PCP
PLANT & CELL PHYSIOLOGY

Enzymatic and Non-Enzymatic Mechanisms Contribute to Lipid Oxidation During Seed Aging

Ayla Oenel[1,3], Agnes Fekete[1,3], Markus Krischke[1], Sophie C. Faul[1], Gabriele Gresser[1], Michel Havaux[2], Martin J. Mueller[1] and Susanne Berger[1,*]

[1]Julius-von-Sachs-Institute, Pharmaceutical Biology, University of Wuerzburg, Julius-von-Sachs-Platz 2, D-97082 Wuerzburg, Germany
[2]CEA, CNRS UMR7265, Aix-Marseille Université, Laboratoire d'Ecophysiologie Moléculaire des Plantes, 13108 Saint-Paul-lez-Durance, France
[3]These authors contributed equally to this work.
*Corresponding author: E-mail, berger@biozentrum.uni-wuerzburg.de; Fax, +49-931-31-86182.
(Received August 24, 2016; Accepted March 5, 2017)

Storage of seeds is accompanied by loss of germination and oxidation of storage and membrane lipids. A lipidomic analysis revealed that during natural and artificial aging of Arabidopsis seeds, levels of several diacylglycerols and free fatty acids, such as linoleic acid and linolenic acid as well as free oxidized fatty acids and oxygenated triacylglycerols, increased. Lipids can be oxidized by enzymatic or non-enzymatic processes. In the enzymatic pathway, lipoxygenases (LOXs) catalyze the first oxygenation step of polyunsaturated fatty acids. Analysis of lipid levels in mutants with defects in the two 9-LOX genes revealed that the strong increase in free 9-hydroxy- and 9-keto-fatty acids is dependent on LOX1 but not LOX5. Fatty acid oxidation correlated with an aging-induced decrease of germination, raising the question of whether these oxylipins negatively regulate germination. However, seeds of the *lox1* mutant were only slightly more tolerant to aging, indicating that 9-LOX products contribute to but are not the major cause of loss of germination during aging. In contrast to free oxidized fatty acids, accumulation of oxygenated triacylglycerols upon accelerated aging was mainly based on non-enzymatic oxidation of seed storage lipids.

Keywords: Arabidopsis • Lipid oxidation • Lipoxygenase • Seed deterioration.

Abbreviations: CID, collision-induced dissociation; HOT(D)E, hydroxy octadecatrienoic (dienoic) fatty acid; KOT(D)E, keto octadecatrienoic (dienoic) fatty acid; LOX, lipoxygenase; ROS, reactive oxygen species; UPLC-ESI-qTOF-MS, ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight-mass spectrometry.

Introduction

The longevity of seeds strongly depends on the plant species and on the storage conditions. Storage of seeds results in aging accompanied by gradual loss of seed quality and a decrease in the germination rate. This is especially relevant when seeds need to sustain longer periods of storage or periods with unfavorable conditions such as high temperatures, high moisture and contamination with microorganisms (Harrington 1972). Loss of germination ability is accompanied by loss of embryo viability and deterioration of storage nutrients. Key factors for the decrease in seed vigor are reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radicals and singlet oxygen. ROS can trigger oxidation and damage of membrane and storage lipids, proteins, DNA and other molecules (McDonald 1999) and can mediate cell death (Hu et al. 2012). Since oilseeds are rich in lipids, lipid oxidation could be a predominant damaging process in aging seeds (Stewart and Bewley 1980). Polyunsaturated fatty acids are particularly prone to oxidation, and most plant seed oils are rich in unsaturated fatty acids. Indeed, it was shown that levels of malondialdehyde, an end-product of oxidation of polyunsaturated fatty acids, increase during natural and artificial aging of seeds in Arabidopsis (Devaiah et al. 2007). In addition, the drastically shortened storability of seeds with a defect in the synthesis of lipophilic antioxidants of the tocopherol family supports the importance of lipid oxidation during aging (Sattler et al. 2004). In addition to non-enzymatic processes, lipids are also enzymatically oxidized. Lipoxygenases (LOXs) and dioxygenases are the most important enzymes oxidizing polyunsaturated fatty acids, with linoleic acid (C18:2) and linolenic acid (C18:3) as the main substrates (Feussner and Wasternack 2002). In plants, LOXs can introduce oxygen at the carbon at position 9 or 13 in C18-fatty acids; accordingly these enzymes are termed 9-LOX and 13-LOX (Andreou and Feussner 2009). The contribution of LOXs to lipid oxidation during seed deterioration has been mainly addressed in rice where it was shown that down-regulation of the activity of different LOX enzymes leads to enhanced storage stability and reduces production of malondialdehyde and lipid hydroperoxides (Gayen et al. 2014, Huang et al. 2014, Ma et al. 2015, Xu et al. 2015). However, it is not clear which portion enzymatic and non-enzymatic processes contribute to lipid oxidation. In addition, it is unclear whether these products of lipid oxidation are causal for the loss of germination.

Here we compared changes in the lipidome of Arabidopsis seeds subjected to accelerated or natural aging. The question of whether formation of oxidized lipids occurs enzymatically or non-enzymatically was addressed by examining aging-induced lipid oxidation of mutants defective in 9- and 13-LOX. Aging

Plant Cell Physiol. 58(5): 925–933 (2017) doi:10.1093/pcp/pcx036, Advance Access publication on 20 March 2017, available online at www.pcp.oxfordjournals.org

was accompanied by an increase in mainly non-enzymatically formed esterified oxygenated fatty acids and enzymatically formed free 9-LOX products.

Results

Aging alters the seed lipidome

First, we addressed the question of how the lipidome of Arabidopsis seeds changes during aging. As a test system for aging effects, accelerated aging is commonly used (Bentsink et al. 2000). Similar to published protocols (Tesnier et al. 2002, Boca et al. 2014), Arabidopsis Columbia (Col-0) seeds were exposed for 4 d to high temperature and humidity (see the Materials and Methods), and total lipid extracts of non-treated and 4 d aged seeds were analyzed with ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight-mass spectrometry (UPLC-ESI-qTOF-MS). Hydroperoxides were converted to their more stable hydroxy derivatives by reduction with triphenylphosphine during lipid extraction. Hence, hydroperoxides and hydroxides were quantified together as hydroxides. To recover a high number of lipid features, the same sample was analyzed using three different UPLC-ESI-qTOF-MS methods (see the Materials and Methods). Multivariate statistical data evaluation for the data sets was applied to identify seed aging markers. Evaluation of the data with principal component analysis showed a clear separation of non-aged and 4 d aged samples (Supplementary Fig. S3–C), proving that seed aging alters the lipidome. Orthogonal partial least squares discriminant analysis was applied to filter out the most dominant lipid features contributing to seed aging (Supplementary Fig. S3E–F). These seed aging markers were verified in an independently performed experiment. Considering all three applied separation methods, levels of 39 out of 4,080 lipid features {CoeffCS[2] >0.001, P(corr)[1]$P > 0.9$} were different in the accelerated aged seeds (**Table 1**). As shown in Supplementary Table S2, the structures of the 39 seed aging markers were identified by their exact mass-to-charge ratios (m/z), elemental compositions and fragmentation patterns using the METLIN and the LIPID MAPS databases. Hydroxylated triacylglycerols, not included in these databases, were identified by their elemental compositions, isotope abundance distributions and characteristic fragmentation patterns (Murphy et al. 2007). Identified seed aging markers (**Table 1**) comprised nine hydroxylated triacylglycerols, 10 oxidized and four non-oxidized fatty acids, eight diacylglycerols and 10 phospholipids. The oxidized fatty acids comprised HO-18:3, HO-18:2, Oxo-18:3 and Oxo-18:2. While levels of some phospholipid species decreased, levels of free fatty acids, diacylglycerols, hydroxylated triacylglycerols and free oxidized fatty acids increased. The strongest increase was found for oxidized fatty acids (between 5- and 138-fold). Levels of linoleic acid, linolenic acid, diacylglycerols and hydroxylated triacylglycerols were elevated in aged seeds between 1.7- and 2.5-fold in comparison with non-treated seeds. To determine whether these changes also occur during natural aging, lipid metabolites in seeds which were stored for 9 and 17 years were analyzed. Overall, an increase in the levels of free fatty acids, diacylglycerols, hydroxylated triacylglycerols and free oxidized fatty acids was also observed in naturally aged seeds (**Table 1**), indicating similarities in the processes occurring during natural and accelerated aging. The increase in the levels of hydroxylated triacylglycerols of 9-year-old seeds was between 2.1- and 6.7-fold; increases were overall higher than the alterations during accelerated aging. The strongest increase was observed in 17-year-old seeds (9.9- to 17.6-fold increase). Elevation in diacylglycerols was observed for some but not all diacylglycerol species. Similar to accelerated aging, natural aging leads to a clear increase in free oxidized and non-oxidized fatty acid, with the exception of one oxidized fatty acid which only increased upon accelerated aging. A decrease in phospholipids was detected for most of the phospholipid species in 17-year-old seeds but not in 9-year-old seeds.

