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Comparative analysis of wild-type accessions reveals novel determinants of Arabidopsis seed longevity

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Abstract

Understanding the genetic factors involved in seed longevity is of paramount importance in agricultural and ecological contexts. The polygenic nature of this trait suggests that many of them remain undiscovered. Here, we exploited the contrasting seed longevity found amongst *Arabidopsis thaliana* accessions to further understand this phenomenon. Concentrations of glutathione were higher in longer-lived than shorter-lived accessions, supporting that redox poise plays a prominent role in seed longevity. However, high seed permeability, normally associated with shorter longevity, is also present in long-lived accessions. Dry seed transcriptome analysis indicated that the contribution to longevity of stored messenger RNA (mRNAs) is complex, including mainly accession-specific mechanisms. The detrimental effect on longevity caused by other factors may be counterbalanced by higher levels of specific mRNAs stored in dry seeds, for instance those of heat-shock proteins. Indeed, loss-of-function mutant analysis demonstrated that heat-shock factors HSF1A and 1B contributed to longevity. Furthermore, mutants of the stress-granule zinc-finger protein TZF9 or the spliceosome subunits MOS4 or MAC3A/MAC3B, extended seed longevity, positioning RNA as a novel player in the regulation of seed viability. mRNAs of proteins with putative relevance to longevity were also abundant in shorter-lived accessions, reinforcing the idea that resistance to ageing is determined by multiple factors.

KEYWORDS

arabidopsis, glutathione; longevity; natural variation; seed; transcriptomics

1 | INTRODUCTION

Understanding why some seeds survive for years or centuries while others die within months is a paramount question in seed science. Further to genetics, environmental cues perceived by the mother plant during seed maturation (He et al., 2014; Nagel et al., 2015), harvesting or postharvesting practices (Probert et al., 2007; Wang et al., 2018), and storage conditions (Walters et al., 2004), all influence seed life-span, or 'longevity'. Regarding the genetic basis, QTL and genome-wide association studies suggest involvement of

many loci, underlining the highly polygenic nature of seed longevity (Agacka-Modoch et al., 2015; Nagel et al., 2015; Nguyen et al., 2012; Renard et al., 2020b; Zuo et al., 2019).

The desiccated state of orthodox seeds restricts metabolism (Buitink & Leprince, 2008), which extends their longevity, well beyond what is capable for desiccation intolerant 'recalcitrant' seeds (Angelovici et al., 2010). Nonetheless, a loss of redox homeostasis, presumably due to excess reactive oxygen species (ROS) production, coincides with viability loss in both recalcitrant and orthodox seeds (Kranner et al., 2006; Roach et al., 2010). Life in a metabolically-inert state entails accumulation of oxidative damage, eventually resulting in cell death. A plethora of metabolites, including antioxidants like glutathione and tocochromanols (Kranner et al., 2006; Nagel et al., 2015; Sattler et al., 2004), and various seed storage (Nguyen et al., 2015) or heat-shock proteins (Bissoli et al., 2012; Tejedor-Cano et al., 2010), help reduce damage while in the desiccated state (Bailly, 2004; Kranner & Birtic, 2005; Sano et al., 2016). Glutathione has a dominant role in maintaining the cellular environment in a reduced redox state, necessary for protection from oxidative damage. Seed ageing is associated with a decrease in GSH and increase in GSSG concentration, in other words, seeds oxidise during ageing as they lose viability. Based on molar concentrations of GSH and GSSG, seed cellular redox state can be quantified *via* the Nernst equation ($E_{GSSG/2GSH}$) (Kranner et al., 2006). The maternal-derived seed coat is a partial barrier to oxygen, minimising oxidation of cellular components (Renard et al., 2021). Mutants affected in seed coat development (Leon-Kloosterziel et al., 1994), or in the composition of the integument layers, have high seed coat permeability and low viability, suggesting that both factors are inversely correlated (Debeaujon et al., 2000; Demonsais et al., 2020; Renard et al., 2020b).

