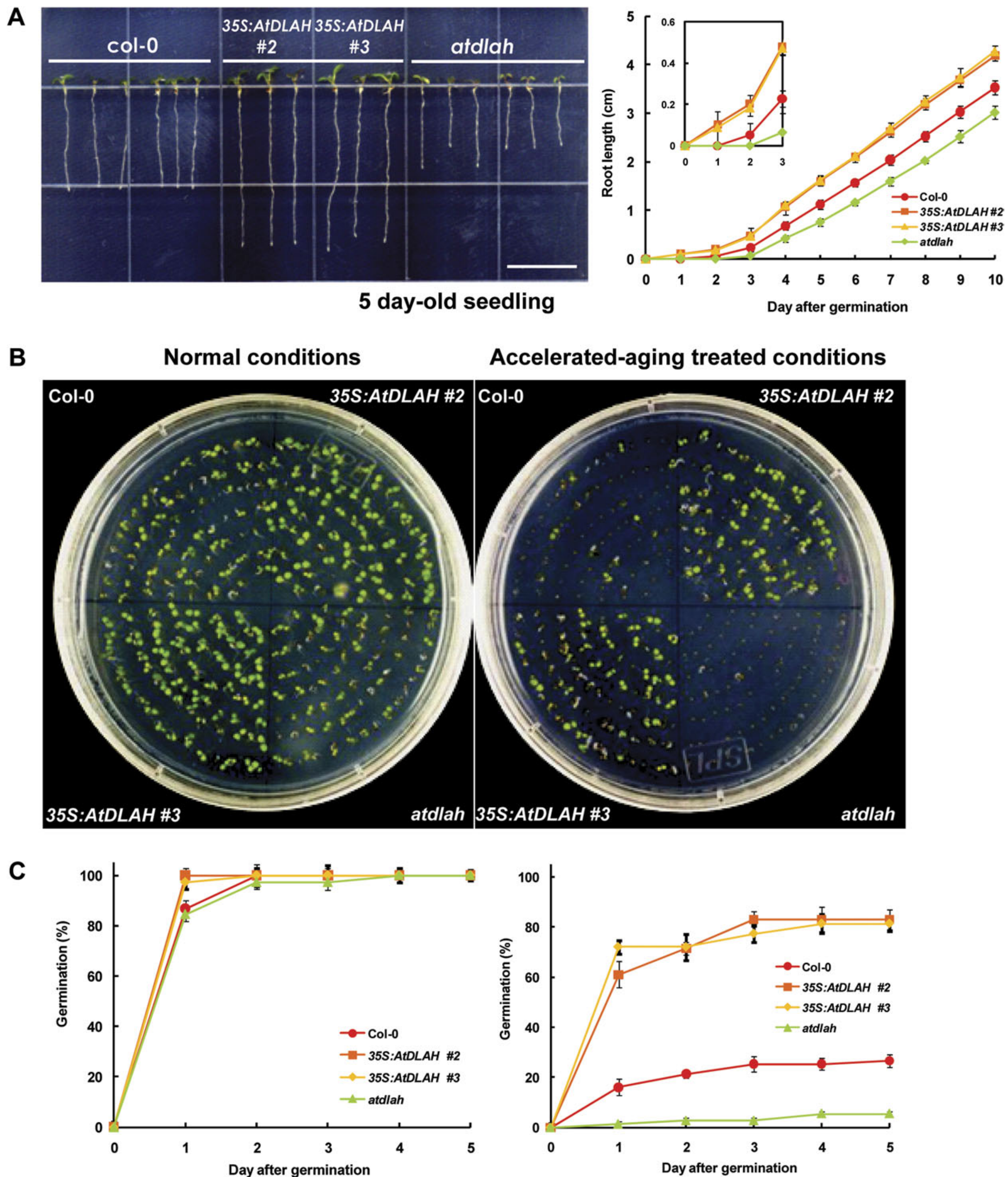

Fig. 5. Phenotypes of wild-type, *AtDLAH*-overexpressing T_4 transgenic, and *atdlah* mutant plants at early stages of development. (A) Morphologies of 5-day-old wild-type, *AtDLAH*-overexpressing transgenic (lines #2 and #3), and *atdlah* mutant seedlings (left panel). Root length was monitored for 10 d after germination (right panel). Results are expressed as means ±SD from three independent experiments. The inset shows the root length for 0–3 d after germination. (B) Germination of wild-type, *AtDLAH*-overexpressing, and *atdlah* mutant seeds under normal germination conditions (left panel) or following accelerated-ageing conditions (43 °C for 48 h at 100% relative humidity) (right panel). (C) Germination percentages of wild-type, *AtDLAH*-overexpressing (lines #2 and #3), and *atdlah* mutant seeds were monitored under normal (left panel) and accelerated-ageing (right panel) conditions for 5 d after germination. Germination was defined as radicle emergence. Results are expressed as means ±SD from three independent experiments.

and Kuiper, 1988; Ouzouline *et al.*, 2009). However, the *35S:AtDLAH* seeds (line #2 and #3) lost only 15–19% of their polar lipids during accelerated-ageing treatments relative to the seeds under normal conditions, indicating that the decrease in polar lipids in *35S:AtDLAH* seeds was not as drastic as those in wild-type and mutant seeds. On the other hand, the levels of neutral storage lipids, including TAGs and diacylglycerols, were not significantly changed in response to

