Abscisic acid-determined seed vigour differences do not influence redox regulation during ageing

Christina Schausberger[1], Thomas Roach[1*], Wolfgang Stöggl[1], Erwann Arc[1], William E. Finch-Savage[2], Ilse Kranner[1]

[1]Department of Botany, Center for Molecular Biosciences Innsbruck (CMBI), Innsbruck, Austria

[2]School of Life Sciences, Wellesbourne Campus, University of Warwick, Wellesbourne, Warwick CV35 9EF, United Kingdom

*corresponding author: Thomas Roach, thomas.roach@uibk.ac.at

telephone number: +43 512 507 51099, ORCiD: 0000-0002-0259-0468

Abstract (word count = 187)

High seed quality is a key trait to achieve successful crop establishment required for optimum yield and sustainable production. Seed storage conditions greatly impact on two key seed quality traits; seed viability (ability to germinate and produce normal seedlings) and vigour (germination performance). Accumulated oxidative damage accompanies loss of seed vigour and viability during ageing, indicating that redox control is key to longevity. Here, we studied the effects of controlled deterioration at 40 °C and 75% relative humidity ("ageing") under two different O_2 concentrations (21 and 78% O_2) in *Brassica oleracea*. Two *B. oleracea* genotypes with allelic differences at two QTL that result in differences in abscisic acid (ABA) signalling and seed vigour were compared. Ageing led to a similar loss in germination speed in both genotypes that was lost faster under elevated O_2. In both genotypes, an equal oxidative shift in the glutathione redox state and a minor loss of α-tocopherol progressively occurred before seed viability was lost. In contrast, ABA levels were not affected by ageing. In conclusion, both ABA signalling and seed ageing impact on seed vigour, but not necessarily through the same biochemical mechanisms.

Abbreviations

A12, *Brassica oleracea* line A12DHd; ABA, Abscisic Acid; ANOVA, analysis of variance; CD, Controlled deterioration at 75% RH and 40 °C; $E_{GSSG/GSH}$, glutathione half-cell reduction potential; FAME, fatty acid methyl esters; FAO, Food and Agriculture Organization; FA, Fatty acid; GA, Gibberellic acid; GC-MS, Gas chromatography-Mass spectrometry; GSH, Glutathione thiol; GR, Glutathione reductase; GSSG, glutathione disulphide; LMW, Low-molecular-weight; CD+O_2, seeds aged with CD under 75% O_2; RH, Relative humidity; ROS / RNS, Reactive oxygen / nitrogen species; SA, Salicylic acid; $T_{25\%}$, time taken to reach 25% germination; TG, Total germination; SL101, *Brassica oleracea* line AGSL101; WC, Water content.

Introduction

Seeds age during storage, and the resulting loss of seed vigour and viability can impact on crop yields and sustainable agricultural production. Seed deterioration rates are accelerated by elevated storage temperatures and seed water contents (WC), which can also be influenced by the O_2 content of the storage environment (1-5). Vigorous germination, resulting in rapid and uniform seedling establishment, is an important agronomic trait (6). Plant hormones are determining factors for seed germination and vigour, with prominent roles for abscisic acid (ABA) and gibberellins (GA) that can delay and advance germination, respectively (6). In seeds, ABA has a well characterised role in maintaining dormancy, whereby germination is restricted in otherwise favourable conditions (7, 8). In addition, ABA signalling is important in coordinating correct embryo development for producing high quality seed. Arabidopsis mutants that are ABA insensitive or ABA deficient display reduced seed longevity (9). An inverse relationship between the level of seed dormancy and longevity in various inbred Arabidopsis lines was revealed (10), but this link is not consistent in Arabidopsis, nor was it found in sunflower seeds (11, 12).

Oxidative processes affect germination, whereby an ‘oxidative window of germination’ has been described (13). On the one hand, applications of reactive oxygen / nitrogen species (ROS / RNS) are often used to break seed dormancy and promote germination, via cross-talk with ABA and GA signalling (14, 15). On the other hand, aged seeds with low germination display symptoms of excess ROS production, such as carbonylated proteins and depleted antioxidant pools (16, 17). A loss of the antioxidant and redox buffer glutathione (GSH), alongside an accumulation of disulphides (i.e. GSSG), occurs during seed ageing, resulting in an oxidative shift in the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) (11, 17-19). Glutathione reductase (GR) converts GSSG to GSH in the presence of NADPH, but

only when seeds are sufficiently hydrated. Glutathione is the most abundant low-molecular-weight (LMW) thiol and an important cellular redox buffer (20), particularly in desiccated seeds that are devoid of ascorbate (21, 22). Seed viability in the Brassicaceae family has also been associated with antioxidant protection of the lipid phase by tocochromanols (12, 23), the collective name for tocopherols and tocotrienols of the vitamin E family. However, a decrease in tocochromanols does not always occur in aged seed (11, 24-26).

Several important crops, including cabbage, kale, kohlrabi, cauliflower, broccoli and Brussel sprouts, belong to the same species, *Brassica oleracea* L., the nearest crop relative to the model plant *Arabidopsis thaliana* L. According to the Food and Agriculture Organization (FAO), 3.8 million hectares of cabbage, cauliflower and broccoli were harvested in 2016, corresponding to a global production of 96.5 million tonnes (www.fao.org/faostat/). Previously, a *B. oleracea* mapping population was investigated, containing lines that had major differences in their reproductive biology (27). An investigation into the variation of germination vigour in these lines identified QTL underlying this trait (28). Further investigation showed that three genes (BoLCVIG1, BoLCVIG2 and RABA1) were responsible for differences in seed vigour within *B. oleracea*. Two lines, namely A12DHd and AGSL101, were identified that differed in seed vigour due to allelic differences in these genes (29, 30). This allelic variation altered both seed ABA content and sensitivity to it, affecting speed of germination. Germination vigour includes all aspects of seed performance. In addition to the initial vigour that is genetically determined and hormone-related, vigour is also affected by seed deterioration that reduces performance. The latter results in a decline in the same germination measures used to indicate initial seed vigour. Therefore, these two aspects can be confounded in research and in commercial estimates of vigour. Here, we used A12DHd and AGSL101 to investigate if genetically determined, ABA-related, differences in initial seed vigour influence mechanisms of seed ageing and ageing-induced losses of seed vigour. Seeds were aged with controlled deterioration (CD) at 40 °C and 75% relative humidity (RH), either under ambient (21%) or elevated (78%) O_2 to further assess the influence of an oxidative environment. Changes in germination rate, levels of LMW thiols and disulphides, plant hormones, tocochromanols and fatty acids (FA), and GR activity were measured before viability loss (0, 4 and 8 d of CD), when total germination (TG) started to be lost (19 d CD), and when around 50% of TG was lost (41 d CD).

Methods

Seed material and germination

Seeds of the slow-germinating *B. oleracea* double haploid Chinese kale parental line (var. *alboglabra* A12DHd; hereafter, A12) and a chromosome substitution line (AGSL101; hereafter, SL101) in the A12 background with introgressions from a fast-germinating doubled haploid Calabrese line (var. *italica* GDDH33) at two seed-performance QTLs were used. GD33 alleles enhance seed performance resulting in more rapid germination and seedling emergence in the field (29). For seed production, A12 and SL101 plants were grown in a glasshouse and pollinated as previously described (30). Desiccated whole seeds, with a water content (WC) of 6.1 ± 1.1% on a fresh weight (FW) basis, were stored at -20 °C before ageing by CD. Seeds were germinated on moistened germination paper (Whatman grade 3644, GE Healthcare, UK) in the dark at 16 °C. A seed was considered germinated when radicle emergence was greater than 2 mm.

Controlled deterioration and seed preparation for biochemical analyses

Seeds were pre-equilibrated at 70% RH over non-saturated LiCl for two days at room temperature to establish a seed WC of 7.7 ± 0.2%, before CD at 40 °C and 75 % RH over saturated NaCl solution to maintain the same seed WC. Four replicate seed lots were aged in separate sealed boxes containing ambient O_2 levels (18-21%) or elevated O_2 levels (75-78 %). The O_2 concentrations were monitored in 1 mL of H_2O contained in the box and readjusted weekly using an Oxygen Dipping Probe connected to a Fibox 3 interface (PreSens, Germany). After various intervals, seeds were removed for germination testing or immediately frozen in liquid nitrogen and stored at -80 °C for biochemical analyses (n = 20 seeds per replicate). Thereafter, seeds were freeze-dried for three days. Freeze-dried seeds (20 seeds = *c.* 100 mg) were ground in a 2 ml Eppendorf tube with a 8 mm metal bead for 10 min at 25 Hz (TissueLyser II, Qiagen).