During germination, pre-existing messenger RNAs (mRNAs), stored in mature dry seeds, are used for protein synthesis. More than 12 000 different types of mRNAs are stored in the dry seed of *Arabidopsis thaliana*. They include mRNAs needed for the repair of cellular damage and for initiation of the germination process (Nakabayashi et al., 2005). Dry seed transcriptomes of the Col-0 and Cvi accessions of Arabidopsis resemble one another, but a fraction of stored mRNAs is accession-specific (Kimura & Nambara, 2010). It is plausible that the nature and abundance of this unique pattern of stored mRNAs between accessions could explain the diversity observed in seed properties. In this regard, Col-0 and Est-1 accessions, which greatly vary in longevity, also differ in a substantial fraction of their dry seed transcriptomes (Sano et al., 2017). Furthermore, proteomes of wheat genotypes with contrasting seed longevities revealed differences in abundance of one-sixth of all detected proteins (Chen et al., 2018). If there are specific proteins within this list contributing to longevity is unknown.

Loss-of-function mutant studies have revealed many cellular components involved in seed longevity in Arabidopsis (Bueso et al., 2014, 2016). Co-expression network analysis identified a list of novel genes and processes likely affecting viability (Righetti et al., 2015); recently, the success of this approach was demonstrated, revealing a novel role for auxin signalling in seed longevity (Pellizzaro et al., 2020). Moreover, large-scale phenotyping coupled with genome-wide association studies identified additional novel genes involved in this trait (Renard et al., 2020a). Comparative analyses of natural accessions with contrasting tolerance is an alternative strategy to elucidate molecular factors involved in stress tolerance (Kusunoki et al., 2017; van Veen et al., 2016). Moreover, natural accessions are an adequate material to investigate how adaptations to different traits are tuned to provide an optimum response for the plant. However, this approach has yet to be applied to seed longevity in a systematic way.

In this study, to investigate attributes of seed longevity from multiple angles, we compared seed coat permeability, dry seed mRNA composition and glutathione contents in wild-type accessions of Arabidopsis with different relative longevity, referred to as 'long-lived' and 'short-lived'. A positive correlation of glutathione contents at seed maturity with seed life span supports that seed oxidation accompanies viability loss, regardless of ageing rate. The importance of heat-shock proteins in resisting ageing was supported in this study, and the transcription factors HSF1A and HSF1B appear to be important regulators in seeds. Moreover, by assaying longevity in T-DNA insertional mutants of genes of the splicing machinery or mRNA packing regulation, we revealed an unexplored role of RNA processing in seed viability, positioning RNA as a key player in the regulation of this trait. However, we also highlight the multifactorial character of seed longevity, and suggest accession-specific processes employed by seeds to resist ageing.

2 | MATERIALS AND METHODS

2.1 | Plant material and germination assays

Arabidopsis thaliana accessions were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (N76309). All Ecotypes were grown simultaneously to obtain fresh seeds in homogeneous conditions (16 h light/8 h dark, at 22°C and 70–75% relative humidity [RH]). For T-DNA insertion lines, the *tzf9* line was kindly donated by Dr. Justin Lee (Maldonado-Bonilla et al., 2014); the *mac3a mac3b* (N69985) and *mos4-1* (N69914) lines were obtained from NASC, and *hsf1a hsf1b* was obtained from Dr. F. Schöffl's laboratory (Lohmann et al., 2004). Seeds were harvested from Arabidopsis plants grown under greenhouse conditions (16 h light/8 h dark, at 23 ± 2°C and 70 ± 5% relative humidity) in pots containing a 1:2 vermiculite:soil mixture.

For germination assays, seeds were surface sterilised with 70% ethanol containing 0.1% Triton X-100 for 15 min followed by rinsing three times with sterile water. Seeds were stratified for 3 days at 4°C. Germination was carried out on plates containing Murashige and Skoog salts with 1% (wt/vol) sucrose, 10 mM 2-(N-morpholino) ethanesulfonic acid and 0.9% (wt/vol) agar, pH was adjusted to 5.7. Percentage of germination was calculated as the mean of three biological replicates (50 seeds per replicate). Germination refers to appearance of open green cotyledons, and was monitored 7 days after sowing.

2.2 | Seed ageing assays

Ambient ageing was performed by storing dry seeds at ambient temperature and humidity (20–25°C, 40–60% RH) for 18 or 30 months, as indicated in each case. The controlled-deterioration treatment (CDT) consisted of maintaining seeds during 14 or 21 days at 37°C or 40°C, as indicated, in an atmosphere of 75% HR above a saturated NaCl solution.

2.3 | Seed coat permeability test

Triphenyltetrazolium reduction assay was used as described in Molina et al. (2008). Briefly, seeds were incubated in the dark in 1% (wt/vol) tetrazolium red at 30°C for 36 h. Then, formazan was extracted and quantified as the absorbance at 485 nm of 1 ml sample. Values shown in figures are means of three biological replicates. Each replicate contains 20 mg of seeds.