In summary, these data indicate that robust changes in lipid levels due to seed aging were detected, which is in agreement with the published data and the expectation that natural as well as accelerated aging results in lipid oxidation. The highest increases were found for oxidized triacylglycerols and oxidized free fatty acids. Therefore, these groups of lipids were chosen for further, detailed analysis to elucidate the oxidation mechanisms during seed aging.

Oxidized triacylglycerols are formed mainly non-enzymatically

To investigate whether oxidation of triacylglycerols occurs enzymatically or non-enzymatically, accumulation of five oxidized triacylglycerols (**Table 1**) was determined in mutants with defects in LOXs. Two 9-LOXs (LOX1 and LOX5) and four 13-LOXs (LOX2, LOX3, LOX4 and LOX6) are present in Arabidopsis. A double mutant with a defect in the expression of the two 9-LOXs (*lox1,5*) showed an increase of all five hydroxylated triacylglycerols upon accelerated aging. Overall, hydroxylated triacylglycerols increased 2-fold in *lox1,5* and 2.5-fold in the corresponding wild type Wassilewskija (WS) (**Fig. 1A** presenting the total of the five hydroxylated triacylglycerols). Levels of each of the hydroxylated triacylglycerols in *lox1,5* and in the single mutants *lox1* and *lox5* also matched the wild type (Supplementary Fig. 4A). Similarly, levels of all five hydroxylated triacylglycerols increased upon aging in the quadruple mutant *lox2,3,4,6* with a defect in all four 13-LOX genes to similar levels as in the wild type Col-0 (**Fig. 1B**). Analysis of the triacylglycerol levels in the *lox2* and *lox6* single mutants and *lox3,4* double mutant revealed that *lox3,4* was also similar to Col-0 (Supplementary Fig. S4B) while levels of most hydroxylated triacylglycerols were reproducibly lower in untreated and in aged seeds of *lox2* (Supplementary Fig. S4C). Unexpectedly, in *lox6*, basal levels were reproducibly almost as high as the levels after aging, with no or only little increase in treated seeds (Supplementary Fig. 4C). The reason why no alteration was detected in the quadruple mutant was probably that lower basal levels in *lox2* compensated higher basal levels in *lox6*.

These results suggest that oxidation of fatty acids esterified in triacylglycerols upon aging is mostly independent of LOX enzymes and mainly based on non-enzymatic processes.

Table 1 Metabolites which increase or decrease during accelerated and natural aging in Col-0

Identified lipid species	Fold change of accelerated aged and non-treated seeds (*P*-value)	Fold change of seeds from 2006 and 2015 (*P*-value)	Fold change of seeds from 1998 and 2015 (*P*-value)
TG (OH-56:6)	1.9 (1.03E-03)	6.1 (8.1E-06)	11.3 (9.2E-07)
TG (OH-54:7)	1.8 (9.5E-04)	5.3 (8.5E-05)	13.4 (1.8E-06)
TG (OH-56:7)	1.9 (8.8E-04)	5.0 (1.6E-05)	9.9 (6.1E-07)
TG (OH-54:8)	2.1 (5.9E-04)	5.8 (2.3E-04)	17.6 (3.8E-0.6)
TG (OH-54:6)	2.0 (5.5E-04)	6.7 (1.9E-05)	16.8 (1.2E-06)
TG (OH-52:5)	2.0 (2.9E-04)	2.4 (1.5E-05)	11.0 (1.7E-06)
TG (OH-54:9)	1.8 (8.7E-04)	4.5 (3.9E-05)	11.6 (1.5E-06)
TG (OH-52:6)	2.0 (7.6E-04)	2.4 (8.6E-05)	13.6 (1.8E-06)
TG (OH-54:5)	2.0 (1.7E-04)	2.1 (3.0E-05)	14.1 (1.7E-06)
DG (36:5)	2.5 (3.3E-05)	3.2 (5.9E-05)	3.7 (4.4E-05)
DG (36:4)	2.5 (3.9E-05)	2.0 (8.1E-04)	2.8 (1.7E-04)
DG (34:2)	4.1 (7.1E-06)	1.3 (1.2E-04)	3.5 (9.3E-05)
DG (36:3)	2.3 (5.5E-06)	1.2 (6.5E-02)	1.8 (1.0E-03)
DG (36:6)	2.2 (4.6E-05)	4.2 (2.5E-04)	5.8 (1.1E-04)
DG (38:3)	1.5 (7.1E-04)	1.4 (1.0E-05)	4.4 (1.8E-05)
DG (38:4)	1.5 (8.8E-04)	1.8 (9.3E-06)	6.0 (1.4E-05)
DG (34:3)	3.9 (1.2E-04)	1.3 (2.3E-03)	3.6 (1.5E-03)
PC (38:3)	0.5 (1.1E-04)	1.1 (1.4E-01)	0.8 (1.3E-01)
PC (36:3)	0.5 (2.4E-05)	0.5 (2.4E-04)	0.6 (1.8E-03)
PC (34:6)	0.1 (9.6E-06)	1.5 (6.2E-03)	0.4 (4.6E-04)
PC (34:3)	0.4 (5.7E-07)	0.8 (1.2E-01)	0.6 (3.0E-03)
PC (34:4)	0.5 (1.3E-05)	1.0 (9.7E-01)	0.8 (2.7E-02)
PC (36:6)	0.2 (1.4E-04)	1.5 (5.0E-03)	0.5 (2.6E-03)
PC (36:4)	0.5 (1.9E-05)	1.0 (9.8E-01)	0.9 (4.7E-01)
PC (36:5)	0.4 (2.3E-06)	1.3 (7.3E-03)	0.9 (2.2E-01)
PE (36:4)	0.6 (2.1E-04)	0.8 (8.9E-04)	0.8 (2.6E-04)
PE (34:2)	0.3 (2.0E-04)	1.0 (7.0E-01)	0.7 (1.5E-04)
Linoleic acid	2.1 (8.7E-05)	5.8 (1.1E-05)	6.0 (3.9E-04)
Linolenic acid	1.7 (1.9E-04)	17.9 (2.0E-06)	13.6 (5.6E-09)
Palmitic acid	1.4 (8.0E-05)	1.8 (1.2E-03)	1.6 (4.3E-3)
Eicosenoic acid	2.2 (3.1E-05)	15. (1.1E-6)	13.1 (1.3E-07)
Oxygenated fatty acid (C18H29O3)	5.0 (1.1E-02)	7.8 (7.1E-06)	6.4 (1.1E-05)
Oxygenated fatty acid (C18H31O3)	137.6 (2.0E-02)	6.6 (3.4E-04)	3.0 (2.4E-03)
Oxygenated fatty acid (C18H33O3)	17.4 (2.0E-02)	0.8 (3.4E-01)	0.8 (3.4E-01)
Oxygenated fatty acid (C18H27O3)	25.5 (1.7E-02)	6.5 (4.4E-05)	3.1 (8.5E-04)
Oxygenated fatty acid (C18H29O3)	24.8 (2.6E-02)	5.1 (5.1E-04)	2.2 (1.2E-02)
Oxygenated fatty acid (C18H31O4)	10.5 (2.3E-03)	44.0 (1.1E-02)	22.0 (1.5E-02)
8.17_379.1572	5.6 (1.6E-02)	34.9 (1.2E-05)	39.2 (7.4E-07)
Oxygenated fatty acid (C18H33O5)	7.4 (2.5E-02)	2.5 (1.1E-02)	1.4 (4.2E-01)
7.15_377.1415	24.7 (3.8E-02)	34.8 (1.9E-05)	25.5 (1.6E-03)
Oxygenated fatty acid (C18H29O3)	8.9 (1.4E-02)	3.8 (8.6E-05)	5.7 (1.0E-03)