LC-MS/MS analysis of ABA and SA

Analyses of ABA and salicylic acid (SA) were carried out according to (31). In brief, 20 mg of lyophilized seed powder were extracted in 1.5 ml of ice-cold acetone/water/acetic acid (80:20:1, v:v:v) after addition of 25 µl stable isotopically labelled internal standard (IS) solution (1 µM ABA-d6, 5 µM SA-d4) by shaking (TissueLyser II, Qiagen, Düsseldorf, Germany) at 30 Hz for 5 min using one 5 mm glass bead per Eppendorf tube, followed by centrifugation at 10,000 × *g*, 4 °C for 12 min. Supernatants were evaporated to dryness using a SpeedVac SPD111 vacuum concentrator (Thermo Fisher Scientific Inc., Waltham, MA, USA), followed by resuspension in 150 µl of ACN/water (50:50, v:v), supported by 5 min ultrasonication in an ice-cooled water bath. The extracts were filtered through 0.2 µm PTFE filters before injection into the UHPLC-MS/MS system. ABA and SA were identified and

quantified by LC-MS, using an ekspert ultraLC 100 UHPLC system coupled to a QTRAP 4500 mass spectrometer (AB SCIEX, Framingham, MA, USA).

HPLC analysis of LMW thiols and disulphides and tocochromanols

LMW thiols and disulphides were determined by HPLC as described in (11). Briefly, LMW thiols / disulphides samples were extracted from 10 mg of freeze-dried and ground seeds in 0.1M HCl containing 10 mg polyvinylpolypyrrolidone (PVPP) and 0.5% (v:v) Triton X 100. Samples were sonicated for 15 min and then centrifuged for 20 min at 15,000 *g* at 4 °C. The supernatant was collected, avoiding the lipid and pellet phases and centrifuged again for 20 min at 15,000 *g* at 4 °C. Thiols were labelled by monobromobimane (mBBr) for fluorescence detection (excitation: 380 nm; emission: 480 nm) and disulphides were determined after blockage of thiols with N-ethylmaleimide and reduction of disulphides with DTT. The half-cell reduction potential of LMW thiols was calculated from the concentrations of thiols and disulphides considering the seed WC (g water per g seed DW) to calculate molar concentration and taking into account deviations from standard conditions in terms of pH (=7.3) and temperature (=298K) using the Nernst equation according to (17) and (32).

Tocochromanols were analysed by HPLC following the procedure described in (33) with slight alterations. Briefly, 20 mg of freeze-dried and ground seeds was extracted three times in 1 ml ice-cold heptane by vortexing for 20 s and centrifugation at 13,000 *g* for 20 min at 4 °C. The combined supernatants were re-centrifuged at 13,000 *g* for 20 min at 4 °C prior to separation on a reversed-phase column (LC-Diol, 250 x 4.6 mm i.d., 5 µm particle size, Supelco Analytical, Supelcosil™) and detection (fluorescence excitation: 295 nm, emission: 325 nm). To quantify high and low abundant tocochromanols, 10 µl and 70 µl of extract were injected, respectively, that were identified and quantified against individual tocopherol and tocotrienol standards.

Colorimetric analysis of glutathione reductase activity

30 mg of freeze-dried and ground seed material was suspended in 1 ml of ice-cold hexane to remove interfering lipids and centrifuged at 6,000 *g* for 15 min. After discarding the hexane, the pellet was rinsed in fresh hexane and air-dried before the seed powder was dissolved in 700 µl Sorensen's phosphate buffer (pH=7.8), containing protease inhibitor. The samples were centrifuged at 20,000 *g* for 15 min at 4 °C, supernatant was collected and re-centrifuged for 15 min at 20,000 *g*. Proteins were precipitated over 1 h at -20 °C with 1.4 ml 100% acetone added directly to the extract. Proteins were collected by centrifugation for 10 min at 6,000 *g* and 4 °C and the acetone was discarded. The step was repeated with 80% acetone. After air-drying the protein pellet, it was suspended in 800 µl of Sorensen's phosphate buffer

(pH 7.8) and 30 µl protease inhibitor cocktail and used immediately. Enzyme kinetics were measured using a Perkin Elmer spectrophotometer (Lambda 800 UV/VIS Spectrometer) with a cuvette holder maintained at 25 °C. Glutathione reductase was measured by the decrease in absorbance at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) with 0.083 mM GSSG, 0.083 mM NADPH and 200 µl enzyme extract in a total reaction volume of 600 µl in 0.05 M Sorenson's buffer at pH 7.8.

GC-MS analysis of FAs

Fatty acids were derivatised to fatty acid methyl esters (FAMEs) as previously described (34). Briefly, 10 mg of freeze-dried seed powder were treated with 2.64 mL of methanol:toluene:sulfuric acid 10:3:0.25 v:v:v containing 0.01% (w:v) butylated hydroxytoluene. 200 µg of heptadecanoic acid (C17:0, dissolved in hexane) were added simultaneously as internal standard. Samples were incubated at 80 °C for 90 min with constant agitation before adding 1 mL of hexane and 3 mL of 0.9 % NaCl (w:v). Samples were vigorously mixed before centrifugation for 10 min at 3,000 *g*. The supernatant was transferred to autosampler vials and kept at −20 °C if not analysed immediately. FAMEs were separated using a Trace 1300 gas chromatograph (GC) (Thermo-Scientific, USA) on a 30 m FAMEWAX column (Restek #12497, Bellefonte, USA) and detected using a TSQ 8000 triple quadrupole detector (Thermo-Scientific, Waltham, USA) operated in full scan mode (50 – 550 m/z). 1 µL of sample was injected in split mode (split ratio 1/100) in a split/splitless (SSL) inlet heated at 230 °C and containing a Mini-Lam 4 mm split liner (Thermo-Scientific #453A2009, Waltham, USA). The temperature gradient was first set to 120 °C for 1 min, then increased by 16 °C per min up to 190 °C, then by 5 °C per min up to 220 °C and finally increased by 2 °C per min up to 235 °C, which was held for 7 min with a carrier gas flow of 1.2 mL min^{-1} of helium. The ion source temperature was set to 250 °C and the transfer line to 240 °C. A commercial FAME mix (Sigma Aldrich ref. 18919, Missouri, USA) was used to confirm the identities of the FAs. External standards of palmitic, stearic, oleic, linoleic and linolenic acid were used in conjunction with the internal standard to estimate the total amount of each fatty acid. Data analysis was performed using the Xcalibur software (Thermo-Scientific, Waltham, USA).

Statistical analysis

Statistical analysis of data was carried out with the SPSS software package (v. 23) via three-way analysis of variance (ANOVA), except FA data that was tested with a Student's t-test. Univariate ANOVA-derived *P*-values for differences in all data are presented in each Figure between genotypes, over time of ageing or in response to level of O_2. For example, when a

significant ageing effect is stated (P<0.05), all data for both genotypes aged under both O_2 levels were considered. For assessing significant changes in viability, the number of seeds that had germinated, and not % TG, was considered to avoid the need for data transformation.

Results

Non-aged A12 seeds took 3.4 ± 0.1 d to reach 25% germination ($T_{25\%}$), slower than the 2.5 ± 0.1 d it took SL101 seeds (Fig. 1A). Values of $T_{25\%}$ increased over the first 19 d of CD during which a significant difference in vigour between the genotypes was maintained (Fig. 1A). The ABA levels in seeds of A12 and SL101 were *c.* 0.5 and 2.5 nmol g^{-1} DW, respectively, and were not significantly altered during 41 d of CD (Fig. 1B). Salicylic acid significantly accumulated during CD, but no significant effect of oxygen concentration and no difference between genotypes was found (Supp. Fig. 1). The TG remained above 90% until 19 d of CD (Fig. 1C), but additional O_2 during CD (CD+O_2) over this period led to a more rapid ageing-induced loss in vigour, as shown by the increased $T_{25\%}$ values (Fig. 1A). However, CD+O_2 had no consistent negative effect on TG, which dramatically declined between 19 and 41 d CD (Fig. 1C), at which point the $T_{25\%}$ values were >20 d and the vigour of the genotypes was indistinguishable (Fig. 1A). Overall, seeds of genotype SL101 had lower ABA contents and faster germination rates than genotype A12. The difference in vigour between the genotypes was maintained during ageing before viability was lost and elevated O_2 during ageing accelerated vigour loss.