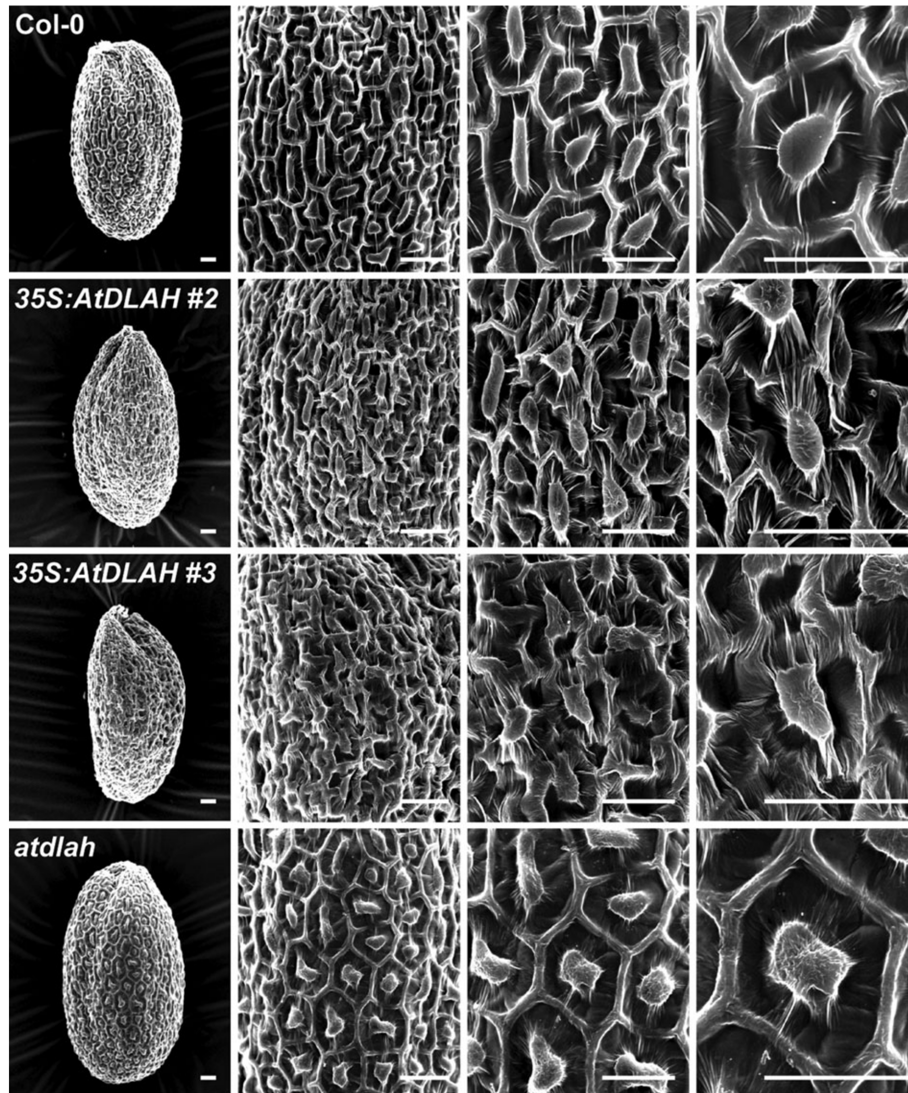

Fig. 6. Structural aspects of the seed coat of wild-type, *AtDLAH*-overexpressing T_4 transgenic, and *atdlah* mutant plants. Scanning electron microscopic examination was performed with wild-type, *AtDLAH*-overexpressing, and *atdlah* mutant seeds. Scale bars=150 μm.

accelerated-ageing treatments (Supplementary Fig. S5 at *JXB* online). These results suggest that enough polar lipids remain in *35S:AtDLAH* seeds to germinate following accelerated-ageing treatment, as overexpression of *AtDLAH* resulted in a smaller reduction of polar lipids.

The loss of polar lipids during ageing is mainly due to lipid peroxidation, and this oxidative stress is a major contributor to seed deterioration (Bailly *et al.*, 1996, 1998; Devaiah *et al.*, 2006, 2007; Ouzouline *et al.*, 2009). To determine the level of lipid peroxidation in wild-type, *35S:AtDLAH*, and *atdlah* mutant seeds, the quantity of LOOHs in seeds was measured using ferrous oxidation–xylenol orange (FOX) assays (DeLong *et al.*, 2002; Zhu *et al.*, 2009). Total lipids were extracted from seeds and incubated with FOX solution, and LOOH levels were measured by spectrophotometry. Because the reactivity of 18:2-derived hydroperoxides with the FOX reagent was reported to be nearly identical to that of hydrogen peroxide (DeLong *et al.*, 2002), different concentrations of H_2O_2 were used for generating a standard curve, and the levels of LOOHs were calculated as H_2O_2 levels. The mean LOOH level in wild-type seeds was 2.42 ± 0.15 mM g^{-1} FW (Fig. 7B). The mean concentrations of LOOHs in *35S:AtDLAH* (lines #2 and #3) and *atdlah* mutant seeds were 1.11 ± 0.068–1.23 ± 0.11 mM g^{-1} FW and 3.08 ± 0.084 mM g^{-1} FW, respectively. Therefore, the *35S:AtDLAH* seeds accumulated a significantly lower level of LOOHs than wild-type seeds, whereas the mutant seeds contained the highest level of lipid hydroperoxidation (Fig. 7B). After accelerated-ageing treatments, this lipid hydroperoxidation trend was maintained, even though the absolute amounts of LOOHs in all seeds were reduced to nearly half of their starting levels (Fig. 7B). In addition, the ratio of LOOH to polar lipids in *35S:AtDLAH* seeds (lines #2 and #3) was further reduced by one-third of that in the wild-type seeds in response to accelerated-ageing treatment, whereas the relative level was increased 1.4 times in *atdlah* mutant seeds (Fig. 7C). These results indicate that propagation of lipid peroxidation during