Metabolites which increase or decrease during accelerated and natural aging in Col-0 were identified by *m/z* and fragmentation pattern (see Supplementary Table S2). Lipid peroxides were reduced to hydroxides prior to analysis. The 'oxygenated fatty acids' were distinguished by their different retention time (see Supplementary Table S2). For oxygenated fatty acids which could not be identified based on the fragmentation pattern, the primary ID is given.
Metabolites are grouped into TG OH (hydroxylated triacylglycerol), DG (diacylglycerol), PC (phosphatidylcholine), PE (phosphaditylethanolamine), free and oxygenated FA (fatty acid) and within a group are sorted by the coefficient (see Supplementary Table S2).

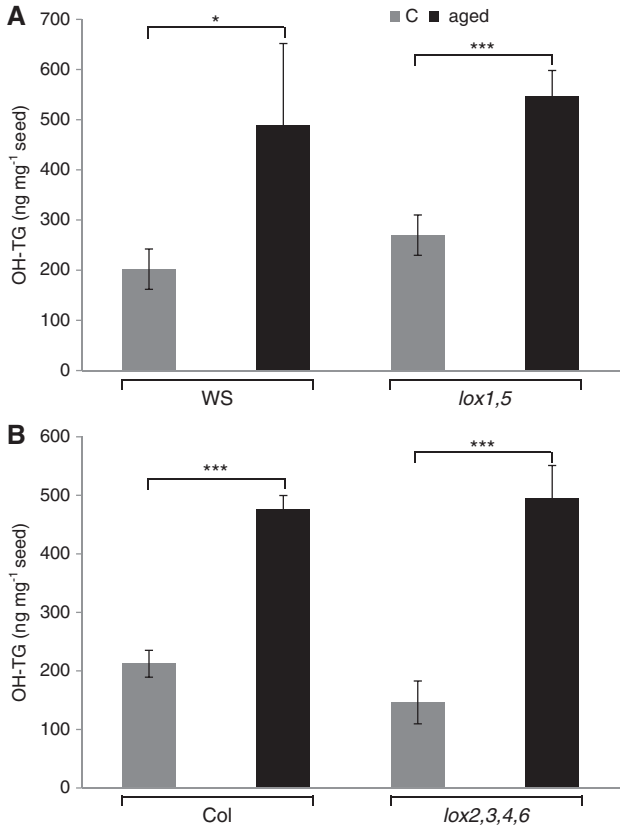

Fig. 1 Oxidation of triacylglycerols occurs mainly non-enzymatically. Levels of hydroxylated triacylglycerols in the double mutant *lox1,5* and the corresponding wild type WS (A), the quadruple mutant *lox2,3,4,6* and the corresponding wild type Col-0 (B). Shown is the sum of the five hydroxylated triacylglycerols which showed a significant increase upon aging (see **Table 1**; Supplementary Fig. S3). Lipid peroxides were reduced to hydroxides prior to analysis. The data were calculated from the mean of three replicates ± SD; values of the individual hydroxylated triacylglycerols are shown in Supplementary Fig. S4. Significant differences are indicated (Student's *t*-test: ***$P < 0.002$; **$P < 0.02$; *$P < 0.05$). There were no significant differences between levels in the mutant and the corresponding wild type.

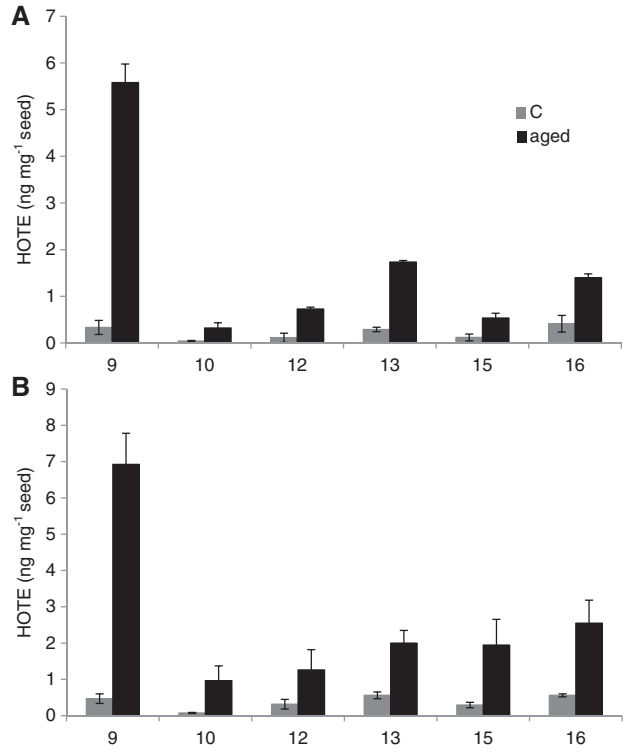

Fig. 2 Levels of 9-HOTE increase more strongly than those of other HOTE species. Levels of HOTE in the wild type WS (A) and Col-0 (B). Lipid peroxides were reduced to hydroxides prior to analysis. Non-treated seeds (gray bars) or seeds subjected to 4 d aging (black bars) were extracted and levels of HOTE were determined. Numbers on the *x*-axis indicate the position of the hydroxy group. Shown is the mean of three replicates ± SD.

Among all LOX enzymes, only LOX2, a 13-LOX, appears to contribute to a minor part.