Non-aged seeds of both genotypes contained *c.* 1.75 µmol g^{-1} DW total glutathione, while possessing a GSH:GSSG ratio of *c.* 2:1 (Fig. 2A) and $E_{GSSG/2GSH}$ values of *c.* -210 mV (Fig. 2B), which is typical for highly viable seeds (17). A significant decrease of GSH levels occurred in response to CD, while GSSG levels increased (Fig. 2A), contributing to an oxidative shift in $E_{GSSG/2GSH}$, which after 19 d of CD rose to *c.* -155 mV. However, no clear effect of CD+O_2 on GSH nor GSSG levels was observed (Fig. 2B). By 41 d CD GSH levels were *c.* 0.2 µmol g^{-1} DW and the GSH:GSSG ratio was *c.*1:5 (Fig. 2A), showing that majority of GSH was converted to GSSG while *c.* 30% of total glutathione was consumed. Glutathione reductase (GR) activities in non-aged seeds were ≤14 nkat g^{-1} DW and were halved after 41 d of CD (Fig. 2C), although overall SL101 had significantly higher GR activity than A12 (Fig. 2C). Concomitantly with a CD-induced accumulation of GSSG, levels of other LMW disulphides also increased, including cystine and bis-γ-glutamylcystine, which after 41 d of CD had increased two-fold and four-fold, respectively (Supp. Fig. 2 and 3). In summary, ageing was associated with an oxidative shift in the LMW thiol-based cellular redox state,

whereby thiols (i.e. GSH) converted to disulphides (i.e. GSSG), but these changes were not influenced by genotype, nor by elevated O_2 during ageing.

Four different types of tocochromanols were identified in whole seeds; γ-tocopherol (70%), α-tocopherol (19% A12; 17% SL101), β-tocotrienol (7% A12; 9% SL101) and γ-tocotrienol (4%), which together amounted to 270 ± 10 µmol g DW^{-1} of total tocochromanols in untreated seeds (Fig. 3). There was a significant effect of CD on α-tocopherol, which decreased over time, while γ-tocopherol and γ-tocotrienol levels tended to increase. A significant difference in α-tocopherol, β- and γ-tocotrienol between genotypes was found when considering all time intervals, while CD+O_2 significantly lowered β-tocotrienol levels relative to CD under ambient O_2. Overall, in response to CD total content of tocochromanols remained stable, but the overall composition changed due to declining levels of α-tocopherol and β-tocotrienol, which was compensated by increasing levels of γ-tocopherol and γ-tocotrienol (Fig. 3).

In total, 18 FAs were detected using GC-MS. The abundance of each FA were approximated from TIC chromatograms (Fig. 4A), whereby the 10 most abundant FAs, representing >95% chromatogram area of the total FAs, were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), eicosanoic acid (20:0), gadoleic acid (20:1), eicosadienoic acid (20:2), docosanoic acid (22:0) and erucic acid (22:1). Comparing the relative FA levels between genotypes, 16:1, an isomer of 18:1, 18:2, 22:0, an isomer of 22:1 and docosadienoic acid (22:2) were significantly higher in non-aged SL101 than A12 (Fig. 4B), indicating a minor effect of ABA signalling during seed development on lipid profiles, but there was no effect of ageing on relative FA contents in either genotype (Supp. Table 1).

Discussion

Following physiological maturity seeds begin to deteriorate, whereby vigour declines prior to viability (1, 6). The extent to which the underlying biochemical pathways leading to losses of vigour and losses of viability are shared is unclear. Here, we compared the effects of ageing by CD on seeds of two *B. oleracea* lines that have allelic differences in key vigour-determining genes (29), but otherwise share a common genetic background. These genes determine ABA content at physiological maturity and sensitivity to ABA and therefore, initial seed vigour (29, 30). Seed vigour is a "catchall" term, comprising all aspects of seed performance. Initial seed vigour is genetically determined, as well as being affected by environmental factors during seed development, but is lost due to seed deterioration (6). These aspects are confounded in many vigour studies and in commercial estimates of

vigour. The A12 (low initial vigour) and SLI101 (high initial vigour) lines, used in this work, allowed us to study how this genetically determined vigour difference may affect seed deterioration and vigour loss induced by ageing.

Seed deterioration due to seed ageing has been linked with oxidative modifications, including to the overall cellular redox state (17, 35, 36). Therefore, it could be expected that seeds age faster under high O_2 concentrations. Studies using elevated partial-pressures of O_2 during CD supported this assumption (4). Within the first 19 d of ageing, prior to the drop in viability, germination speed was slowed by elevated O_2 (Fig. 1A). Interestingly, the ABA-associated genetic differences that determine initial speed of germination had no influence on the O_2-associated loss of vigour. Elevated O_2 during CD also resulted in a loss of vigour in non-dormant *H. annuus* and *H. vulgare* seeds (11, 25). However, in this study, as also shown previously for *Helianthus annuus* (11) or *Hordeum vulgare* seeds (25), elevated O_2 during CD had limited impact on viability loss. Other studies support the idea that seed ageing can be influenced by O_2 (4, 37-39), but viability loss also includes O_2-independent factors. One explanation for inconsistent effects of O_2 on seed viability loss is the variability of molecular mobility. Seed WC appears to be decisive for seed responsivity to O_2 during ageing. At higher WC O_2 may promote seed longevity by allowing O_2-mediated metabolism, whereas decreasing O_2 levels increases seed survival at slower ageing rates (3, 5, 37, 40). The minor effects of O_2 levels on viability loss observed here (Fig. 1C) indicates that the potentially deteriorative effects of O_2 were outweighed by its potential benefit for O_2-dependent metabolism.

During ageing, and before viability loss, levels of GSSG rose while GSH contents and GR activity decreased, which occurred equally for both genotypes (Fig. 2). It is unlikely that GR is able to efficiently reduce GSSG during CD due to restricted molecular mobility in seed equilibrated to 75% RH. However, decreased GR activity in aged seeds would affect the thiol-based cellular redox state of seeds during imbibition when GSSG is converted back to GSH (41). Changes in glutathione redox state can be expressed through $E_{GSSG/2GSH}$, a Nernst equation-derived calculation that considers molar concentrations of both GSH and GSSH. The changes in GSH and GSSG amounts during seed ageing led to an oxidative shift of the $E_{GSSG/2GSH}$ by 60 mV to *c.* -160 mV during the first 19 d of CD (Fig. 2B). A zone between -180 to -160 mV has been associated with a 50 % viability loss of a seed population (17) and $E_{GSSG/2GSH}$ values did not change between 19-41d of CD when viability dropped to *c.* 50%. In both genotypes, a shift of $E_{GSSG/2GSH}$ to less negative values appears to be associated with the loss of seed vigour (Fig. 1A). However, the weak significant effect of O_2 on $E_{GSSG/2GSH}$ values during CD (P=0.28) is insufficient to be able to directly associate the enhanced O_2-associated decrease in germination vigour with an oxidative shift of $E_{GSSG/2GSH}$.

The differences in FA and tocochromanol composition between the genotypes (Fig. 3; Fig. 4) indicate that ABA signalling can influence lipid-related pathways. In *Medicago truncatula* an interconnection between tocochromanol synthesis and ABA signalling has been demonstrated (42, 43). Tocochromanol-deficient mutants of *A. thaliana* were not able to retain viable embryos (23) and incurred an elevated level of lipid peroxidation during seed storage, germination, and early seedling development (44). In *A. thaliana* and *B. oleracea* γ-tocopherol is the dominant tocochromanol, but in this study levels of γ-tocopherol did not decline in response to CD (Fig. 3A). In contrast, an increase in tocochromanols in both genotypes occurred within the first 4 d of CD, indicating either tocochromanol synthesis or conversion from existing precursors. A lack in change of FA profiles of aged seed (Supp. Tab. 1) indicate that PUFA were well protected from peroxidation during CD. The second most abundant tocochromanol, α-tocopherol, was significantly affected by CD in both genotypes, decreasing after 8 d (Fig. 3B). This different response of γ- and α-tocopherol is consistent with the faster degradation of α-tocopherol than of γ-tocopherol observed in seeds of *B. napus* (26), *Pinus sylvestris* (45) and *Suaeda maritima* (46), suggesting that either α-tocopherol is more efficient at radical scavenging than γ-tocopherol or that it is located in lipid domains more affected during seed ageing.

Summary and conclusion

As summarised in Figure 5, the differences in ABA levels and signalling between the investigated genotypes had a significant impact on germination speed, which were maintained during CD before viability was lost. Other more subtle genotype-associated differences in tocochromanols, FA profiles and GR activity were found. However, these had no apparent impact on rates of seed ageing and associated biochemistry; a loss of GSH, α-tocopherol and GR activity, an oxidative shift in $E_{GSSG/2GSH}$ and an increase in SA all occurred to a similar extent in both of *B. oleracea* genotypes. Therefore, low seed vigour due to ABA signalling is based on different biochemical mechanisms from those resulting from deterioration.

Author contribution

TR and IK conceived the study. All authors contributed to the experimental design. CS, TR, WS and EA conducted the experiments and TR analysed the data. All authors wrote the manuscript.

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Competing interests

The authors declare that there no competing interests associated with the manuscript.