2.4 | Glutathione thiol and disulphide measurements

Seeds (15–20 mg/replicate) were freeze-dried for 5 days before grinding with two 3 mm quartz beads at 30 Hz for 2 min at −80°C. A total of 1 ml of ice-cold 0.1 M HCl and polyvinylpolypyrrolidone equal to seed weight was added before extracting at 30 Hz for 2 min. The extract was centrifuged at 20 000*g* for 10 min at 4°C and 700 µl of the supernatant was transferred, avoiding the lipid phase, for re-centrifugation as before. LMW thiols and disulphides were analysed by HPLC modified from (Kranner, 1998). Briefly, for the determination of total LMW thiols and disulphides (e.g., GSH + GSSG), 120 µl of the supernatant was pH-adjusted to 8.0 with 180 µl of 200 mM bicine buffer, reduced with dithiothreitol (DTT) for 1 h, before labelling thiol groups with monobromobimane (mBBr) for 15 min. To quantify disulphides only (e.g., GSSG), thiols (e.g., GSH) in 400 µl of supernatant were blocked for 15 min with N-ethylmaleimide (NEM), which was added just before pH-adjustment with 600 µl bicine buffer. Excess NEM was removed with an equal volume of toluene four times. Disulphides in 300 µl of NEM-treated extract were reduced with DTT and labelled with mBBr, as before. mBBR-labelled compounds were separated by reversed-phase HPLC, using an Agilent 1100 HPLC system (Agilent Technologies), on a ChromBudget 120-5-C18 column (5.0 µm, BISCHOFF GmbH), and detected by a fluorescence detector (ex: 380 nm, em: 480 nm). Amount of each disulphide was calculated by halving the number of thiol equivalents measured (e.g., 1 mol GSSG = 2 mol GSH), and amount of each LMW thiol was calculated by subtracting the amount of LMW thiol equivalents of each disulphide from total amounts of LMW thiol equivalents of both disulphides and thiols, e.g., GSH = (mol GSH + (mol GSSG × 2)) − (mol GSSG × 2).

The half-cell reduction potential (E_{hc}) for each thiol-disulphide couple was calculated according to the Nernst equation (Kranner et al., 2006; Schafer & Buettner, 2001):

$$E_i = E^{0'} - \frac{RT}{nF}\ln\frac{[\text{LMW thiol}]^2}{[\text{LMW disulphide}]}$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}); T, temperature in K; n, number of transferred electrons (=2); F, Faraday constant (9.6485×10^4 C mol^{-1}); $E^{0'}$, standard half-cell reduction potential of a thiol-disulphide redox couple at an assumed cellular pH of 7.3 ($E^{0'}_{GSSG/2GSH}$ = −258 mV). E_i is the half-cell reduction potential of an individual redox couple i, and $[\text{reduced species}]_i$ is the concentration of the reduced species in that redox pair. The molar concentrations of LMW thiols and disulphides were calculated based on seed water content.

2.5 | RNA extraction and qRT-PCRs

Extraction of total RNA from dry seeds was performed according to Oñate-Sánchez and Vicente-Carbajosa (2008). DNA removal was performed with DNAse kit (E.Z.N.A). About 1 µg RNA was reverse-transcribed using the Maxima first-strand cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the PyroTaq EvaGreen qPCR Mix Plus (ROX; Cultek S.L.U.) in a total volume of 20 µl using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific). Data are the mean of three replicates. PCR amplification specificity was confirmed with a heat-dissociation curve (from 60–95°C). The internal standard used was ASAR1 (AT4G02080), specifically selected for its stable expression in seeds (Czechowski et al., 2005). Relative mRNA abundance was calculated using the comparative ΔCt method. Primers for qRT-PCR are listed in Table S1.