LOX1 is responsible for the production of enzymatically oxidized free fatty acids

To elucidate the mechanism of generation of free oxidized fatty acids, at first the position of the hydroxy group in free hydroxy octadecatrienoic fatty acids (HOTEs) was analyzed because the oxidation pattern can reveal information on the processes involved. Hydroxylated fatty acids with a hydroxy group at position 9, 10, 12, 13, 15 and 16 can be formed from linolenic acid triggered by singlet oxygen, at position 9, 12, 13 and 16 by free radicals and at position 9 and 13 by LOXs (Triantaphylides et al. 2008). To analyze the position of the hydroxy group, targeted analyses employing triple quadrupole-MS was performed. Lipid peroxides were reduced to hydroxides prior to analysis. Hence, hydroperoxides and hydroxides were quantified together as hydroxides. If—similar to the oxidation of esterified fatty acids—non-enzymatic oxidation is the most important process, free fatty acids were expected to exhibit a random pattern of hydroxylated fatty acids typical for singlet oxygen/free radical-triggered formation. Basal levels of all HOTEs were low, mostly close to the detection limit. Upon aging, the content of all six possible HOTE species increased, suggesting that non-enzymatic oxidation occurred. However, 9-HOTE displayed by far the strongest increase; levels in 4 d aged seeds reached 5.6 to 7 ng mg^{-1} in WS and Col-0, respectively (**Fig. 2**). The other HOTEs only accumulated to levels below 3 ng mg^{-1}, with 16- and 13-HOTEs reaching the highest and 10-HOTE the lowest levels. This pattern indicates that, in addition to some non-enzymatic oxidation triggered by free radicals and singlet oxygen, 9-LOXs in particular are involved in oxidation of free fatty acids during aging. To elucidate which of the two 9-LOXs is responsible for the production of these compounds, mutants with defects in LOX1 and LOX5 were investigated. In addition to HOTEs, metabolites derived from linoleic acid (HODE and KODE) and linolenate metabolites containing a keto group at position 9 (9-KOTE) were also analyzed. Also 9-HODE, 9-KODE and 9-KOTE levels accumulated from very low basal levels strongly in response to aging treatment, reaching levels of 15, 33 and 10 ng mg^{-1} seeds, respectively, in the wild type (**Fig. 3**).

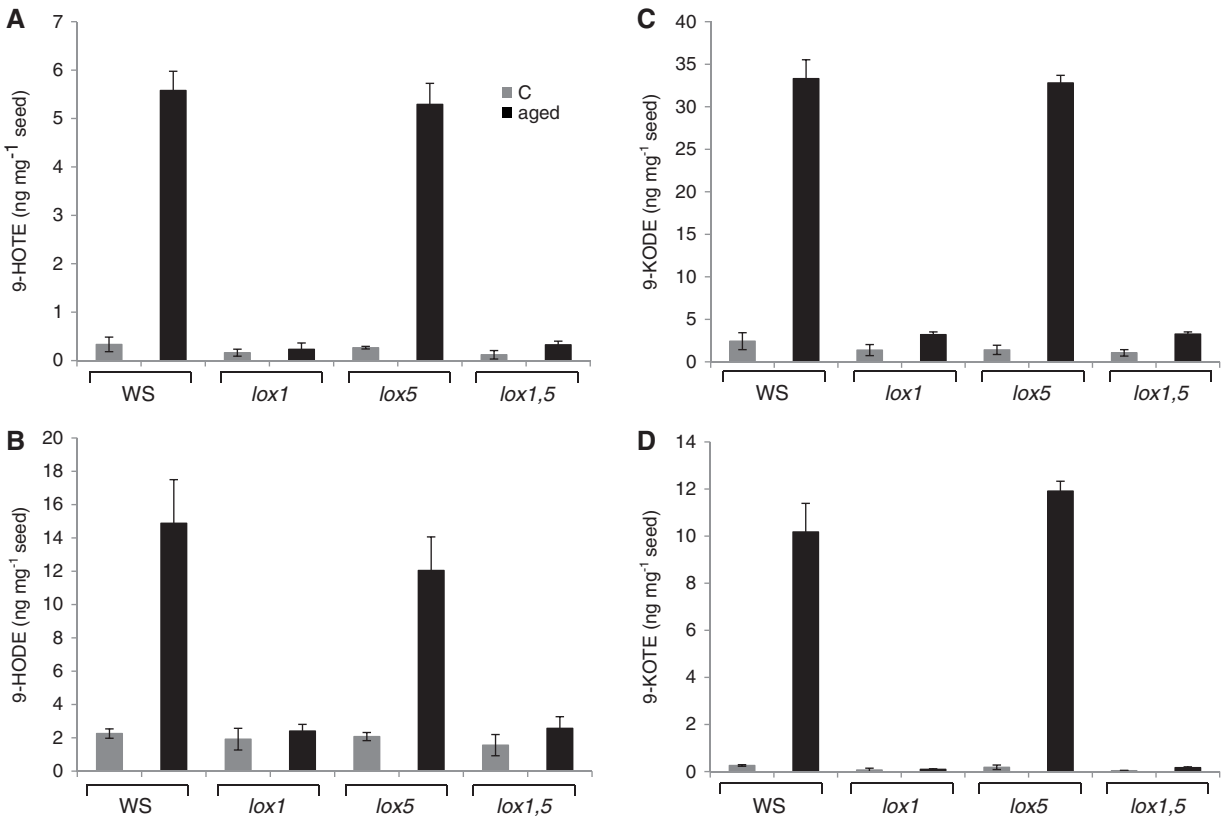

Fig. 3 Increase in 9-oxygenated compounds depends on LOX1. Levels of 9-LOX products in non-treated and aged seeds of the mutants *lox1*, *lox5*, the double mutant *lox1,5* and the corresponding wild type WS. Lipid peroxides were reduced to hydroxides prior to analysis. Shown is the mean of three replicates ± SD.

Levels of these 9-LOX products in untreated and aged seeds of the *lox5* mutant were similar to wild type levels (**Fig. 3**), indicating that LOX5 is not involved in formation of these compounds in response to aging. In contrast, only a very minor increase upon aging to levels between 0.1 and 3 ng mg^{-1} seed was detectable in the *lox1* and *lox1,5* mutants, indicating that LOX1 is responsible for formation of 9-LOX products during seed aging.

It was also investigated whether 13-LOXs are involved in the formation of free oxidized fatty acid derivatives during aging. In addition to 13-HOTE, 13-HODE and 13-KODE were analyzed; 13-KOTE was below the limit of detection. Similar to 13-HOTE, levels of 13-HODE and 13-KODE showed a small increase upon aging (Supplementary Fig. S5). The rather small increase (similar to the increase in 16-HOTE) suggests that formation of 13-oxygenated lipids was non-enzymatic. In the quadruple mutant *lox2,3,4,6*, these oxylipins accumulated to levels which tended to be even higher than in the wild type. This suggests that 13-LOXs are not involved in formation of these compounds in aging seeds but rather non-enzymatic processes lead to the formation of fatty acid derivatives oxidized at position 13.

LOX1 products only slightly contribute to loss of germination

The correlation between loss of germination and accumulation of 9-LOX products during aging raises the question of whether the oxidized lipids are causal for lower germination rates. To test this, it was determined whether the lack of accumulation of 9-LOX products in aged seeds of the *lox1* mutant results in altered germination rates. If the accumulation of 9-LOX products is responsible for the decrease in germination upon aging, a higher germination rate in aged *lox1* seeds in comparison with the wild type would be expected. In four out of six independent experiments, the germination of aged *lox1* seeds was slightly higher than in the wild type. The mean of all six experiments is shown in **Fig. 4**, with a germination rate of 7% for the wild type and 15% for *lox1*. The difference was significant; however, the effects were always rather small, with 21% being the highest germination rate observed for 4 d aged *lox1* seeds.