References

1. Roberts EH. (1973) Predicting the storage life of seeds. Seed Science and Technology. **1**, 499-514
2. Harrington JF. (1963) Practical instructions and advice on seed storage. Proceedings of the International Seed Testing Association: 989-94
3. Ibrahim AE, Roberts EH, Murdoch AJ. (1983) Viability of Lettuce Seeds. J Exp Bot. **34**, 631-40
4. Groot SPC, Surki AA, de Vos RCH, Kodde J. (2012) Seed storage at elevated partial pressure of oxygen, a fast method for analysing seed ageing under dry conditions. Ann Bot. **110**, 1149-59.
5. Schwember AR, Bradford KJ. (2011) Oxygen interacts with priming, moisture content and temperature to affect the longevity of lettuce and onion seeds. Seed Sci Res. **21,** 175-85.
6. Finch-Savage WE, Bassel GW. (2016) Seed vigour and crop establishment: extending performance beyond adaptation. J Exp Bot. **67,** 567-91.
7. Koornneef M, Bentsink L, Hilhorst H. (2002) Seed dormancy and germination. Curr Opin Plant Biol. **5,** 33-6.
8. Finch-Savage WE, Leubner-Metzger G. (2006) Seed dormancy and the control of germination. New Phytol. **171,** 501-23.
9. Clerkx EJM, Blankestijn-De Vries H, Ruys GJ, Groot SPC, Koornneef M. (2004) Genetic differences in seed longevity of various Arabidopsis mutants. Physiol Plant. **121,** 448-61.
10. Nguyen TP, Keizer P, van Eeuwijk F, Smeekens S, Bentsink L. (2012) Natural variation for seed longevity and seed dormancy are negatively correlated in Arabidopsis. Plant Physiol. **160,** 2083-92.
11. Morscher F, Kranner I, Arc E, Bailly C, Roach T. (2015) Glutathione redox state, tocochromanols, fatty acids, antioxidant enzymes and protein carbonylation in sunflower seed embryos associated with after-ripening and ageing. Ann Bot-London. **116,** 669-78.
12. Nguyen T-P, Cueff G, Hegedus DD, Rajjou L, Bentsink L. (2015) A role for seed storage proteins in Arabidopsis seed longevity. Journal Exp Bot. **66**, 6399-413.
13. Bailly C, El-Maarouf-Bouteau H, Corbineau F. (2008) From intracellular signaling networks to cell death: The dual role of reactive oxygen species in seed physiology. Cr Biol. **331,** 806-14.
14. Arc E, Sechet J, Corbineau F, Rajjou L, Marion-Poll A. (2013) ABA crosstalk with ethylene and nitric oxide in seed dormancy and germination. Front Plant Sci. **4**:63.
15. Wojtyla Ł, Lechowska K, Kubala S, Garnczarska M. (2016) Different Modes of Hydrogen Peroxide Action During Seed Germination. Front Plant Sci. **7**, 66.
16. Rajjou L, Lovigny Y, Groot SP, Belghazi M, Job C, Job D. (2008) Proteome-wide characterization of seed aging in Arabidopsis: a comparison between artificial and natural aging protocols. Plant Physiol. **148**, 620-41.
17. Kranner I, Birtic S, Anderson KM, Pritchard HW. (2006) Glutathione half-cell reduction potential: a universal stress marker and modulator of programmed cell death? Free Radical Bio Med. **40**, 2155-65.

18. Roach T, Beckett RP, Minibayeva FV, Colville L, Whitaker C, Chen H, et al. (2010) Extracellular superoxide production, viability and redox poise in response to desiccation in recalcitrant *Castanea sativa* seeds. Plant Cell Environ. **33**, 59-75.
19. Nagel M, Kranner I, Neumann K, Rolletschek H, Seal CE, Colville L, et al. (2015) Genome-wide association mapping and biochemical markers reveal that seed ageing and longevity are intricately affected by genetic background and developmental and environmental conditions in barley. Plant Cell Environ. **38**, 1011-22.
20. Foyer CH, Noctor G. (2011) Ascorbate and Glutathione: the heart of the redox hub. Plant Physiol. **155**, 2-18.
21. Tommasi F, Paciolla C, Arrigoni O. (1999) The ascorbate system in recalcitrant and orthodox seeds. Physiological and Molecular Plant Pathol. **105**, 193-8.
22. Kranner I, Minibayeva FV, Beckett RP, Seal CE. (2010) What is stress? Concepts, definitions and applications in seed science. New Phytol. **188,** 655-73.
23. Mène-Saffrané L, Jones AD, DellaPenna D. (2010) Plastochromanol-8 and tocopherols are essential lipid-soluble antioxidants during seed desiccation and quiescence in Arabidopsis. PNAS USA. **107,** 17815-20.
24. Seal CE, Zammit R, Scott P, Nyamongo DO, Daws MI, Kranner I. (2010) Glutathione half-cell reduction potential as a seed viability marker of the potential oilseed crop *Vernonia galamensis*. Ind Crop Prod. **32,** 687-91.
25. Roach T, Nagel M, Börner A, Eberle C, Kranner I. (2018) Changes in tocochromanols and glutathione reveal differences in the mechanisms of seed ageing under seedbank conditions and controlled deterioration in barley. Environ Exper Bot. **156**, 8-15.
26. Goffman FD, Mollers C. (2000) Changes in tocopherol and plastochromanol-8 contents in seeds and oil of oilseed rape (*Brassica napus* L.) during storage as influenced by temperature and air oxygen. J Agric Food Chem. **48,** 1605-9.
27. Rae AM, Howell EC, Kearsey MJ. (1999) More QTL for flowering time revealed by substitution lines in *Brassica oleracea*. Heredity. **83**, 586-96.
28. Bettey M, Finch-Savage WE, King GJ, Lynn JR. (2000) Quantitative genetic analysis of seed vigour and pre-emergence seedling growth traits in *Brassica oleracea*. New Phytol. **148**, 277-86.
29. Morris K, Barker GC, Walley PG, Lynn JR, Finch-Savage WE. (2016) Trait to gene analysis reveals that allelic variation in three genes determines seed vigour. New Phytol. **212**, 964-76.
30. Awan S, Steven F, E. FSW. (2018) Interaction of maternal environment and allelic differences in seed vigour genes determines seed performance in *Brassica oleracea*. Plant J. **94**, 1098-108.
31. Buchner O, Roach T, Gertzen J, Schenk S, Karadar M, Stoggl W, et al. (2017) Drought affects the heat-hardening capacity of alpine plants as indicated by changes in xanthophyll cycle pigments, singlet oxygen scavenging, alpha-tocopherol and plant hormones. Environ Exper Bot. **133**, 159-75.
32. Schafer FQ, Buettner GR. (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radical Bio Med. **30**, 1191-212.
33. Bagci E, Vural M, Dirmenci T, Bruehl L, Aitzetmuller K. (2004) Fatty acid and tocochromanol patterns of some *Salvia* L. species. Z Naturforsch C. **59**, 305-9.
34. Li-Beisson Y, Shorrosh B, Beisson F, Andersson MX, Arondel V, Bates PD, et al. (2013) Acyl-lipid metabolism. The Arabidopsis book; 11:e0161.
35. Bailly C (2004). Active oxygen species and antioxidants in seed biology. Seed Sci Res. **14**, 93-107.
36. Rajjou L, Debeaujon I. (2008) Seed longevity: Survival and maintenance of high germination ability of dry seeds. Cr Biol. **331**, 796-805.
37. Roberts EH, Abdalla FH. (1968) The influence of temperature, moisture, and Oxygen on period of seed viability in barley, broad beans, and peas. Ann Bot. **32**, 97-117.
38. Ohlrogge JB, Kernan TP. (1982) Oxygen-Dependent Aging of Seeds. Plant Physiol. **70**, 791-4.

39. Nagel M, Kodde J, Pistrick S, Mascher M, Börner A, Groot PC. (2016) Barley seed ageing: genetics behind the dry elevated pressure of oxygen ageing and moist controlled deterioration. Front Plant Sci. **7**, 388
40. Groot SPC, de Groot L, Kodde J, van Treuren R. (2015) Prolonging the longevity of ex situ conserved seeds by storage under anoxia. Plant Gen Resour. **13**, 18-26.
41. Kranner I, Grill D. (1993) Content of low-molecular-weight Thiols during the Imbibition of pea-seeds. Physiol Plant. **88**, 557-62.
42. Zinsmeister J, Lalanne D, Terrasson E, Chatelain E, Vandecasteele C, Ly Vu B, et al. (2016) ABI5 is a Regulator of Seed Maturation and Longevity in Legumes. Plant Cell. **28**, 2735-54
43. Jiang J, Chen Z, Ban L, Wu Y, Huang J, Chu J, et al. (2017) P-HYDROXYPHENYLPYRUVATE DIOXYGENASE from *Medicago sativa* is involved in vitamin E biosynthesis and abscisic acid-mediated seed germination. Sci Rep. **7**, 40625.
44. 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. Plant Cell. **16**, 1419-32.
45. Tammela P, Salo-Vaananen P, Laakso I, Hopia A, Vuorela H, Nygren M. (2005) Tocopherols, tocotrienols and fatty acids as indicators of natural ageing in *Pinus sylvestris* seeds. Scand J Forest Res. **20**, 378-84.
46. Seal CE, Zammit R, Scott P, Flowers TJ, Kranner I. (2010) Glutathione half-cell reduction potential and α-tocopherol as viability markers during the prolonged storage of *Suaeda maritima* seeds. Seed Sci Res. **20**, 47-53.