2.6 | Transcriptomic assays and RNA data analysis

RNA was extracted from dry seeds as mentioned before. Twenty-million 50 nt reads per library were sequenced. Two or three replicates were used per accession. After adaptor removal and low-quality trimming of raw reads with cutadapt (Martin, 2011), clean reads were quality assessed with FastQC (Andrews, 2010) and mapped to the TAIR10 *Arabidopsis thaliana* genome using HISAT2 (Kim et al., 2015). Gene counts were then obtained with htseq-count (Anders et al., 2015) and used for differential expression analysis with DESeq. 2 (Love et al., 2014). For PCA and clustering analysis, average expression (read-per-kilobase-million reads, RPKM) was used per accession, displaying only genes with standard deviation higher than 500 between accessions. UPGMA was used for cluster analysis with average linkage and Pearson correlation. Upset plots was performed using Intervene (Khan & Mathelier, 2017). Functional analysis was performed using AgriGOv2 (Tian et al., 2017) and redundant gene ontology (GO) terms were removed and remaining GO terms visualised using ReviGO (Supek et al., 2011). For differential splicing analysis, quantification of AtRTD2 transcript isoforms (Zhang et al.,

2017) was done with salmon in mapping-based mode (Patro et al., 2017) and used for alternative splicing analysis with IsoformSwitch-AnalyzeR (Vitting-Seerup & Sandelin, 2019).

3 | RESULTS

3.1 | Arabidopsis accessions differ in seed longevity

For the present study 30 accessions used in Renard et al. (2020a) were selected for further analysis, 14 'long-lived' accessions with higher total germination (TG) and 16 'short-lived' ones with lower TG than Col-0 after 18 months of ambient ageing (Figure 1a). After 30 months, seed longevity was re-assayed in triplicate for 14 of these accessions (8 with TG > and 5 with TG < than Col-0) (Figure 1b, left panel). The same 14 genotypes were also aged much more rapidly using CDT. TG of these 14 genotypes after these contrasting ageing regimes significantly correlated ($R^2 = 0.911$; Pearson $R = 0.955$, Figure 1b, right panel). As all plants were grown under the same controlled environmental conditions during seed development, these results suggest that genetic differences affecting seed traits were responsible for the distinct longevity responses.

3.2 | High seed coat permeability and low longevity does not always correlate

The tetrazolium penetration assay conducted on seed batches showing 100% TG is a standard method to quantify seed coat permeability (Molina et al., 2008). To test if an inverse association of seed coat permeability with seed longevity applies to wild-type accessions, we assayed tetrazolium permeability in 30 accessions with contrasting seed longevity. No correlation was observed (Figure 2b, $r = -0.28$). High permeability ($A_{485} > 0.6$) was associated with shortened longevity, although accessions Bs-2, Da-0, Bsch-0 and TDr-3 are long-lived despite being quite permeable ($A_{485} > 0.5$). On the other hand, although very low permeability ($A_{485} < 0.2$) was