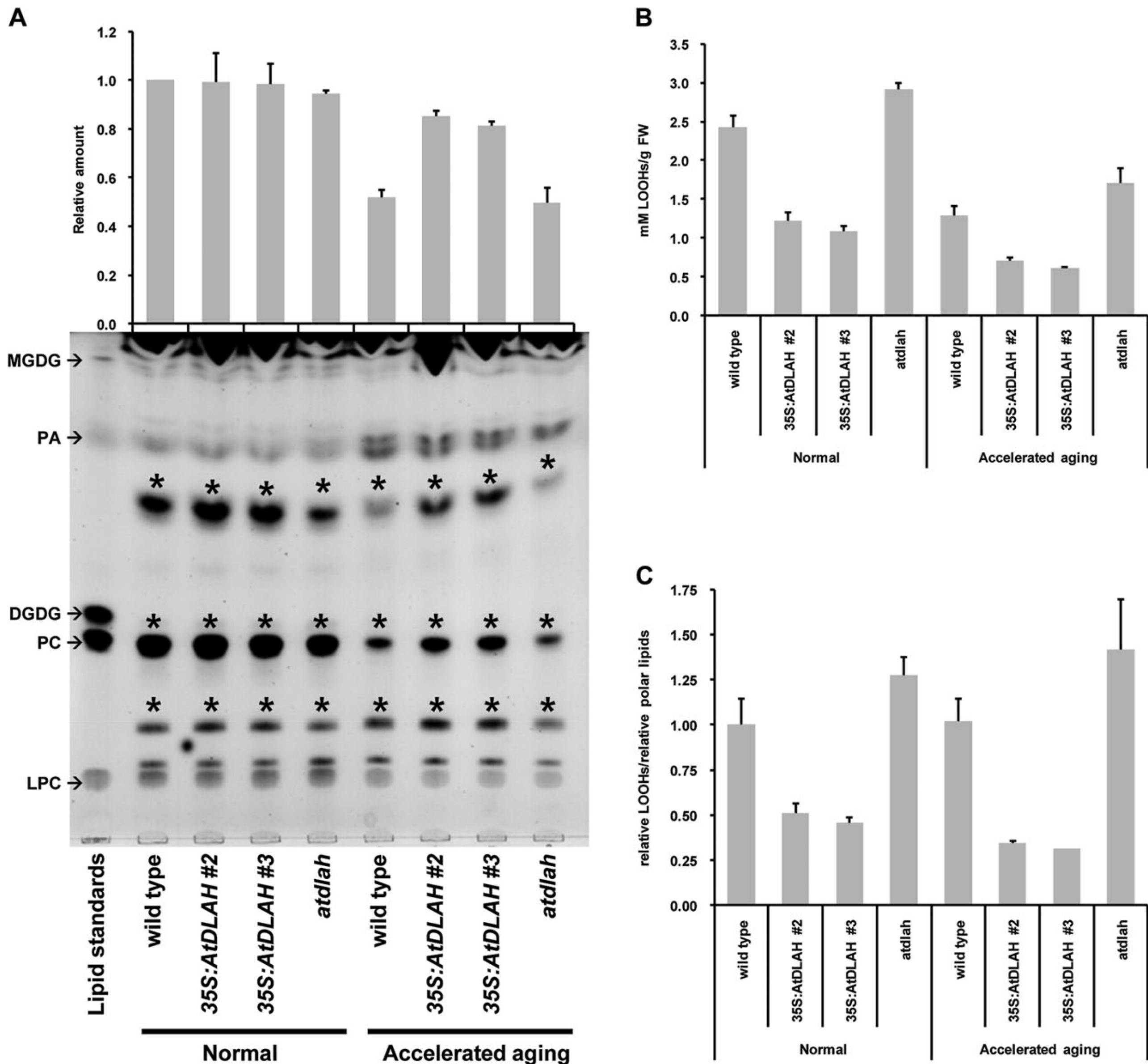

Fig. 7. Lipid peroxidation and lipid content in wild-type, *AtDLAH*-overexpressing T_4 transgenic, and *atdlah* mutant seeds. (A) Total lipid content in wild-type, *AtDLAH*-overexpressing (lines #2 and #3), and *atdlah* mutant seeds under normal and accelerated-ageing conditions. Total polar lipids were separated by TLC and visualized with iodine vapour. The lipid standards from top to bottom are MGDG, PA, DGDG, PC, and LPC. The amounts of individual polar lipids (represented above the plate) were quantified from the TLC plates using Multi Gauge v.3.1 (Fuji Film, Tokyo, Japan). The lipid bands which showed the detectable differences in intensity on the TLC plate among wild-type, *35S:AtDLAH*, and *ataldh* are indicated by asterisks (*). Results are expressed as means ±SD from three independent experiments. (B) Absolute levels of lipid hydroperoxide in wild-type, *AtDLAH*-overexpressing (lines #2 and #3), and *atdlah* mutant seeds under normal and accelerated-ageing conditions. The levels of lipid hydroperoxides (LOOHs) were measured by FOX assay. The different amounts of H_2O_2 were used for generating a standard curve and the amount of LOOH in seeds was calculated as a H_2O_2 level. LPC (40 μg) was used as a control for lipid extraction from each seed sample. Results are expressed as means ±SD from three independent experiments. (C) Relative levels of LOOHs per polar lipid in wild-type, *AtDLAH*-overexpressing (lines #2 and #3), and *atdlah* mutant seeds under normal and accelerated-ageing conditions. Each ratio was calculated by dividing the amount of LOOH by the relative amount of polar lipids. Results are expressed as means ±SD from three independent experiments.

seed ageing was repressed by overexpression of *AtDLAH* and accelerated by ablation of the gene. Thus, overexpression of *AtDLAH* decreased the reduction of polar lipids and reduced lipid peroxidation, which probably led to the increased seed viability.