These data indicate that 9-LOX products slightly contribute to but are not the major cause for loss of germination during aging.

Discussion

Enzymatic and non-enzymatic lipid oxidation during seed aging

It has been proposed for a long time that lipid oxidation is an important factor in seed deterioration (Harman and Mattick 1976). Consistent with this assumption, changes in membrane

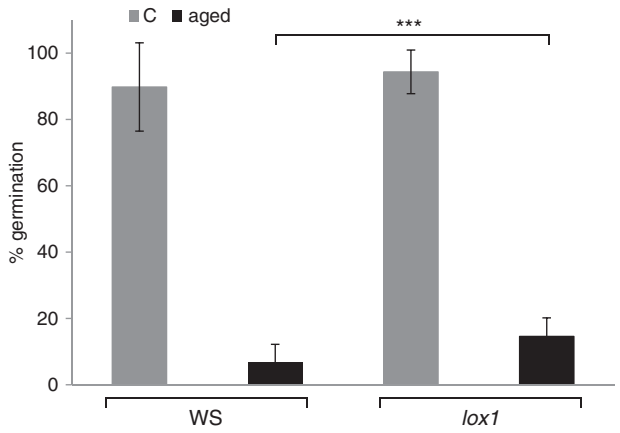

Fig. 4 Aging-induced loss of germination is only slightly affected by a defect in LOX1. Germination rate of the mutant *lox1* and the corresponding wild type WS. Non-treated seeds (gray bars) or seeds subjected to 4 d aging (black bars) were sown on agar plates containing 3% sucrose, and germinated seeds were counted after 8 d. Shown is the mean of six independent experiments with three plates with 40 seeds each ± SD. The difference between non-treated seeds of *lox1* and WS was not significant according to *t*-test; the difference in germination of 4 d aged seeds was significant, with $P = 0.0001$.

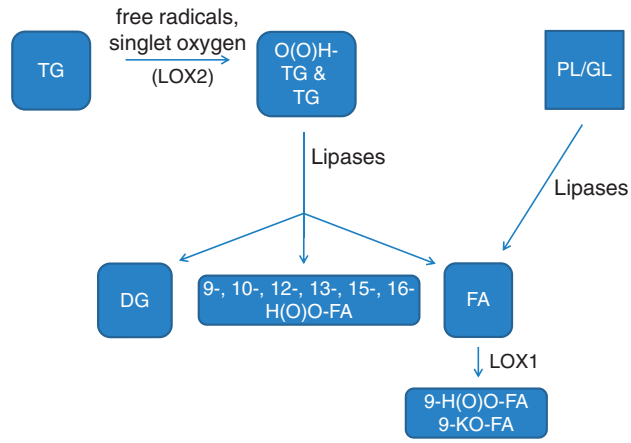

Fig. 5 Pathways for the generation of oxidized lipids. DG, diacylglycerol; FA, fatty acid; GL, galactolipid; O(O)H, hydroxy/peroxy; KO, keto PL, phospholipid; TG, triacylglycerol.

lipids as well as storage lipids during aging and especially an increase in oxidation products have been reported: upon natural or accelerated aging, the oil content as well as the amount of total fatty acids and of phospholipids in Arabidopsis seeds decreases, while levels of malondialdehyde, lipid peroxides (determined by FOX assay) and oxidized triacylglycerols have been reported to increase (Devaiah et al. 2007, Boca et al. 2014, Gayen et al. 2014). In order to obtain a comprehensive picture of changes in the lipidome, we performed an untargeted analysis in Arabidopsis. In agreement with the published data, levels of several hydroxylated triacylglycerols increased (upon both aging treatments). Boca et al. (2014) reported an increase in six hydroxylated triacylglycerols in seeds of an Arabidopsis mutant with a defect in two lipocalins which correlated with a much faster decrease in the germination rate of this double mutant during storage or upon artificial aging. This indicates that these hydroxylated triacylglycerols are suitable markers reflecting aging of seeds. Based on the fact that the increase in hydroxylated triacylglycerols was also detectable in a mutant lacking both 9-LOXs as well as in a mutant lacking all four 13-LOXs, it can be concluded that the oxidation of esterified fatty acids occurs mainly non-enzymatically (**Fig. 1**). LOX2 was the only LOX which seemed to contribute to the generation of hydroxylated triacylglycerol. While most LOXs prefer free fatty acids as substrates, 13-LOXs which are localized at the membrane of lipid bodies can use esterified fatty acids, as has been shown for mobilization of storage triacylglycerols in oilseed lipid bodies (Holtman et al. 1997, Feussner et al. 1998, Feussner et al. 2001). In addition, LOX2 is one of the few LOXs reported to oxidize esterified fatty acids, strongly contributing to the oxidation of galactolipids of the plastid membrane in leaves in response to different stress conditions such as wounding, pathogen infection and sorbitol treatment (Glauser et al. 2009, Seltmann et al. 2010, Zoeller et al. 2012). However, the plastidal localization of LOX2 raises the question of how LOX2 can be involved in the generation of oxidized triacylglycerols. Plastids of some tissues, in particular leaves, can contain triacylglycerols stored in plastoglobules. Plastoglobules share similarities with lipid bodies in seeds (Kessler and Vidi 2007). However, so far, LOX2 has not been found directly associated with plastoglobules (Lundquist et al. 2012). Another possible mechanism is that oxygenated fatty acids which are formed by LOX2 in plastidal membrane lipids are removed by a lipase and re-esterified into triacylglycerols. This mechanism of oxidation, hydrolysis and re-esterification has been reported for ricinoleate. In castor bean, the hydroxy fatty acid ricinoleate is formed esterified to the *sn*-2 position of phosphatidylcholine, released by a phospholipase and incorporated into triacylglycerols (Bafor et al. 1991, Bayon et al. 2015).

Some non-enzymatic oxidation was also detectable within the pool of free oxidized fatty acids, as indicated by a small increase in the levels of randomly oxidized HOTEs. However, the majority of free oxidized fatty acids were formed enzymatically. For the non-enzymatically formed free oxylipins, two sources are possible: these compounds could be generated by non-enzymatic oxidation of free fatty acids or triacylglycerols, followed by release through the activity of lipases. The latter scenario is more likely since the pool of oxygenated fatty acids esterified in triacylglycerols is comparatively large and triacylglycerols apparently become partially hydrolyzed during aging, thereby releasing pre-formed oxygenated fatty acids in addition to non-oxygenated fatty acids and diacylglycerols (**Fig. 5**). Strikingly, there was a very strong increase in the levels of the 9-LOX products 9-KODE, 9-HODE, 9-KOTE and 9-HOTE derived from linoleic and linolenic acid, and this increase was absent in the *lox1* mutant line (**Fig. 3**), revealing that formation of these oxylipins during seed aging is enzymatically catalyzed and identifying LOX1 as the isozyme that is responsible for the production of these oxylipins during seed aging. LOX1 has also been reported to be involved in the response to other stresses in Arabidopsis. LOX1 is responsible for the accumulation of 9-LOX products, especially 9-KOTE, upon infection with

Pseudomonas syringae (Montillet et al. 2013). LOX1/LOX5 also play a role in generating signaling compounds by oxidizing *N*-acylethanolamines to *N*-linoleoylethanolamides which inhibit germination and seedling growth. (Keereetaweep et al. 2015). LOX1 probably uses free fatty acids as substrates. During aging, the content of free linoleic and linolenic acid increased in Arabidopsis seeds (**Table 1**), generating an increased pool of substrates. These free fatty acids might be released from triacylglycerols or membrane lipids by the activity of lipases (**Fig. 5**). This would be in agreement with the observed aging-induced accumulation of diacylglycerols and a decline in phospholipid levels which was detectable in our accelerated aging experiment.