Figure legends

Figure 1. Ageing-induced changes on abscisic acid (ABA) levels, germination speed and losses of total germination. (A) Time for 25% of seeds to germinate (T_{25}), **(B)** seed ABA amounts and **(C)** total germination in genotype A12 (closed symbols) and genotype SL101 (open symbols) before and after controlled deterioration at 40 °C and 75% RH under ambient O_2 (circles) or elevated O_2 (squares), n=4 reps of 20 seeds ± SD. *P*-values were calculated from a univariate ANOVA of all data over 0-41 d ageing (Age), additionally between 0-19 d in (A), of all data between genotypes (Gen), or of all data in response differences in O_2 environment (O_2).

Figure 2. Ageing-induced changes in glutathione metabolism. (A) Levels of GSH (white bars) and GSSG (black bars). Values of **(B)** $E_{GSSG/2GSH}$ and **(C)** GR activity in A12 (closed symbols) and genotype SL101 (open symbols) before and after controlled deterioration at 40 °C and 75% RH under ambient O_2 (circles) or elevated O_2 (squares), n=4 reps of 20 seeds ± SD. *P*-values were calculated from a univariate ANOVA of all data over 0-41 d ageing (Age), of all data between genotypes (Gen), or of all data in response differences in O_2 environment (O_2).

Figure 3. Ageing-induced changes in tocochromanols. Content of **(A)** γ-tocopherol, **(B)** α-tocopherol, **(C)** β-tocotrienol and **(D)** γ- tocotrienol in genotype A12 (closed symbols) and genotype SL101 (open symbols) before and after controlled deterioration at 40 °C and 75% RH under either ambient O_2 (circles) or elevated O_2 (squares), n=4 reps of 20 seeds ± SE. *P*-values were calculated from a univariate ANOVA of all data over 0-41 d ageing (Age), of all data between genotypes (Gen), or of all data in response differences in O_2 environment (O_2).

Figure 4. Relative fatty acid (FA) contents in non-aged seed. (A) Representative total ion current (TIC) chromatogram with the main FAs labelled. **(B)** FA abundance in non-aged seeds of genotype A12 (black bars, below) relative to SL101 levels (above, error bars only), as shown on a $\log_2$ scale. Palmitic acid (C16:0), palmitoleic acid (16:1), hexadecadienoic acid (C16:2), stearic acid (C18:0), oleic acid (C18:1), an isomer of oleic acid (C18:1i), linoleic acid (C18:2), linolenic acid (C18:3), eicosanoic acid (C20:0), gadoleic acid (C20:1), an isomer of gadoleic acid (C20:1i), eicosadienoic acid (C20:2), docosanoic acid (C22:0), erucic acid (C22:1), an isomer of erucic acid (C22:1i), docosadienoic acid (C22:2), lignoceric acid (C24:0) and nervonic acid (C24:1). Significant relative differences ($P<0.05$) of FAs between genotypes are denoted by an asterisk, as detected by Student's t-test, n=4 reps of 20 seeds ± SD.

Figure 5. Summary of the seed properties associated to ABA signalling and ageing in *Brassica oleracea*. Significant associations with ABA in non-aged seeds (upper) separated by dotted lines from associations with CD-induced ageing (lower), using data from Figures 1-4 & Supp. Fig. 1.

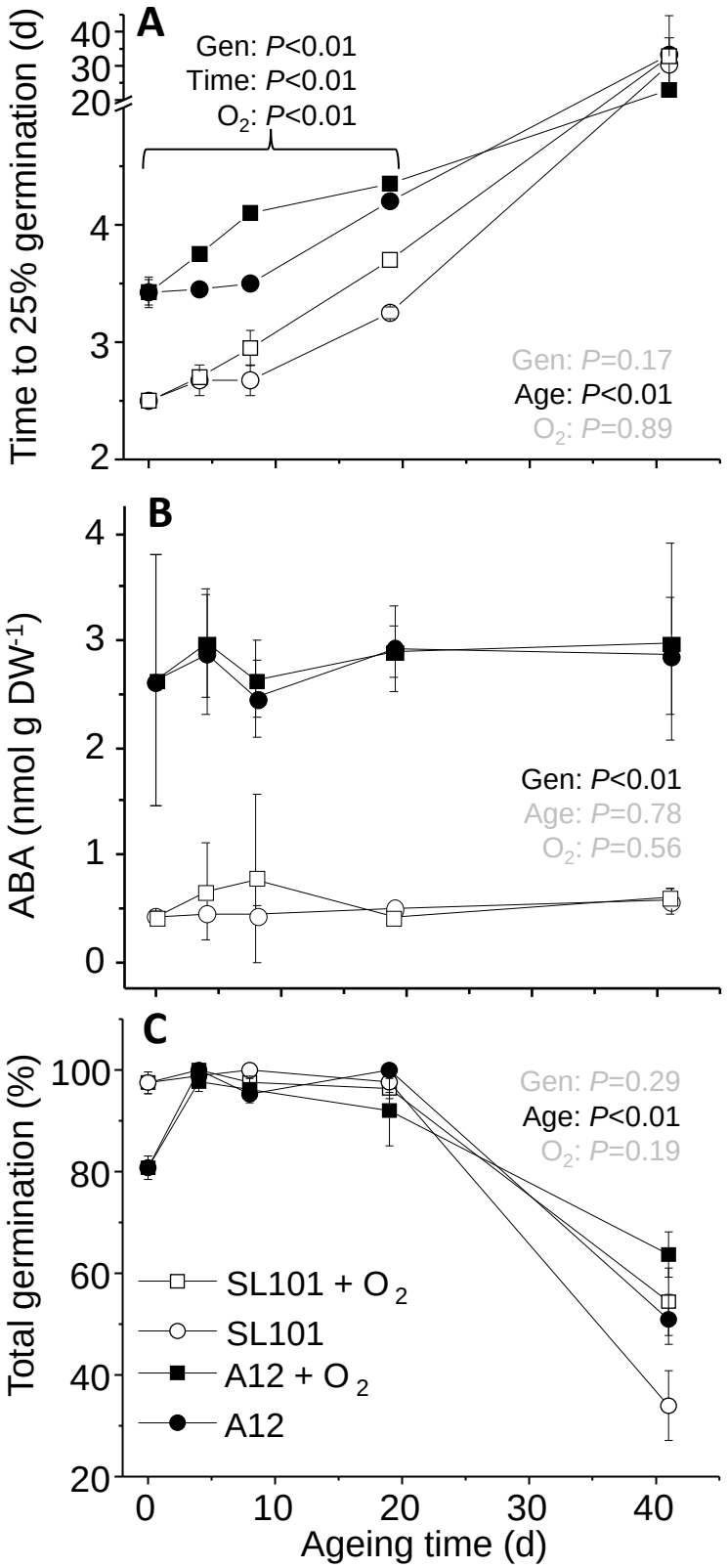

A
Gen: P<0.01
Time: P<0.01
O2: P<0.01
Gen: P=0.17
Age: P<0.01
O2: P=0.89
Time to 25% germination (d)
B
Gen: P<0.01
Age: P=0.78
O2: P=0.56
ABA (nmol g DW-1)
C
Gen: P=0.29
Age: P<0.01
O2: P=0.19
SL101 + O2
SL101
A12 + O2
A12
Total germination (%)
Ageing time (d)