Discussion

In this study, AtDLAH, an *Arabidopsis* DAD1-like acylhydrolase homologue encoded by the *At1g30370* gene, was characterized. AtDLAH has a well-conserved GHSLG lipase consensus sequence and a catalytic triad similar to

fungal and animal lipases (Fig. 1A) (Brady *et al.*, 1990; Winkler *et al.*, 1990). Similar to DAD1, AtDLAH also has a highly conserved lipase 3 domain, and therefore, it belongs to the *Arabidopsis* PLA1 family (Ryu, 2004). Several recent reports suggested that DAD1-like PLA1 family members contain lipase activities in addition to their PLA1 activity (Padham *et al.*, 2007; Hyun *et al.*, 2008; Seo *et al.*, 2009). Consistently, bacterially expressed AtDLAH had PLA1 activity for catalysing the hydrolysis of phospholipids at the *sn*-1 position (Fig. 2A); however, it displayed an even stronger preference toward 1-LPC, 1-monodiacylglycerol, and PA (Fig. 2B). These results suggest that AtDLAH has lipase activity toward a broad range of lipid substrates with a preferential acylhydrolase activity on the *sn*-1 position of its lipid substrates.

In silico analyses indicated that AtDLAH is a mitochondria-localized class III PLA1 (Ishiguro *et al.*, 2001; Ryu, 2004). AtDLAH was predominantly found in the mitochondria in both the protoplast transient assay (Fig. 1C) and the cellular fractionation study (Fig. 4A). In addition, *35S:AtDLAH* transgenic lines had significantly higher lipase activities in their mitochondrial fractions relative to those of the wild-type and *atdlah* mutant plants (Fig. 4B). Thus, AtDLAH is most probably a mitochondria-localized DAD1-like acylhydrolase. A number of mitochondrial lipases with critical cellular functions, such as preventing oxidant-induced lipid peroxidation and rescuing cells from death, have been identified in various species, including humans, animals, and yeast (Claycomb and Kilsheimer, 1971; Schousboe, 1976; Demant, 1978; Andersen *et al.*, 2009). In contrast, only a few plant lipases, such as peanut lipase and potato PLA, have been identified as mitochondria-targeted lipases (Jacks *et al.*, 1967; Hasson and Laties, 1976). Research elucidating functional roles of these putative plant mitochondrial lipases is scarce.

These results prompted this investigation of the cellular functions of AtDLAH. This initial phenotypic analysis demonstrated that *AtDLAH*-overexpressing young seedlings contained significantly longer roots than wild-type and *atdlah* seedlings (Fig. 5A). This apparent phenotype was reminiscent of the transgenic *Arabidopsis* plants that constitutively expressed a hot pepper phospholipase 1 (*CaPLA1*). The root length of *35S:CaPLA1* seedlings was longer than that of wild-type plants, and was caused by promotion of the cell cycle and enhanced fatty acid metabolism (Seo *et al.*, 2008). However, the longer-root phenotype of *35S:AtDLAH* was unlikely to be due to increased cell elongation or cell cycle progression (Supplementary Fig. S2 at *JXB* online), but was probably due to enhanced seed germination under normal conditions (Fig. 5B, C). More importantly, *35S:AtDLAH* seeds were markedly tolerant to accelerated-ageing treatments, while most *atdlah* seeds failed to germinate under the accelerated-ageing conditions. Because *35S:AtDLAH* and *atdlah* seeds displayed very similar sensitivities to ABA in terms of germination percentages (Supplementary Fig. S3 at *JXB* online), AtDLAH probably plays a positive role in protecting and/or maintaining seed contents that are important for germination. This view is further supported by the finding that *AtDLAH*-overexpressing seeds were more effectively stained by tetrazolium (Supplementary Fig. S4A) and contained more densely populated epidermal cells with amorphous shapes (Fig. 6) in comparison with the wild-type and *atdlah* mutant seeds.

Seed deterioration during storage is accompanied by a progressive loss of membrane lipids (Stewart and Bewley, 1980; Samama and Pearce, 1993; Al-Maskri *et al.*, 2003; Sattler *et al.*, 2006; Devaiah *et al.*, 2007). This type of polar lipid degradation is mainly due to lipid peroxidation caused by natural ageing and environmental factors, such as temperature, humidity, and oxygen (Smirnoff, 1993; Bailly *et al.*, 1996, 1998; Rajjou and Debeaujon, 2008; Ouzouline *et al.*, 2009; Mene-Saffrane *et al.*, 2010). A previous study using accelerated-ageing-treated, tocopherol-deficient *Arabidopsis* mutants (*vte1* and *vte2* plants), indicated that tocopherols can prevent membrane lipid peroxidation during seed storage, germination, and early seedling development, thus preserving seed viability (Sattler *et al.*, 2004). The *35S:AtDLAH* seeds harboured greater amounts of polar lipids following accelerated-ageing treatments than wild-type and *atdlah* knockout mutant seeds (Fig. 7A). Furthermore, the lipid peroxidation level of *35S:AtDLAH* seeds was almost 2-fold lower than that of wild-type and mutant seeds under normal conditions, and this difference was increased to 3-fold following accelerated-ageing treatments (Fig. 7B). Therefore, cellular levels of AtDLAH are inversely correlated to the peroxidation of polar lipids in mature seed embryos and, in turn, the reduction of lipid hydroperoxide content enhances ageing tolerance and seed viability.