Relevance of lipid oxidation/LOX products for seed aging

What so far is the evidence that enzymes involved in lipid oxidation or metabolism are involved in processes during seed aging and deterioration? In Arabidopsis, two phospholipases have been reported to play a role in seed viability and aging. Knock down of the phospholipase D PLDα1 enhanced the tolerance of seeds to accelerated aging which correlated with a lower decrease in oil content and less production of lipid peroxides. It was concluded that the PLDα1-mediated release of phosphatidic acid results in higher generation of ROS and subsequent lipid peroxidation. In addition, this enzyme might contribute to degradation of the phospholipid monolayer of lipid bodies, thereby exposing triacylglycerols to oxidants (Devaiah et al. 2007). In contrast, a DAD1-like phospholipase A1 was reported to have a protective role for seed viability during aging. Loss of function of this mitochondrial-localized acyl hydrolase resulted in enhanced susceptibility, and overexpression of this enzyme led to reduced susceptibility to accelerated aging which correlated with higher lipid peroxidation in the mutant and lower peroxidation in the overexpressing plants (Seo et al. 2011).

In rice, there is direct evidence for a function for lipid peroxidation and especially LOXs in seed deterioration. However, for comparing Arabidopsis and rice, it has to be taken into account that these plants follow different strategies with respect to the seed storage compounds: Arabidopsis accumulates oil while rice is a starch accumulator. Down-regulation or lack of the rice 9-LOXs LOX1, LOX2 and LOX3 led to lower levels of lipid peroxides as well as improved storability and germination upon accelerated aging in comparison with the wild type (Gayen et al. 2014, Huang et al. 2014, Xu et al. 2015). The effect of reduced expression of multiple LOX proteins was much stronger compared with the effect of down-regulation of only one of these LOXs (Song et al. 2007, Ma et al. 2015). These data together with the known inhibitory effect of the oxylipin 12-oxophytodienoic acid on seed germination (Dave et al. 2011, Dave et al. 2016) as well as the regulation of lateral root initiation and induction of root waving by exogenously applied 9-LOX products (Vollenweider et al. 2000, Vellosillo et al. 2007) suggested that LOX1 products might negatively regulate seed longevity and germination. Indeed, we found that in Arabidopsis lack of synthesis of 9-LOX products in *lox1* during seed aging significantly increased the germination rate; however, the effect was only small. In addition, we tested whether germination is inhibited by exogenous application of oxidized lipids. No negative effect on germination was detectable when seeds were incubated with 9-KODE, 9-HODE or 9-HOTE in a concentration of 10 or 50 μM (data not shown). However, for evaluation of these results, it has to be taken into account that it is not clear to what extent exogenously administered oxylipins actually penetrated the seed coat and how rapidly these compounds are metabolized within the seed. Nevertheless, based on both results, 9-hydroxy- and 9-keto-oxylipins appear not to be crucial for the reduction of the germination rate upon aging, suggesting that in Arabidopsis, processes other than enzymatic lipid oxidation are more important for loss of germination. Also results with safflower indicate that lipid oxidation is not a major cause of seed deterioration (Ohlrogge and Kernan 1982). The oxidation of proteins is also thought to contribute to the decline in the germination rate (Rajjou et al. 2008, Sano et al. 2016). However, oxidation of lipids and proteins cannot be looked at separately. Lipid peroxidation will permanently generate radicals which can damage proteins (Farmer and Mueller 2013). Following this line, the increase in oxidized lipids or the decrease in the oil content might not be the most harmful processes per se but indicate lipid peroxidation-associated ROS production that contributes to seed deterioration via protein damage.

Materials and Methods

Plant material

The *Arabidopsis thaliana* 13-LOX mutant lines *lox2* single, *lox3lox4* double (*lox3,4*), *lox6* single, *lox2lox3lox4lox6* quadruple (*lox2,3,4,6*) with the corresponding wild type ecotype Col-0, and 9-LOX mutants *lox1-1* single, *lox5-1* single and *lox1-1lox5-1* double (*lox1,5*) with the corresponding wild-type ecotype WS were used. The *lox2* (Glauser et al. 2009), *lox3,4*, *lox6* mutants and *lox2,3,4,6* (Caldelari et al. 2011, Chauvin et al. 2013) as well as the *lox1-1*, *lox5-1* and *lox1,5* mutants were kindly provided by E. Farmer. The *lox6* mutant analyzed throughout the manuscript corresponds to the line SALK_138907. To generate seeds of *lox2,3,4,6* and *lox3,4* mutants, flowers were sprayed with methyl jasmonate (Grebner et al. 2013). Seeds used for comparison with these mutants were harvested from wild-type plants which were treated simultaneously with methyl jasmonate. The identity of the mutant lines was verified by male sterility of *lox2,3,4,6* and *lox3,4*; lack of wound-induced formation of jasmonic acid and 12-oxo-phytodienoic acid in *lox2,3,4,6*; and quantitative reverse tanscription–PCR (qRT–PCR) or PCR as described in Grebner et al. (2013). The T-DNA insertion of *lox1*, *lox5* and *lox1,5* in the respective gene was verified by PCR (Supplementary Fig. S1). The age of the seeds used for the experiments shown was 62 d after harvest. Experiments have been repeated with seeds of ages between 4 weeks and 15 months. Seeds were stored at 4.6°C and 79% relative humidity.

Accelerated aging treatment and natural aging

Arabidopsis seeds were exposed for different time periods to 42°C and 84% relative humidity. The germination rate was monitored. In seeds of the ecotype WS, germination decreased from 94% to 24, 10 and 4% after 2, 3 and 4 d, respectively; in Col-0, germination decreased from 95% to 48, 20 and 4% after 2, 3 and 4 d, respectively (Supplementary Fig. S2A). Natural aged Col-0

seeds had been stored in the refrigerator for 9 and 17 years, respectively. The germination rate of 9- and 17-year-old seeds was 80% and 2%, respectively. (Supplementary Fig. S2B)

Determination of germination rates

Seeds were surface-sterilized and sown on agar plates with Murashige and Skoog medium containing 3% sucrose. After incubation for 2 d at 4 °C in the dark, plates were placed in a plant cabinet with 22 °C and a 9 h photoperiod (100 µmol photons $m^{-2}s^{-1}$). Germination was monitored routinely after 8 d using a binocular. A seed was classified as germinated when the radicula was at least 0.5 mm long.

Lipidomics

Lipids were extracted from 3 mg of seeds per sample using Folch solvent (chloroform : methanol 2 : 1, v/v) as described in Boca et al. (2014). Reversed-phase chromatographic separation was performed using an ACQUITY Ultra Performance LC system (UPLC; Waters) with an ACQUITY UPLC BEH C18, 1.7 µm particle size, 2.1×100 mm column (Waters). To recover a high number of lipid features, the same sample was analyzed using different UPLC-ESI-qTOF-MS conditions. For the 'lipidomic method' for middle-apolar and apolar lipid compounds, the column temperature was set to 60°C; 5 µl of each sample were injected and eluted with a linear gradient of 30–100% B over 10 min at a flow rate of 0.3 ml min^{-1}. Eluent A consisted of 10 mM ammonium acetate in water : acetonitrile (60 : 40, v/v) and eluent B consisted of 10 mM ammonium acetate in 2-propanol : acetonitrile (90 : 10, v/v). For the 'fatty acid method' for more polar compounds, the column was maintained at 30 °C and the injected sample (5 µl) was eluted using a binary linear gradient of 50–100% B over 10 min at a flow rate of 0.3 ml min^{-1} with eluent A consisting of 0.1% formic acid in water and eluent B being acetonitrile.