Figure 1

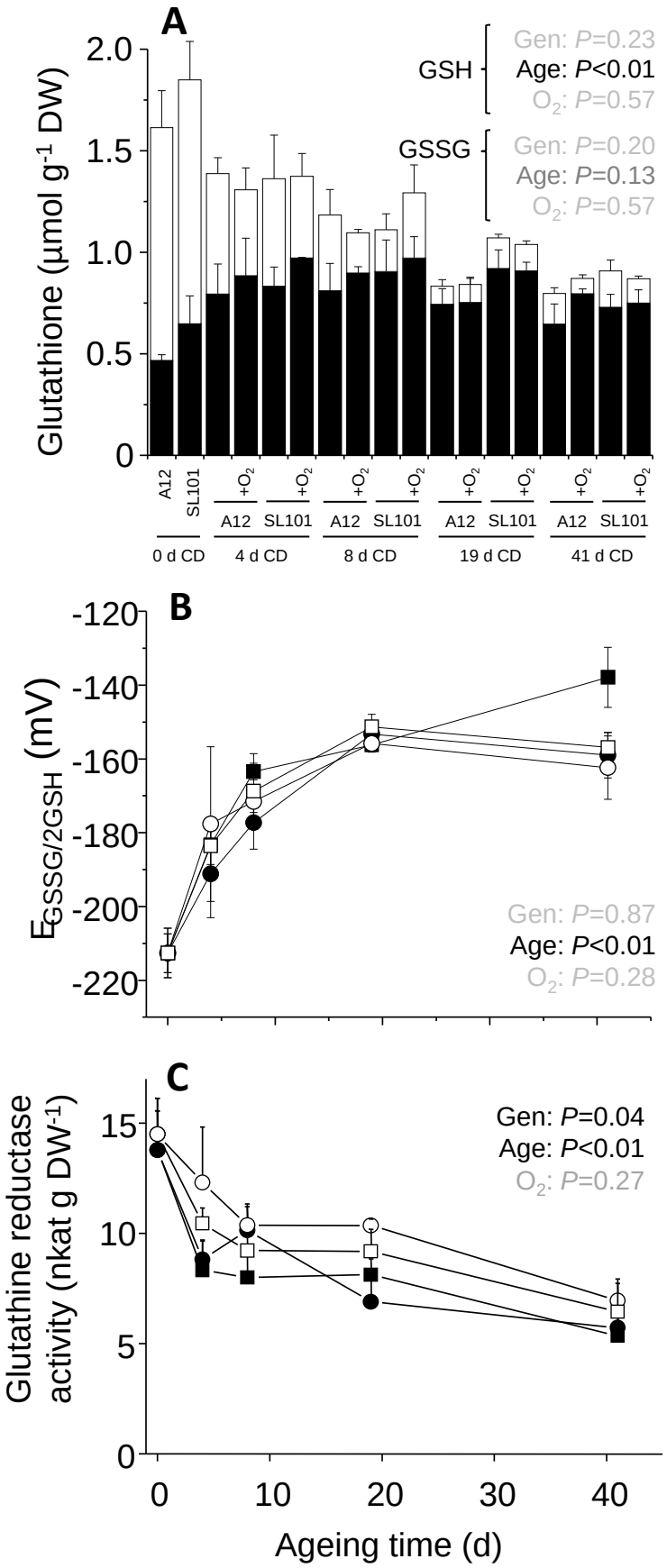

A
Glutathione (µmol g-1 DW)
2.0
1.5
1.0
0.5
0
GSH
Gen: P=0.23
Age: P<0.01
O2: P=0.57
GSSG
Gen: P=0.20
Age: P=0.13
O2: P=0.57
A12
SL101
+O2
0 d CD
4 d CD
8 d CD
19 d CD
41 d CD
B
EGSSG/2GSH (mV)
-120
-140
-160
-180
-200
-220
Gen: P=0.87
Age: P<0.01
O2: P=0.28
C
Glutathine reductase activity (nkat g DW-1)
15
10
5
0
Gen: P=0.04
Age: P<0.01
O2: P=0.27
0
10
20
30
40
Ageing time (d)

Figure 2

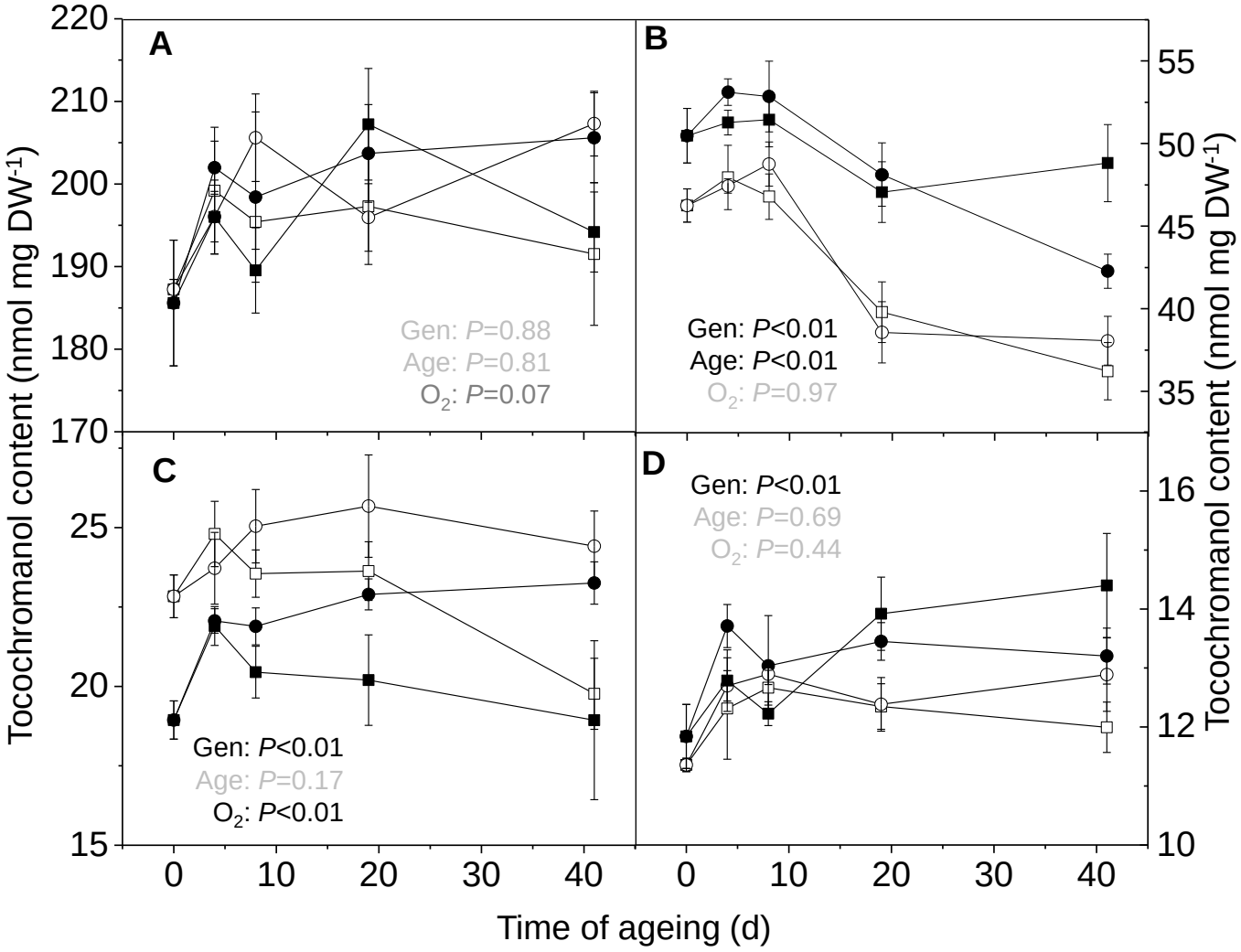

A
B
C
D
Gen: P=0.88
Age: P=0.81
O2: P=0.07
Gen: P<0.01
Age: P<0.01
O2: P=0.97
Gen: P<0.01
Age: P=0.17
O2: P<0.01
Gen: P<0.01
Age: P=0.69
O2: P=0.44
Tocochromanol content (nmol mg DW-1)
Tocochromanol content (nmol mg DW-1)
Time of ageing (d)
220
210
200
190
180
170
55
50
45
40
35
25
20
15
16
14
12
10
0
10
20
30
40