For germination, *Arabidopsis* seeds obtain most of their metabolic energy from sucrose in the cotyledons of mature embryos, rather than from storage lipids (Baud *et al.*, 2002; Dekkers *et al.*, 2004; Cernac *et al.*, 2006; Andre and Benning, 2007). On the other hand, storage lipid mobilization is essential for subsequent seedling establishment, including elongation of the hypocotyl and root, greening of the cotyledons, and transition from a heterotroph to a photoautotroph (Eastmond, 2006). Therefore, the increased ageing tolerance of *35S:AtDLAH* seeds may have been caused by limiting the peroxidation and degradation of the polar lipids in structural embryonic membranes, not by reduction of oxidative damage in neutral storage lipids. The results of the current study suggest that there was no difference in the quantity of neutral lipids among wild-type, *35S:AtDLAH*, and *atdlah* seeds following accelerated-ageing treatments (Supplementary Fig. S5 at *JXB* online). Additionally, root elongation rates were constant in these seedlings 1–10 d after germination (Fig. 5A), suggesting that the differences in seed viabilities were not a result of the mobilization ability of the storage lipids. In addition, although the total amounts of the polar lipids seemed to be indistinguishable among wild-type, overexpressors, and mutant seeds, the intensities of 2–3 polar lipid bands on TLC plates were apparently different among these lines under normal conditions (Fig. 7A). These results suggest that the differences in some kinds of polar lipid contents resulting from the changes in expression of *AtDLAH* are possibly attributed to germination under normal conditions (Fig. 5A). These differences were further

exacerbated by accelerated-ageing treatment (Fig. 7) sufficient to produce large differences in germination ability (Fig. 5B, C).

Recently, polar lipid profiling and lipid peroxidation analyses with a *PLDα1* knockout mutant indicated that the activity of PLDα1 may promote membrane lipid degradation and reduce seed viability (Devaiah *et al.*, 2006, 2007). After storage and accelerated ageing, the *pldα1* mutant seeds exhibited higher germination percentages, decreased reduction in oil content, and significantly lower lipid hydroperoxide levels than did wild-type seeds that were similar to the *35S:AtDLAH* seeds. Therefore, it is possible that PLDα1 and AtDLAH have conflicting roles in peroxidation and degradation of polar lipids. One possible mechanism is that PLDα1 and AtDLAH compete for the same phospholipid substrates. This hypothesis is partially supported by the finding that PLDα1 is found not only in the plasma membrane fractions but also in the mitochondria (Fan *et al.*, 1999) where AtDLAH is predominantly localized (Figs 1C, 4).

PA is a strong candidate for a signalling molecule for generating reactive oxygen species (ROS) and inducing lipid peroxidation (Sang *et al.*, 2001; Park *et al.*, 2004; Devaiah *et al.*, 2007). In *Arabidopsis* leaves, PA activates NADPH oxidase activity to produce superoxide, which is immediately converted to H_2O_2. These PA-induced ROS, in turn, promote the death of leaf cells (Sang *et al.*, 2001; Park *et al.*, 2004). There is circumstantial evidence that PA produced by PLDα1 enhances production of lipid peroxidation in *Arabidopsis* seeds, thereby decreasing seed quality during natural and accelerated ageing processes (Devaiah *et al.*, 2007). Under the *in vitro* lipase enzyme assay conditions used in this study, PA was one of the preferred substrates for AtDLAH (Fig. 2B). Therefore, PA produced by natural and accelerated ageing conditions could be degraded by AtDLAH in transgenic *35S:AtDLAH* seeds, resulting in increased seed longevity.

Alternatively, PLDα1 and AtDLAH may work independently. The overall level of PA detected by TLC was similar in wild-type, *35S:AtDLAH*, and *atdlah* seeds (Fig. 7A). Thus, more detailed quantifications of PA and other signalling lipids are required to elucidate the roles of PLDα1 and AtDLAH further. Current efforts are focused on analysing total and mitochondrial lipid profiles in wild-type, *35S:AtDLAH*, and *atdlah* seeds using an electrospray ionization tandem mass spectrometer (ESI-MS/MS), rather than TLC, to elucidate the effect of *AtDLAH* expression on both qualitative and quantitative traits of seed lipids. In addition, analysis of phenotypes and cellular functions of AtDLAH with fine-regulated ageing treatments under lower relative humidity conditions, which may be more similar to natural ageing conditions (Oge *et al.*, 2008), is also being conducted. These results will clarify the mode of action of mitochondrial DAD1-like acylhydolase in seed viability and longevity.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Sequence analysis of *Arabidopsis* AtDLAH.

Figure S2. Expression levels of cell cycle- and cell elongation-related genes in wild-type, *35S:AtDLAH*, and *atdlah* mutant seedlings.

Figure S3. Germination analysis of wild-type, *35S:AtDLAH*, and *atdlah* mutant seeds in response to ABA.

Figure S4. Vital staining with tetrazolium and mucilage release of wild-type, *AtDLAH*-overexpressing T_4 transgenic, and *atdlah* mutant seeds.

Figure S5. Neutral lipid content of wild-type, *AtDLAH*-overexpressing T_4 transgenic, and *atdlah* mutant seeds.

Table S1. Primer sequences used for cloning *AtDLAH* cDNA, construction of the MBP fusion protein, the GFP fusion protein, and transgenic plants, genotyping PCR, and RT-PCR.

Acknowledgements

This work was supported by grants from the Technology Development Program for Agriculture and Forestry (Project no. 309017-5 funded by the Ministry for Agriculture, Forestry and Fisheries, Republic of Korea), the National Research Foundation (Project no. 2009-0078317 funded by the Ministry of Education, Science, and Technology, Republic of Korea), and the National Center for GM Crops (Project no. PJ008152 of the Next Generation BioGreen 21 Program funded by the Rural Development Administration, Republic of Korea) to WTK.

References

Al-Maskri AY, Khan MM, Khan IA, Al-Habsi K. 2003. Effect of accelerated aging on viability, vigor (RGR), lipid peroxidation and leakage in carrot (*Daucus carota* L.) seeds. *International Journal of Agriculture and Biology* **5,** 580–584.

Andersen AD, Poulsen KA, Lambert IH, Pedersen SF. 2009. HL-1 mouse cardiomyocyte injury and death after simulated ischemia and reperfusion: roles of pH, Ca2+-independent phospholipase A(2), and Na+/H+ exchange. *American Journal of Physiology-Cell Physiology* **296,** C1227–C1242.