The UPLC was coupled to a hybrid quadrupole orthogonal time of-flight mass spectrometer (qTOF-MS, SYNAPT G2 HDMS; Waters). Positive and negative electrospray ionization (ESI) mode was used with capillary voltage and cone voltage of 0.8 kV and 25 V, respectively. The desolvation temperature was maintained at 350°C and the flow rate of the desolvation gas (nitrogen) was 800 l h^{-1}. The system was equipped with an integral LockSpray unit with its own reference sprayer. As the internal reference, leucine encephalin was used. The quadrupole was operated in a wide band radiofrequency (RF) mode. The mass range of data acquisition was between 50 and 1,200 Da. Two discrete and independent interleaved acquisition functions were automatically created. The first function collected the low-energy data where molecule ions are acquired. The second function collected the high-energy data or the fragments of the molecule ion by using a collision energy ramp from 15 to 35 eV. Argon gas was used for collision-induced dissociation (CID) in both functions. Multivariate statistical analysis of the low-energy data was carried out using MarkerLynx software (Waters Corporation). First, principal component analysis were performed to identify lipid features that correlate with aging. Since the seed aging was determined as the first component (Supplementary Fig. S3A–C), orthogonal partial least squares analysis was applied to filter out the most dominant lipid features contributing to seed aging termed seed-aging markers (Supplementary Fig. S3E, F) which were verified in an independently performed experiment. For targeted analysis of hydroxylated triacylglycerols, the compound C30:0 was added as internal reference. For calculation of concentrations, a response factor of 1 was used.

Free oxidized fatty acids (HODE/HOTE and KODE/KOTE) were analyzed by UPLC-MS/MS using a Waters Acquity UPLC system coupled to a Waters Quattro Premier triple quadrupole mass spectrometer equipped with an electrospray interface (UPLC; Waters). Seeds were extracted as described above; in addition, solvents contained 1 mg ml^{-1} triphenyl phosphine and 1 mg ml^{-1} butylated hydroxytoluene, to prevent further oxidation of polyunsaturated fatty acids. For quantitation of oxidized fatty acids, 150 ng of each 15-oxo-eicosadienoic acid (15-KEDE) and 15-hydroxy-eicosadienoic acid (15-HEDE) were added as internal standards. A 7.5 µl aliquot of the extract was separated by reversed-phase chromatography using an Acquity BEH C18 column (1.7 µm particle size, 2.1×50 mm; Waters) equipped with a VanGuard pre-column (BEH C18, 1.7 µm particle size, 2.1×5 mm) at 40 °C and a linear gradient starting from 65% (v/v) eluent A, consisting of 1 mM ammonium acetate in water to 70% (v/v) eluent B (acetonitrile) within 6 min at a flow rate of 0.25 ml min^{-1}. Oxylipins were detected by Multiple Reaction Monitoring (MRM) in the negative ESI mode with a capillary voltage of 3 kV for hydroxy- and 3.25 kV for oxo-fatty acids. The source was set at 120 °C and nitrogen was used as desolvation gas with a flow rate of 800 l h^{-1} at 350 °C and cone gas with a flow rate of 50 l h^{-1}. Argon was used for CID (flow rate of 0.3 ml min^{-1}, 3×10^{-3} mBar). The cone voltage and collision energy were optimized for all compounds, and specific mass transitions were monitored for each compound for 0.025 s. Mass transitions of parent and daughter ions, cone voltage and collision energy are given in Supplementary Table S1. For accurate quantification of oxylipins, response factors for each compound were determined with commercially available reference compounds (CaymanChemical) or with a mixture of hydroxy fatty acids, prepared as described in Triantaphylides et al. (2008).

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the German Research Foundation [GK 1342 to A.O., S.B. and M.M.].

Acknowledgments

The authors are especially grateful to Fleur Köstler, Rebecca Blumroeder, Beeke Tappe and Michelle Dorenkamp for their for support in analysis of lipid levels and germination rates.

Disclosures

The authors have no conflicts of interest to declare.

References

Andreou, A. and Feussner, I. (2009) Lipoxygenases—structure and reaction mechanism. *Phytochemistry* 70: 1504–1510.

Bafor, M., Smith, M.A., Jonsson, L., Stobart, K. and Stymne, S. (1991) Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor-bean (*Ricinus communis*) endosperm. *Biochem. J.* 280: 507–514.

Bayon, S., Chen, G., Weselake, R.J. and Browse, J. (2015) A small phospholipase A2-alpha from castor catalyzes the removal of hydroxy fatty acids from phosphatidylcholine in transgenic Arabidopsis seeds. *Plant Physiol.* 167: 1259–1270.

Bentsink, L., Alonso-Blanco, C., Vreugdenhil, D., Tesnier, K., Groot, S.P. and Koornneef, M. (2000) Genetic analysis of seed-soluble oligosaccharides in relation to seed storability of Arabidopsis. *Plant Physiol.* 124: 1595–1604.

Boca, S., Koestler, F., Ksas, B., Chevalier, A., Leymarie, J., Fekete, A., et al. (2014) Arabidopsis lipocalins AtCHL and AtTIL have distinct but overlapping functions essential for lipid protection and seed longevity. *Plant Cell Environ.* 37: 368–381.

Caldelari, D., Wang, G., Farmer, E.E. and Dong, X. (2011) Arabidopsis *lox3 lox4* double mutants are male sterile and defective in global proliferative arrest. *Plant Mol. Biol.* 75: 25–33.

Chauvin, A., Caldelari, D., Wolfender, J.L. and Farmer, E.E. (2013) Four 13-lipoxygenases contribute to rapid jasmonate synthesis in wounded *Arabidopsis thaliana* leaves: a role for lipoxygenase 6 in responses to long-distance wound signals. *New Phytol.* 197: 566–575.

Dave, A., Hernandez, M.L., He, Z., Andriotis, V.M., Vaistij, F.E., Larson, T.R., et al. (2011) 12-Oxo-phytodienoic acid accumulation during seed development represses seed germination in Arabidopsis. *Plant Cell* 23: 583–599.

Dave, A., Vaistij, F.E., Gilday, A.D., Penfield, S.D. and Graham, I.A. (2016) Regulation of *Arabidopsis thaliana* seed dormancy and germination by 12-oxo-phytodienoic acid. *J. Exp. Bot.* 67: 2277–2284.

Devaiah, S.P., Pan, X., Hong, Y., Roth, M., Welti, R. and Wang, X. (2007) Enhancing seed quality and viability by suppressing phospholipase D in Arabidopsis. *Plant J.* 50: 950–957.

Farmer, E.E. and Mueller, M.J. (2013) ROS-mediated lipid peroxidation and RES-activated signaling. *Annu. Rev. Plant Biol.* 64: 429–450.

Feussner, I., Bachmann, A., Hohne, M. and Kindl, H. (1998) All three acyl moieties of trilinolein are efficiently oxygenated by recombinant His-tagged lipid body lipoxygenase in vitro. *FEBS Lett.* 431: 433–436.

Feussner, I., Kuhn, H. and Wasternack, C. (2001) Lipoxygenase-dependent degradation of storage lipids. *Trends Plant Sci.* 6: 268–273.