Figure 3

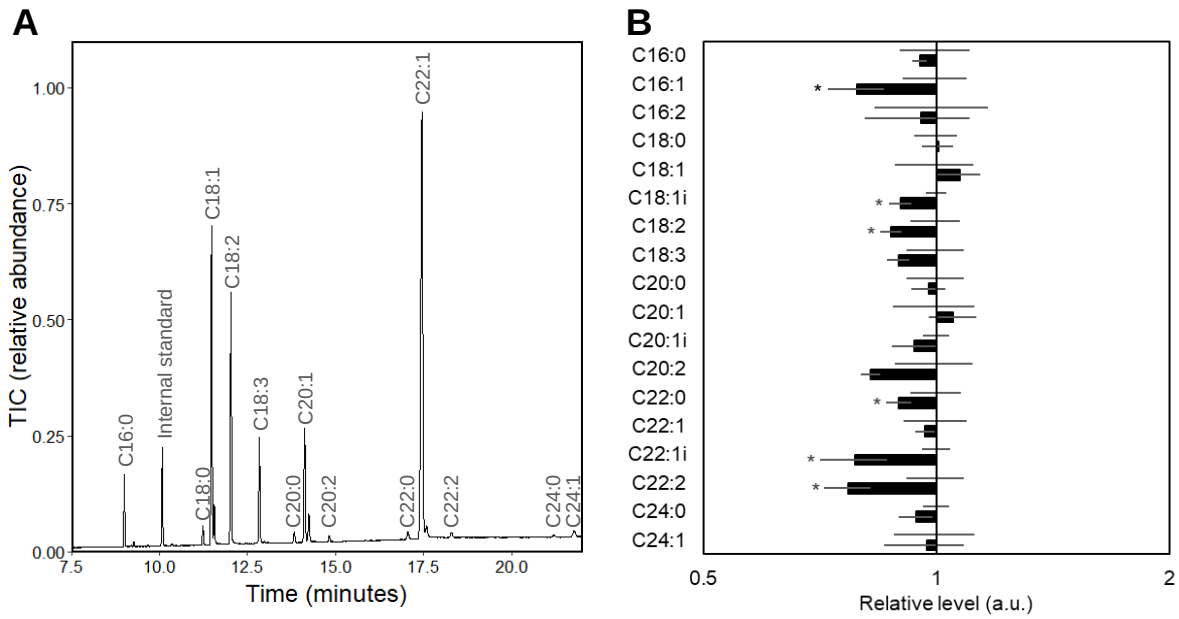

A
TIC (relative abundance)
1.00
0.75
0.50
0.25
0.00
7.5
10.0
12.5
15.0
17.5
20.0
Time (minutes)
C16:0
Internal standard
C18:0
C18:1
C18:2
C18:3
C20:0
C20:1
C20:2
C22:0
C22:1
C22:2
C24:0
C24:1
B
C16:0
C16:1
C16:2
C18:0
C18:1
C18:1i
C18:2
C18:3
C20:0
C20:1
C20:1i
C20:2
C22:0
C22:1
C22:1i
C22:2
C24:0
C24:1
0.5
1
2
Relative level (a.u.)

Figure 4

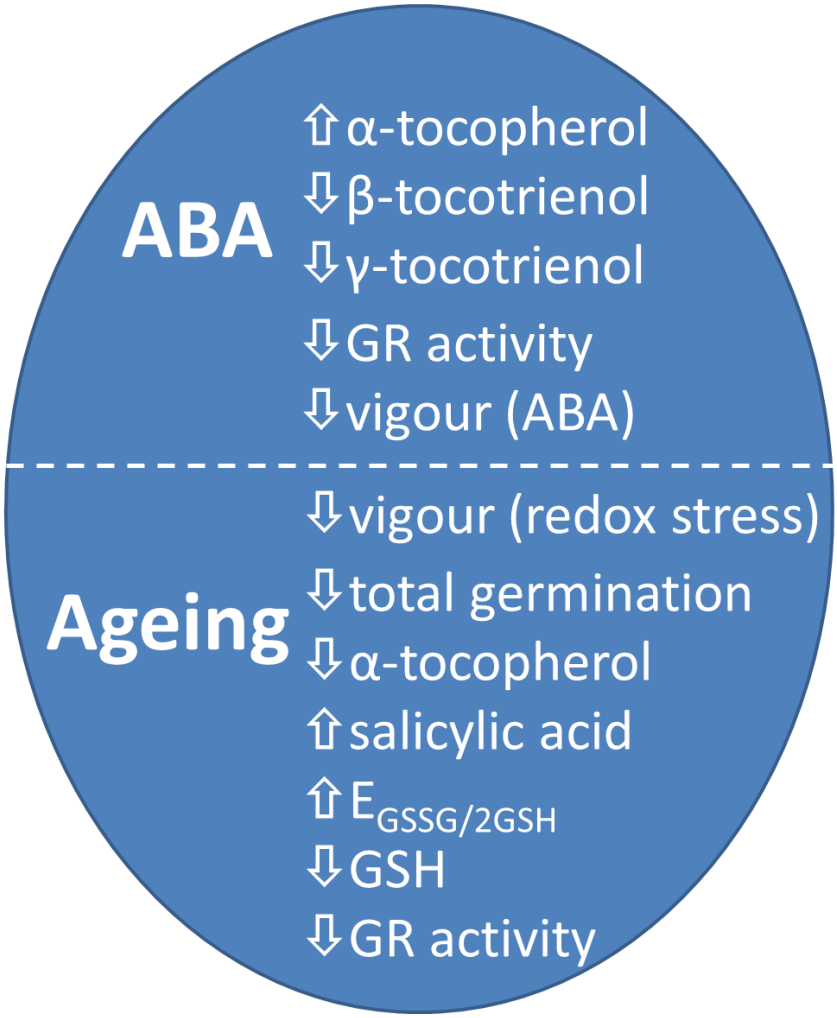
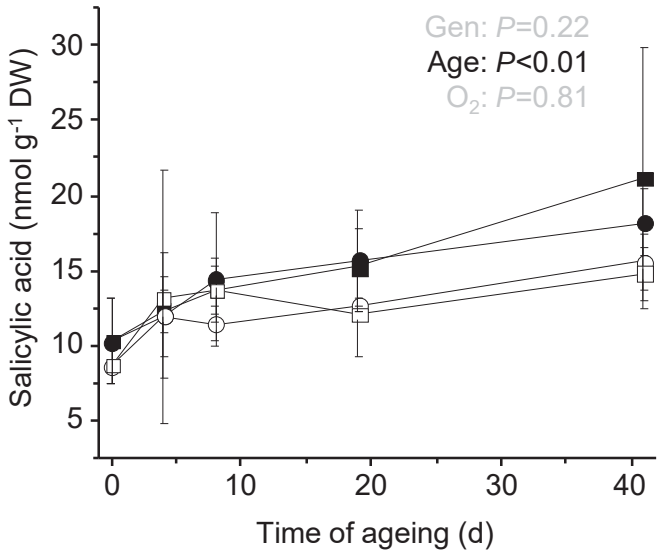
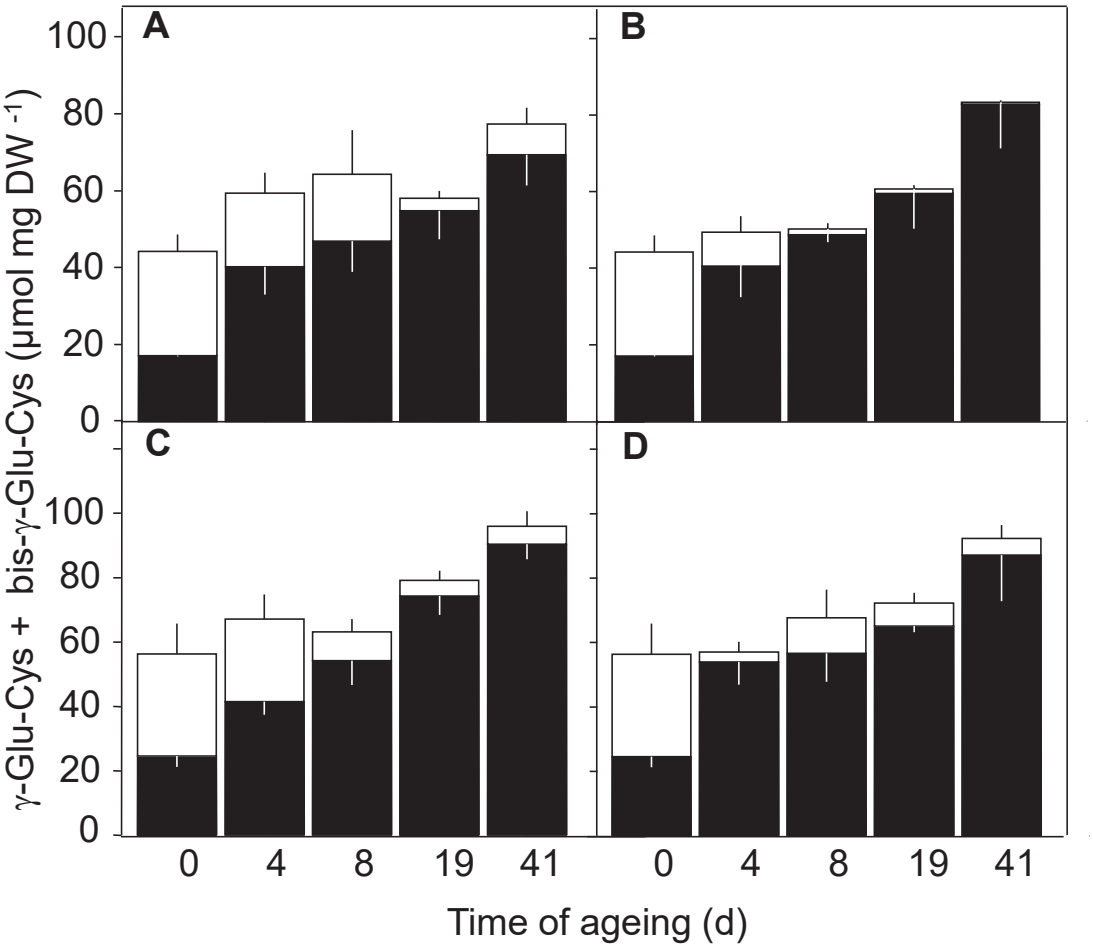