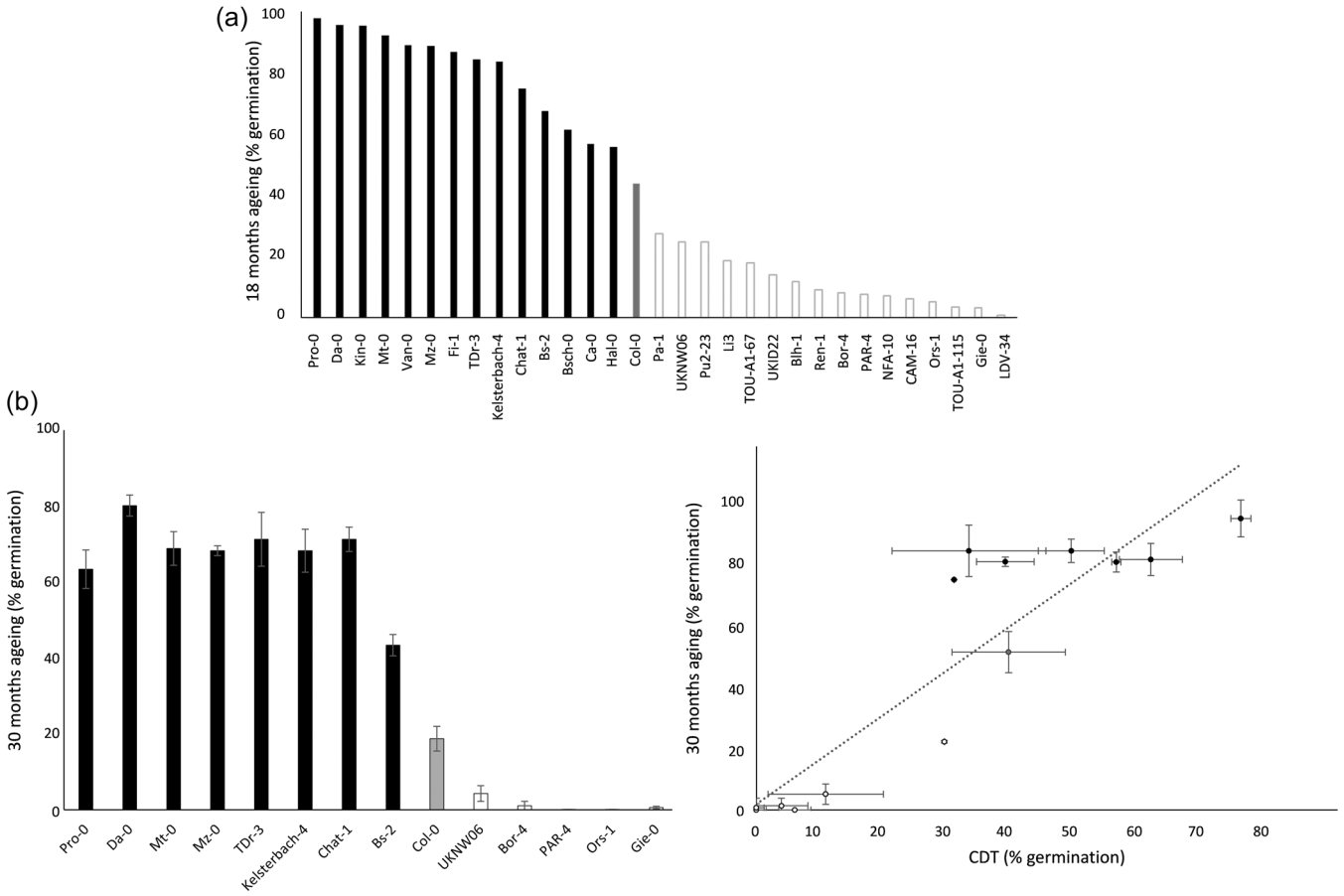

FIGURE 1 Phenotypic response to seed deterioration in *Arabidopsis thaliana* accessions. (a) Percentage of total germination of long-lived (black bars) and short-lived (white bars) accessions after 18 months of ambient ageing according to Renard et al. (2020a). Col-0 has intermediate seed longevity and is included for reference (grey bar). (b) Left panel: Percentage of germination of 8 long-lived and 5 short-lived accessions after 30 months of ambient ageing (grey bars). Data represent the average of three biological replicates. Right panel: Correlation of germination data for 30-month ambient ageing and controlled-deterioration (CDT) for Arabidopsis accessions. Black solid circles: long-lived accessions; open circles: short-lived accessions; grey circle: Col-0

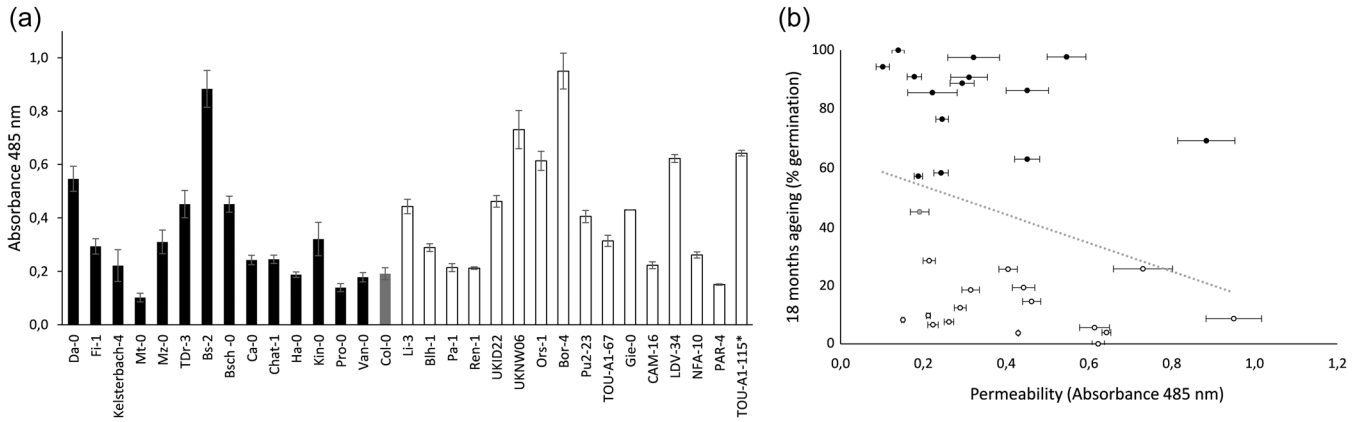

FIGURE 2 (a) Tetrazolium assay in long-lived (black bars) and short-lived (white bars) accessions. Col-0 has an intermediate response for seed longevity (grey bar). (b) Correlation between total germination after 18 months of ambient ageing and permeability data according to the tetrazolium assay in Figure 2a