Feussner, I. and Wasternack, C. (2002) The lipoxygenase pathway. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 53: 275–297.

Gayen, D., Ali, N., Ganguly, M., Paul, S., Datta, K. and Datta, S.K. (2014) RNAi mediated silencing of lipoxygenase gene to maintain rice grain quality and viability during storage. *Plant Cell Tissue Org. Cult.* 118: 229–243.

Glauser, G., Dubugnon, L., Mousavi, S.A., Rudaz, S., Wolfender, J.L. and Farmer, E.E. (2009) Velocity estimates for signal propagation leading to systemic jasmonic acid accumulation in wounded Arabidopsis. *J. Biol. Chem.* 284: 34506–34513.

Grebner, W., Stingl, N.E., Oenel, A., Mueller, M.J. and Berger, S. (2013) Lipoxygenase 6-dependent oxylipin synthesis in roots is required for abiotic and biotic stress resistance of Arabidopsis. *Plant Physiol.* 161: 2159–2170.

Harman, G.E. and Mattick, L.R. (1976) Association of lipid oxidation with seed ageing and death. *Nature* 260: 323–324.

Harrington, J.F. (1972) Seed storage and longevity. In Seed Biology. Edited by Kozlowski, T.T. pp. 145–245. Academic Press, New York.

Holtman, W.L., Vredenbregt-Heistek, J.C., Schmitt, N.F. and Feussner, I. (1997) Lipoxygenase-2 oxygenates storage lipids in embryos of germinating barley. *Eur. J. Biochem.* 248: 452–458.

Hu, D., Ma, G., Wang, Q., Yao, J., Wang, Y., Pritchard, H.W., et al. (2012) Spatial and temporal nature of reactive oxygen species production and programmed cell death in elm (*Ulmus pumila* L.) seeds during controlled deterioration. *Plant Cell Environ.* 35: 2045–2059.

Huang, J., Cai, M., Long, Q., Liu, L., Lin, Q., Jiang, L., et al. (2014) OsLOX2, a rice type I lipoxygenase, confers opposite effects on seed germination and longevity. *Transgenic Res.* 23: 643–655.

Keereetaweep, J., Blancaflor, E.B., Hornung, E., Feussner, I. and Chapman, K.D. (2015) Lipoxygenase-derived 9-hydro(pero)xides of linoleoylethanolamide interact with ABA signaling to arrest root development during Arabidopsis seedling establishment. *Plant J.* 82: 315–327.

Kessler, F. and Vidi, P.A. (2007) Plastoglobule lipid bodies: their functions in chloroplasts and their potential for applications. *Adv. Biochem. Eng. Biotechnol.* 107: 153–172.

Lundquist, P.K., Poliakov, A., Bhuiyan, N.H., Zybailov, B., Sun, Q. and van Wijk, K.J. (2012) The functional network of the Arabidopsis plastoglobule proteome based on quantitative proteomics and genome-wide coexpression analysis. *Plant Physiol.* 158: 1172–1192.

Ma, L., Zhu, F., Li, Z., Zhang, J., Li, X., Dong, J., et al. (2015) TALEN-based mutagenesis of lipoxygenase LOX3 enhances the storage tolerance of rice (*Oryza sativa*) seeds. *PLoS One* 10: e0143877.

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

Montillet, J.L., Leonhardt, N., Mondy, S., Tranchimand, S., Rumeau, D., Boudsocq, M., et al. (2013) An abscisic acid-independent oxylipin pathway controls stomatal closure and immune defense in Arabidopsis. *PLoS Biol.* 11: e1001513.

Murphy, R.C., James, P.F., McAnoy, A.M., Krank, J., Duchoslav, E. and Barkley, R.M. (2007) Detection of the abundance of diacylglycerol and triacylglycerol molecular species in cells using neutral loss mass spectrometry. *Anal. Biochem.* 366: 59–70.

Ohlrogge, J.B. and Kernan, T.P. (1982) Oxygen-dependent aging of seeds. *Plant Physiol.* 70: 791–794.

Rajjou, L., Lovigny, Y., Groot, S.P., Belghazi, M., Job, C. and Job, D. (2008) Proteome-wide characterization of seed aging in Arabidopsis: a comparison between artificial and natural aging protocols. *Plant Physiol.* 148: 620–641.

Sano, N., Rajjou, L., North, H.M., Debeaujon, I., Marion-Poll, A. and Seo, M. (2016) Staying alive: molecular aspects of seed longevity. *Plant Cell Physiol.* 57: 660–674.

Sattler, S.E., Gilliland, L.U., Magallanes-Lundback, M., Pollard, M. and DellaPenna, D. (2004) Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. *Plant Cell* 16: 1419–1432.

Seltmann, M.A., Stingl, N.E., Lautenschlaeger, J.K., Krischke, M., Mueller, M.J. and Berger, S. (2010) Differential impact of lipoxygenase 2 and jasmonates on natural and stress-induced senescence in Arabidopsis. *Plant Physiol.* 152: 1940–1950.

Seo, Y.S., Kim, E.Y. and Kim, W.T. (2011) The Arabidopsis sn-1-specific mitochondrial acylhydrolase AtDLAH is positively correlated with seed viability. *J. Exp. Bot.* 62: 5683–5698.

Song, M., Yuelin, W., Zhang, Y., Liu, B.M., Jiang, J.Y., Xu, X., et al. (2007) Mutation of rice (*Oryza sativa* L.) LOX-1/2 near-isogenic lines with ion beam implantation and study of their storability. *Nucl. Instrum. Methods Phys. Res. Sect. B* 265: 495–500.

Stewart, R.R. and Bewley, J.D. (1980) Lipid peroxidation associated with accelerated aging of soybean axes. *Plant Physiol.* 65: 245–248.

Tesnier, K., Strookman-Donkers, H.M., van Pijlen, J.G., van der Geest, A.H.M., Bino, R.J. and Groot, S.P. (2002) A controlled deterioration test for *Arabidopsis thaliana* reveals genetic variation in seed quality. *Seed Sci. Technol.* 30: 149–165.

Triantaphylides, C., Krischke, M., Hoeberichts, F.A., Ksas, B., Gresser, G., Havaux, M., et al. (2008) Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants. *Plant Physiol.* 148: 960–968.

Vellosillo, T., Martinez, M., Lopez, M.A., Vicente, J., Cascon, T., Dolan, L., et al. (2007) Oxylipins produced by the 9-lipoxygenase pathway in Arabidopsis regulate lateral root development and defense responses through a specific signaling cascade. *Plant Cell* 19: 831–846.

Vollenweider, S., Weber, H., Stolz, S., Chetelat, A. and Farmer, E.E. (2000) Fatty acid ketodienes and fatty acid ketotrienes: Michael addition acceptors that accumulate in wounded and diseased Arabidopsis leaves. *Plant J.* 24: 467–476.

Xu, H., Wei, Y., Zhu, Y., Lian, L., Xie, H., Cai, Q., et al. (2015) Antisense suppression of LOX3 gene expression in rice endosperm enhances seed longevity. *Plant Biotechnol. J.* 13: 526–539.

Zoeller, M., Stingl, N., Krischke, M., Fekete, A., Waller, F., Berger, S., et al. (2012) Lipid profiling of the Arabidopsis hypersensitive response reveals specific lipid peroxidation and fragmentation processes: biogenesis of pimelic and azelaic acid. *Plant Physiol.* 160: 365–378.