Andre C, Benning C. 2007. Arabidopsis seedlings deficient in a plastidic pyruvate kinase are unable to utilize seed storage compounds for germination and establishment. *Plant Physiology* **145,** 1670–1680.

Atia A, Debez A, Barhoumi Z, Abdelly C, Smaoui A. 2009. Histochemical localization of essential oils and bioactive substances in the seed coat of the halophyte *Crithmum maritimum* L. (Apiaceae). *Journal of Plant Biology* **52,** 448–452.

Arsovski AA, Villota MM, Rowland O, Subramaniam R, Western TL. 2009. MUM ENHANCERS are important for seed coat mucilage production and mucilage secretory cell differentiation in *Arabidopsis thaliana*. *Journal of Experimental Botany* **60,** 2601–2612.

Bailly C, Benamar A, Corbineau F, 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. *Physiologia Plantarum* **97,** 104–110.

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

Baud S, Boutin JP, Miquel M, Lepiniec L, Rochat C. 2002. An integrated overview of seed development in Arabidopsis thaliana ecotype WS. *Plant Physiology and Biochemistry* **40,** 151–160.

Beeckman T, De Rycke R, Viane R, Inze D. 2000. Histological study of seed coat development in Arabidopsis thaliana. *Journal of Plant Research* **113,** 139–148.

Beisson F, Koo AJ, Ruuska S, *et al*. 2003. Arabidopsis genes involved in acyl lipid metabolism. A 2003 census of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based database. *Plant Physiology* **132,** 681–697.

Bewley JD, Black M. 1994. *Seeds: physiology of development and germination*. New York: Plenum Press.

Brady L, Brzozowski AM, Derewenda ZS, Dodson E, Dodson G, Tolley S, Turkenburg JP, Christiansen L, Huge-Jensen B, Norskov L. 1990. A serine protease triad forms the catalytic centre of a triacylglycerol lipase. *Nature* **343,** 767–770.

Cernac A, Andre C, Hoffmann-Benning S, Benning C. 2006. WRI1 is required for seed germination and seedling establishment. *Plant Physiology* **141,** 745–757.

Cho SK, Ryu MY, Song C, Kwak JM, Kim WT. 2008. Arabidopsis PUB22 and PUB23 are homologous U-box E3 ubiquitin ligases that play combinatory roles in response to drought stress. *The Plant Cell* **20,** 1899–1914.

Chrispeels MJ, Sadava DE. 2003. *Plants, genes, and crop biotechnology*. Boston: Jones and Bartlett Publishers, Inc.

Clausen C, Ilkavets I, Thomson R, Philippar K, Vojta A, Mohlmann T, Neuhaus E, Fulgosi H, Soll J. 2004. Intracellular localization of VDAC proteins in plants. *Planta* **220,** 30–37.

Claycomb WC, Kilsheimer GS. 1971. Purification and properties of a lipase from rat liver mitochondria. *Journal of Biological Chemistry* **246,** 7139–7143.

Clerkx EJM, Blankestijn-De Vries H, Ruys GJ, Groot SPC, Koornneef M. 2004. Genetic differences in seed longevity of various Arabidopsis mutants. *Physiologia Plantarum* **121,** 448–461.

Coolbear P. 1995. Mechanism of seed deteroration. In: Basra AS, ed. *Seed quality: basic mechanisms and agricultural implications*. New York: Food Product Press, 223–277.

Debeaujon I, Koornneef M. 2000. Gibberellin requirement for Arabidopsis seed germination is determined both by testa characteristics and embryonic abscisic acid. *Plant Physiology* **122,** 415–424.

Dekkers BJ, Schuurmans JA, Smeekens SC. 2004. Glucose delays seed germination in Arabidopsis thaliana. *Planta* **218,** 579–588.

DeLong JM, Prange RK, Hodges DM, Forney CF, Bishop MC, Quilliam M. 2002. Using a modified ferrous oxidation–xylenol orange (FOX) assay for detection of lipid hydroperoxides in plant tissue. *Journal of Agricultural and Food Chemistry* **50,** 248–254.

Demant EJ. 1978. Fatty acid inhibition of triacylglycerol lipase in mitochondrial fractions from baker's yeast. *FEBS Letters* **85,** 109–113.

Devaiah SP, Pan XQ, Hong YY, Roth M, Welti R, Wang XM. 2007. Enhancing seed quality and viability by suppressing phospholipase D in Arabidopsis. *The Plant Journal* **50,** 950–957.

Devaiah SP, Roth MR, Baughman E, Li M, Tamura P, Jeannotte R, Welti R, Wang X. 2006. Quantitative profiling of polar glycerolipid species from organs of wild-type Arabidopsis and a phospholipase Dalpha1 knockout mutant. *Phytochemistry* **67,** 1907–1924.

Eastmond PJ. 2006. SUGAR-DEPENDENT1 encodes a patatin domain triacylglycerol lipase that initiates storage oil breakdown in germinating Arabidopsis seeds. *The Plant Cell* **18,** 665–675.

Eastmond PJ. 2007. MONODEHYROASCORBATE REDUCTASE4 is required for seed storage oil hydrolysis and postgerminative growth in Arabidopsis. *The Plant Cell* **19,** 1376–1387.

Ellinger D, Stingl N, Kubigsteltig II , Bals T, Juenger M, Pollmann S, Berger S, Schuenemann D, Mueller MJ. 2010. DONGLE and DEFECTIVE IN ANTHER DEHISCENCE1 lipases are not essential for wound- and pathogen-induced jasmonate biosynthesis: redundant lipases contribute to jasmonate formation. *Plant Physiology* **153,** 114–127.