generally found in long-lived accessions, there were exceptions to the rule, e.g., the short-lived PAR-4 had very low permeability. Moreover, no clear trend was observed in the intermediate range (A_{485} = 0.2–0.5), with accessions of contrasting longevity (Figure 2a). In summary, these results indicate that other mechanisms likely exist that compensate highly permeable seed coats in accessions that are also relatively long-lived. Similarly, other seed properties may be responsible for the short-lived phenotype of accessions with less permeable seed coats.

3.3 | Stored mRNAs composition of long- and short-lived dry seeds suggests accession-specific processes to resist ageing

Next, we conducted a transcriptome analysis to compare stored mRNAs composition in long- and short-lived accessions. In total, six accessions with long-lived seeds (Da-0, Pro-0, TDr-3, Chat-1, Kelsterbach-4 and Fi-1) and six with short-lived seeds (Ors-1, Bor-0, PAR-4, UKNW06-386, CAM-16 and Ren-1), relative to Col-0, were analysed (Table S2). Around 12 000 genes were expressed in dry seeds (considering expression when RPKM [read-per-kilobase-per-million-bases] >1 in at least two replicates per accession). Transcriptomes of different accessions resembled one another, as previously reported (Nakabayashi et al., 2005): around 80% of the genes identified in the seed were expressed in all accessions (see Table 1), and only 737 genes were accession-specific. However, for all accessions, a non-negligible fraction of the dry seed transcriptome (10–28%), depending on the accession, see Table 1, was differentially expressed as compared to Col-0, indicating differences in transcript abundance between them.

To have a first estimation of the transcriptome landscape for long- and short-lived accessions, we compared genes differentially-expressed with Col-0 (false discovery rate adjusted $p < 0.05$ and $\log_2$FoldChange <−1 or >1) between all the accessions, searching for shared and unique differences. Forty-three genes were more expressed than in Col-0 in all the six long-lived accessions, and 40 were less expressed. Extending this search to genes more (or less) expressed than in Col-0 in at least five long-lived accessions increased the numbers to 133 (more expressed than in Col-0) and 127 (less expressed than in Col-0). Only 25 and 9 of those genes, respectively, were not found differential in more than two of the short-lived accessions, and might constitute a group of genes whose higher (or lower) expression in dry seeds is relevant for longevity (Figure 3a,b and Table 2) However, no functional enrichment or annotation related to seed longevity processes was observed for these genes, making at this point speculative to estimate their precise role in this trait. Conversely, most of the differentially-expressed genes in the long-lived accessions were differential in only one accession, or shared by just two accessions, as shown in Figure 3c,d. The same trend is observed for differentially-expressed genes in short-lived accessions (Figure S1). This suggests that transcriptome contributions to longevity should be mediated by different mechanisms in every accession.

To evaluate how differences in transcriptome composition could influence seed longevity in every accession, differentially-expressed genes with Col-0 were analysed for functional enrichment. The top 20 enriched functional categories per accession are listed in Figure 4. These results suggest that different long-lived accessions modulate their biochemical potential to resist seed ageing in different ways. Among the transcripts more expressed in seeds of the long-lived accession than in Col-0, the category 'response to heat' (mainly including heat-shock proteins) was highlighted in Da-0 and TDr-3, whereas the category 'DNA repair' or 'response to DNA damage stimulus' (including genes such as RAD51 or ATM, involved in maintaining genome stability in seeds; Waterworth et al., 2016) were highlighted in Pro-0, and the category 'response to oxidative stress' was highlighted in Fi-1 and TDr-3. For Kelsterbach-4 and Chat-1, no clear longevity-related gene category was observed, making more difficult the interpretation. Considering genes more expressed in

TABLE 1 mRNAs present in dry seeds of wild-type accessions of Arabidopsis, and differentially-expressed genes compared to Col-0