ABA
⇧α-tocopherol
⇩β-tocotrienol
⇩γ-tocotrienol
⇩GR activity
⇩vigour (ABA)
Ageing
⇩vigour (redox stress)
⇩total germination
⇩α-tocopherol
⇧salicylic acid
⇧E GSSG/2GSH
⇩GSH
⇩GR activity

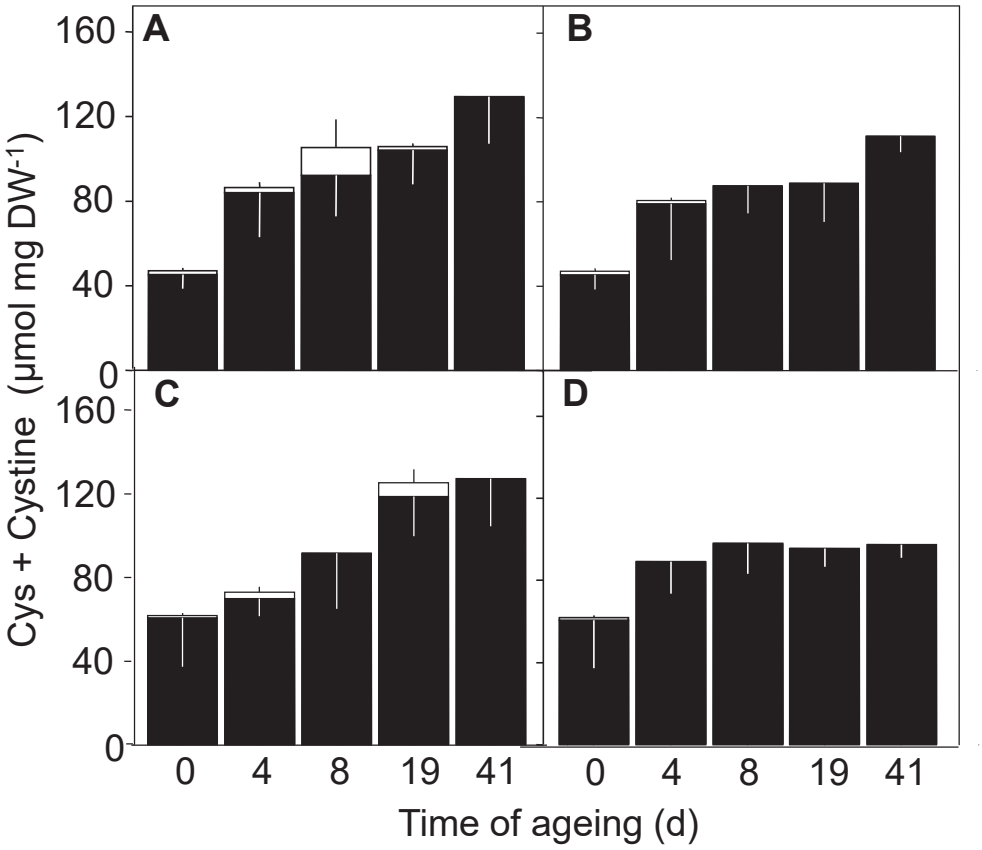
Figure 5



Supplementary Figure 1. Seed salicylic acid amounts in A12 (closed symbols) and genotype SL101 (open symbols) before and after ageing at 40°C and 75% RH under ambient O_2 (21%; circles) or elevated O_2 (78%; squares), n=4 reps of 20 seeds ± SD. *P*-values were calculated from a univariate ANOVA of all data over 0-41 d ageing (Age), of all data between genotypes (Gen), or of all data in response differences in O_2 environment (O_2).



Supplemetary Figure 2. The thiol γ-Glutamyl-cysteine (γ-Glu-Cys; white bars) and disulphide bis-γ-glutamyl-cystine (bis-γ-Glu-Cys; black bars) in genotype A12 **(A, B)** and genotype SI101 **(C, D)** in response to ageing at 40°C and 75% RH under either ambient oxygen (18-21%) **(A,C)** or elevated oxygen (75-78%) **(B,D)**. Data represent means ± SE (n=4).



Supplementary Figure 3. The thiol cysteine (Cys; white bars) and disulphide cystine (black bars) in genotype A12 **(A, B)** and genotype SI101 **(C, D)** in response to ageing at 40°C and 75% RH under either ambient oxygen (18-21%) **(A,C)** or elevated oxygen (75-78%) **(B,D)**. Data represent means ± SE (n=4).

Supplemental Table 1. Relative abundance of fatty acids in comparison to non-aged seeds of genotype Sl101 in *B. oleracea* seeds. Palmitic acid (16:0), palmitoleic acid (16:1), hexadecadienoic acid (16:2), stearic acid (18:0), oleic acid(18:1) and an isomer of oleic acid, linoleic acid (18:2), linolenic acid (18:3), eicosanoic acid (20:0), gadoleic acid (20:1) and an isomer of gadoleic acid, eicosadienoic acid (20:2), docosanoic acid (22:0),erucic acid (22:1)and an isomer of erucic acid, docosadienoic acid (22:2), lignoceric acid (24:0) and nervonic acid (24:1) after 41 days (A12) and 56 days (Sl101) of ageing at 40°C and 75% RH and either ambient oxygen (18%) or high oxygen (78%)."0" shows non-aged seeds (controls). Data show means ± SD (n=4).

	0 d Sl101	0 d A12	41 d A12 Ambient O_2	41 d A12 High O_2	56 d Sl101 Ambient O_2	56 d Sl101 High O_2
C16:0	1 ± 0.1	0.95 ± 0.02	0.98 ± 0.01	1.01 ± 0.06	0.97 ± 0.03	0.98 ± 0.05
C16:1	1 ± 0.1	0.79 ± 0.07	0.80 ± 0.08	0.88 ± 0.08	1.03 ±0.13	1.03 ± 0.04
C16:2	1 ± 0.17	0.96 ± 0.15	1.08 ± 0.04	0.98 ± 0.14	1.02 ± 0.11	1.07 ± 0.13
C18:0	1 ± 0.06	1.01 ± 0.05	0.98 ± 0.02	1.05 ± 0.05	1.02 ± 0.05	1.06 ± 0.07
C18:1	1 ± 0.12	1.07 ± 0.07	1.04 ± 0.03	1.06 ± 0.04	0.99 ± 0.02	1.02 ± 0.08
C18:1 iso	1 ± 0.03	0.90 ± 0.03	0.90 ± 0.03	0.91 ± 0.03	0.97 ± 0.05	1.02 ± 0.04
C18:2	1 ± 0.07	0.88 ± 0.03	0.91 ±0.03	0.92 ± 0.06	0.99 ± 0.03	0.97 ± 0.03
C18:3	1 ± 0.09	0.89 ± 0.03	0.96 ± 0.03	0.92 ± 0.05	0.90 ± 0.02	0.87 ± 0.03
C20:0	1 ± 0.09	0.98 ± 0.05	0.99 ± 0.05	1.04 ± 0.04	1.03± 0.06	1.08 ± 0.09
C20:1	1 ± 0.12	1.05 ± 0.07	1.04 ± 0.06	1.03 ± 0.04	0.97 ± 0.01	0.95 ± 0.08
C20:1 iso	1 ± 0.04	0.94 ± 0.06	0.96 ± 0.07	0.91 ± 0.07	1.00 ± 0.08	0.93 ± 0.04
C20:2	1 ± 0.12	0.82 ± 0.02	0.91 ± 0.08	0.93 ± 0.06	0.95 ± 0.04	0.94 ± 0.08
C22:0	1 ± 0.08	0.89 ± 0.03	0.89 ± 0.05	0.96 ± 0.06	1.06 ± 0.04	1.06 ± 0.07
C22:1	1 ± 0.09	0.97 ± 0.03	0.96 ± 0.04	0.98 ± 0.02	1.01 ± 0.05	0.96 ± 0.06
C22:1 iso	1 ±0.04	0.79 ± 0.08	0.82 ± 0.11	0.83 ± 0.09	1.03 ± 0.06	0.90 ± 0.11
C22:2	1 ± 0.09	0.77 ± 0.05	0.81 ± 0.07	0.78 ± 0.04	0.96 ± 0.09	0.86 ± 0.13
C24:0	1 ± 0.04	0.94 ± 0.05	0.97 ± 0.05	1.00 ± 0.05	1.08 ± 0.07	1.06 ± 0.11
C24:1	1 ± 0.12	0.97 ± 0.11	1.04 ± 0.06	0.97 ±0.03	0.98 ± 0.02	0.98 ± 0.11