Fan L, Zheng S, Cui D, Wang X. 1999. Subcellular distribution and tissue expression of phospholipase Dα, D$\beta\Delta$, and Dγ in Arabidopsis. *Plant Physiology* **119,** 1371–1378.

Griffiths G, Leverentz M, Silkowski H, Gill N, Sanchez-Serrano JJ. 2000. Lipid hydroperoxide levels in plant tissues. *Journal of Experimental Botany* **51,** 1363–1370.

Hasson EP, Laties GG. 1976. Purification and characterization of an a type phospholipase from potato and its effect on potato mitochondria. *Plant Physiology* **57,** 148–152.

He YH, Gan SS. 2002. A gene encoding an acyl hydrolase is involved in leaf senescence in Arabidopsis. *The Plant Cell* **14,** 805–815.

Hyun Y, Choi S, Hwang HJ, *et al*. 2008. Cooperation and functional diversification of two closely related galactolipase genes for jasmonate biosynthesis. *Developmental Cell* **14,** 183–192.

Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K. 2001. The DEFECTIVE IN ANTHER DEHISCIENCE gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. *The Plant Cell* **13,** 2191–2209.

Jacks TJ, Yatsu LY, Altschul AM. 1967. Isolation and characterization of peanut spherosomes. *Plant Physiology* **42,** 585–597.

Kabeya Y, Sato N. 2005. Unique translation initiation at the second AUG codon determines mitochondrial localization of the phage-type RNA polymerases in the moss Physcomitrella patens. *Plant Physiology* **138,** 369–382.

Kim EY, Seo YS, Lee H, Kim WT. 2010. Constitutive expression of CaSRP1, a hot pepper small rubber particle protein homolog, resulted in fast growth and improved drought tolerance in transgenic Arabidopsis plants. *Planta* **232,** 71–83.

Kim EY, Seo YS, Kim WT. 2011. AtDSEL, an *Arabidopsis* cytosolic DAD1-like acylhydrolase, is involved in negative regulation of storage oil mobilization during seedling establishment. *Journal of Plant Physiology* **168,** 1705–1709.

Lee HY, Bahn SC, Kang YM, Lee KH, Kim HJ, Noh EK, Palta JP, Shin JS, Ryu SB. 2003. Secretory low molecular weight

phospholipase A2 plays important roles in cell elongation and shoot gravitropism in Arabidopsis. *The Plant Cell* **15,** 1990–2002.

Leung J, Giraudat J. 1998. Abscisic acid signal transduction. *Annual Review of Plant Physiology* **49,** 199–222.

Li D, Pritchard H. 2009. The science and economics of *ex situ* plant conservation. *Trends in Plant Science* **14,** 614–621.

Lo M, Taylor C, Wang L, Nowack L, Wang TW, Thompson J. 2004. Characterization of an ultraviolet B-induced lipase in Arabidopsis. *Plant Physiology* **135,** 947–958.

McDonald MB. 1999. Seed deterioration: physiology, repair and assessment. *Seed Science and Technology* **27,** 177–237.

Mene-Saffrane L, Jones AD, Dellapenna D. 2010. Plastochromanol-8 and tocopherols are essential lipid-soluble antioxidants during seed desiccation and quiescence in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **107,** 17815–17820.

Mohamed-Yasseen Y, Barringer SA, Splittstoesser WE, Costanza S. 1994. The role of seed coats in seed viability. *Botanical Review* **60,** 426–439.

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

Nelson B, Cai X, Nebenfuhr A. 2007. A multicolored set of *in vivo* organelle markers for co-localization studies in Arabidopsis and other plants. *The Plant Journal* **51,** 1126–1136.

Oge L, Bourdais G, Bove J, Collet B, Godin B, Granier F, Boutin JP, Job D, Jullien M, Grappin P. 2008. Protein repair l-isoaspartyl methyltransferase 1 is involved in both seed longevity and germination vigor in Arabidopsis. *The Plant Cell* **20,** 3022–3037.

Osborne DJ. 1980. *Senescence in plants*. Boca Raton, FL: CRC Press.

Ouzouline M, Tahani N, Demandre C, El Amrani A, Benhassaine-Kesri G, Caid HS. 2009. Effects of accelerated aging upon the lipid composition of seeds from two soft wheat varieties from Morocco. *Grasas Y Aceites* **60,** 367–374.

Padham AK, Hopkins MT, Wang TW, McNamara LM, Lo M, Richardson LG, Smith MD, Taylor CA, Thompson JE. 2007. Characterization of a plastid triacylglycerol lipase from Arabidopsis. *Plant Physiology* **143,** 1372–1384.

Park J, Gu Y, Lee Y, Yang ZB, 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 Physiology* **134,** 129–136.

Pearce RS, Abdel-Samad IM. 1980. Change in fatty acid content of polar lipids during ageing of seeds of peanut (*Arachis hypogea* L.). *Journal of Experimental Botany* **31,** 1283–1980.

Penfield S, Meissner RC, Shoue DA, Carpita NC, Bevan MW. 2001. MYB61 is required for mucilage deposition and extrusion in the Arabidopsis seed coat. *The Plant Cell* **13,** 2777–2791.

Pukacka S. 1998. Changes in membrane fatty acid composition during desiccation of seeds of silver maple. *Seed Science and Technology* **26,** 535–540.

Pukacka S, Kuiper PJC. 1988. Phospholipid composition and fatty acid peroxidation during ageing of Acer platanoides seeds. *Physiologia Plantarum* **72,** 89–93.

Rajjou L, Debeaujon I. 2008. Seed longevity: survival and maintenance of high germination ability of dry seeds. *Comptes Rendus Biologies* **331,** 796–805.

Rajjou L, Lovigny Y, Groot SPC, Belghaz M, Job C, Job D. 2008. Proteome-wide characterization of seed aging in Arabidopsis. A comparison between artificial and natural aging protocols. *Plant Physiology* **148,** 620–641.