Accession	Expressed genes	% Genes in all accessions	Differentially expressed genes (vs. Col-0)	% Differentially expressed genes (vs. Col-0)
Long-lived				
Pro-0	12756	77.5	3104	24.3
Da-0	12689	77.9	2258	17.8
TDr-3	12230	80.9	1721	14
Kelsterbach-4	11987	82.5	1305	10.8
Chat-1	11942	82.8	1783	14.9
Fi-1	12022	82.3	1578	13.1
Col-0	11994	82.5		
Short-lived				
UNKNOW06	12130	81.5	3122	25.7
Ren-1	11843	83.5	1899	16
Bor-4	11122	88.9	3146	28.2
PAR-4	12574	78.7	2463	19.5
CAM16	11698	84.5	1783	15.2
Ors-1	11064	89.4	2565	23.1
Mean	**12003.9**	**82.5**	**2227.3**	**18.6**

Note: Expressed genes: Number of genes representing at least two replicates with RPKM > 1 (reads per kilobase per million reads); % of genes in all accessions: percentage of expressed genes with transcripts in all accessions (including Col-0); differentially-expressed genes (vs. Col-0): differentially expressed genes with FDR adjusted $p < 0.05$ and log2FoldChange <−1 or >1; % differentially expressed genes (vs. Col-0): percentage of differentially expressed genes versus Col-0 from the total number of genes expressed in each accession.

Abbreviations: FDR, false discovery rate; mRNA, messenger RNA.

seeds of the short-lived accession than in Col-0, it is remarkable that the category 'lipid localisation' was highlighted in the six accessions analysed. This category includes lipid-transfer proteins (LTPs), many of them belonging to the glycosylphosphatidylinositol (GPI)-anchored subfamily (LTPGs), needed for proper development of the suberin layer in the seed coat (Edstam & Edqvist, 2014).

PCA analysis confirms the partition of samples according to longevity, some long-lived accessions mapping to the top-left quadrant of the plot, and most short-lived accessions distributed along the middle- and bottom-right quadrants (Figure 5). This again indicates that differences in abundance of stored mRNAs between these accessions could help to explain their differences in seed longevity. However, many long- and short-lived accessions grouped close to Col-0, reinforcing the idea that there is not a core group of regulated genes that explains the longevity phenotypes for all accessions.

In summary, these results suggest that: (1) there is not a clear core transcriptome in all long-lived accessions that determines longevity, and (2) for some accessions, transcriptome composition correlates well with seed longevity, suggesting that a detailed view of stored mRNAs in those accessions could provide insights into new components involved in this trait.

3.4 | Both short- and long-lived seeds store mRNAs important for seed longevity

The long-lived Da-0 and the short-lived Ors-1 and Bor-4 accessions mapped very apart in the PCA plot, with Col-0 in an intermediate position between them along PC1, indicating that they differ considerably in their transcriptome profiles. Cluster analysis highlighted groups of genes with different expression profiles among those accessions. As shown in Figure 6a, clusters 1 and 2 include genes more expressed in the long-lived Da-0 than in the short-lived Ors-1 and Bor-4 accessions. Functional analysis using GO indicated that these clusters are enriched in genes belonging to five groups of categories (see Figure 6b and Table S3). Group 1 categories are related to RNA processing and translational initiation. Genes presenting this pattern of expression include a substantial number of RNA-binding proteins, including the polyA-binding protein PAB2, helicases and splicing factors such as PRP8, SMP2, RBM25, or components of the MOS4-associated complex such as MAC3B, as well as translational initiation factors, such as SUI1, IF2/IF5 and several subunits of eIF4 and eIF3. Group 2 includes categories involved in gene regulation, including 'regulation of seed germination', or 'regulation of circadian rhythm', with some genes important