ReuZeau C, Goffner D, Cavalie G. 1992. Relations between protein composition and germination capacity of sunflowers seeds. *Seed Science Research* **2,** 223–230.

Rossetto M, Gross CL, Jones R, Hunter J. 2004. The impact of clonality on an endangered tree (Elaeocarpus williamsianus) in a fragmented rainforest. *Biological Conservation* **117,** 33–39.

Ruuska SA, Ohlrogge JB. 2001. Protocol for small-scale RNA isolation and transcriptional profiling of developing Arabidopsis seeds. *Biotechniques* **31,** 752–758.

Ryu MY, Cho SK, Kim WT. 2009. RNAi suppression of RPN12a decreases the expression of type-A ARRs, negative regulators of cytokinin signaling pathway, in Arabidopsis. *Molecules and Cells* **28,** 375–382.

Ryu SB. 2004. Phospholipid-derived signaling mediated by phospholipase A in plants. *Trends in Plant Science* **9,** 229–235.

Samama AM, Pearce RS. 1993. Ageing of cucumber and onion seeds—phospholipase-D, lipoxygenase activity and changes in phospholipid content. *Journal of Experimental Botany* **44,** 1253–1265.

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

Sattler SE, Gilliland LU, Magallanes-Lundback M, Pollard M, DellaPenna D. 2004. Vitamin E is essential for seed longevity, and for preventing lipid peroxidation during germination. *The Plant Cell* **16,** 1419–1432.

Sattler SE, Mene-Saffrane L, Farmer EE, Krischke M, Mueller MJ, DellaPennaa D. 2006. Nonenzymatic lipid peroxidation reprograms gene expression and activates defense markers in *Arabidopsis* tocopherol-deficient mutants. *The Plant Cell* **18,** 3706–3720.

Schousboe I. 1976. Properties of triacylglycerol lipase in a mitochondrial fraction from baker's yeast (*Saccharomyces cerevisiae*). *Biochimica et Biophysica Acta* **450,** 165–174.

Seo YS, Kim EY, Kim JH, Kim WT. 2009. Enzymatic characterization of class I DAD1-like acylhydrolase members targeted to chloroplast in Arabidopsis. *FEBS Letters* **583,** 2301–2307.

Seo YS, Kim EY, Mang HG, Kim WT. 2008. Heterologous expression, and biochemical and cellular characterization of CaPLA1 encoding a hot pepper phospholipase A1 homolog. *The Plant Journal* **53,** 895–908.

Sheahan MB, McCurdy DW, Rose RJ. 2005. Mitochondria as a connected population: ensuring continuity of the mitochondrial genome during plant cell dedifferentiation through massive mitochondrial fusion. *The Plant Journal* **44,** 744–755.

Smirnoff N. 1993. The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytologist* **125,** 27–58.

Son O, Cho SK, Kim EY, Kim WT. 2009. Characterization of three Arabidopsis homologs of human RING membrane anchor E3 ubiquitin ligase. *Plant Cell Reports* **28,** 561–569.

Stewart RRC, Bewley JD. 1980. Lipid peroxidation associated with accelerated aging of soybean axes. *Plant Physiology* **65,** 245–248.

Sun WO, Leopold AC. 1995. The Millard reaction and oxidative stress during aging of soybean seeds. *Physiologia Plantarum* **94,** 94–104.

Tanaka N, Fujita M, Handa H, *et al*. 2004. Proteomics of the rice cell: systematic identification of the protein populations in subcellular compartments. *Molecular Genetics and Genomics* **271,** 566–576.

Tesnier K, Strookman-Donkers HM, Van Pijlen JG, Van der Geest AHM, Bino RJ, Groot SPC. 2002. A controlled deterioration test for Arabidopsis thaliana reveals genetic variation in seed quality. *Seed Science and Technology* **30,** 149–165.

Thapliyal RC, Connor KF. 1997. Effects of accelerated ageing on viability, leachate exudation, and fatty acid content of Dalbergia sissoo Roxb seeds. *Seed Science and Technology* **25,** 311–319.

Thompson JE. 1988. *Senescence and aging in plants*. New York: Academic Press, 52–84.

Walters C, Wheeler L, Grotenhuis J. 2005. Longevity of seeds stored in a genebank: species characteristics. *Seed Science Research* **15,** 1–20.

Welti R, Li W, Li M, Sang Y, Biesiada H, Zhou HE, Rajashekar CB, Williams TD, Wang X. 2002. Profiling membrane lipids in plant stress responses. Role of phospholipase D alpha in freezing-induced lipid changes in Arabidopsis. *Journal of Biological Chemistry* **277,** 31994–32002.

Western TL, Skinner DJ, Haughn GW. 2000. Differentiation of mucilage secretory cells of the Arabidopsis seed coat. *Plant Physiology* **122,** 345–355.

Windsor JB, Symonds VV, Mendenhall J, Lloyd AM. 2000. Arabidopsis seed coat development: morphological differentiation of the outer integument. *The Plant Journal* **22,** 483–493.

Winkler FK, D'Arcy A, Hunziker W. 1990. Structure of human pancreatic lipase. *Nature* **343,** 771–774.

Woo HR, Kim JH, Kim J, Lee U, Song IJ, Lee HY, Nam HG, Lim PO. 2010. The RAV1 transcription factor positively regulates leaf senescence in Arabidopsis. *Journal of Experimental Botany* **61,** 3947–3957.

Zhu W, Zhao D-X, Miao Q, Xue T-T, Li X-Z, Zheng C-C. 2009. *Arabidopsis thaliana* metallothionein, AtMT2a, mediates ROS balance during oxidative stress. *Journal of Plant Biology* **52,** 582–592.