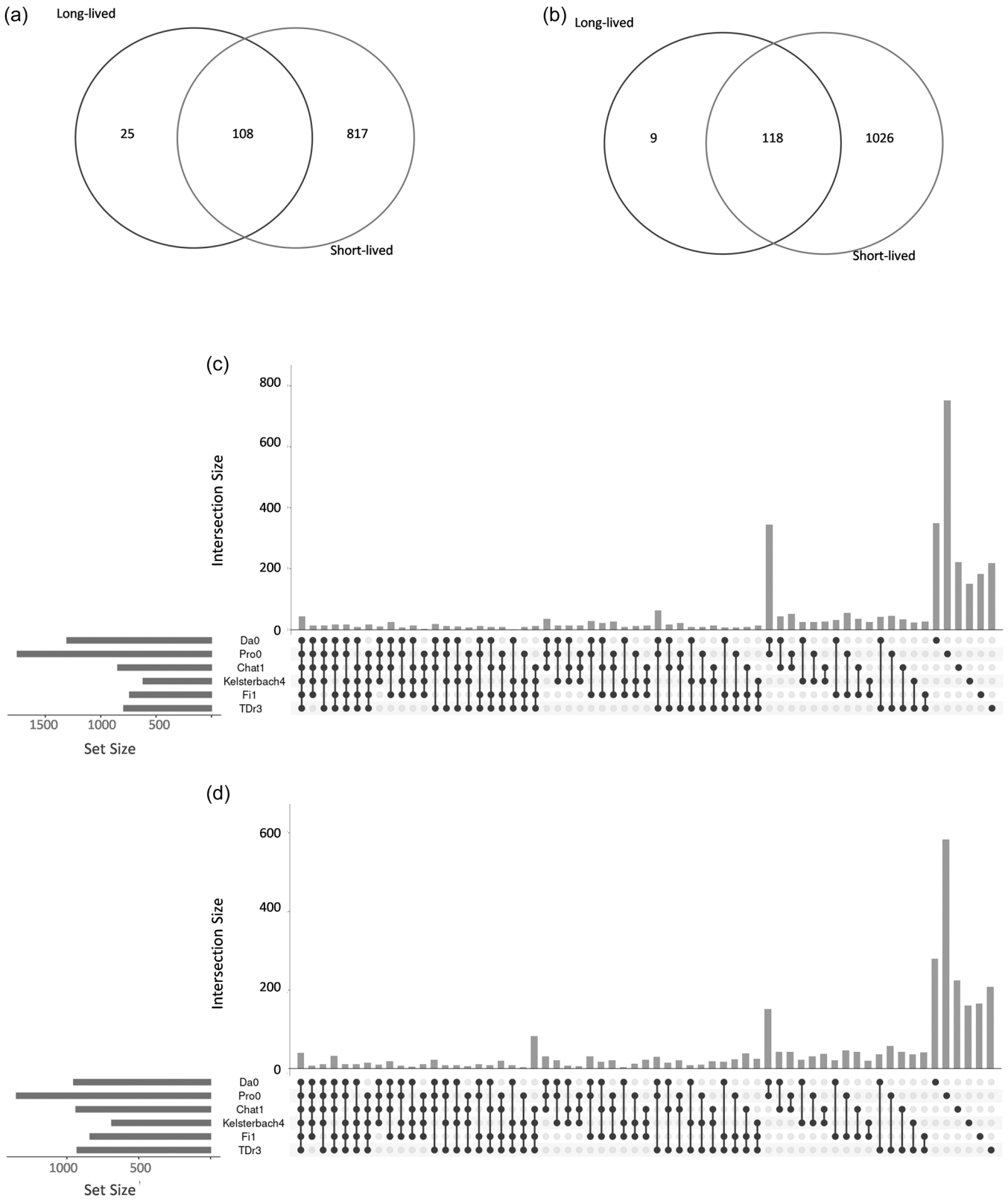

FIGURE 3 (a, b) Venn diagrams showing unique differentially-expressed genes in dry seeds of long-lived accessions (compared to Col-0). (a) Intersection of transcripts more expressed than in Col-0 in at least five of the six long-lived accessions (long-lived), with transcripts more expressed than in Col-0 in at least three of the six short-lived accessions (short-lived). (b) Intersection of transcripts less expressed than in Col-0 in at least five of the six long-lived accessions (long-lived), with transcripts less expressed than in Col-0 in at least three of the six short-lived accessions (short-lived). (c, d) Upset plots showing overlapping number of differentially-expressed genes (compared to Col-0) in each of the six long-lived accessions. (c) transcripts more expressed in long-lived accessions, (d) transcripts less expressed in long-lived accessions. Set size: number of differentially-expressed genes (vs. Col-0) in every accession. Intersection size: number of genes intersected in accessions highlighted (solid circles) in x-axis. Upset plot were generated using the Intervene software (Khan & Mathelier, 2017)

TABLE 2 (a) Transcripts more expressed in long-lived accessions than in Col-0. Differentially-expressed genes (more expressed than in Col-0) in at least five out of the six long-lived accessions, and not differentially-expressed (more expressed than in Col-0) in at least four out of the six short-lived accessions. (b) mRNAs less expressed in long-lived accessions than in Col-0. Differentially-expressed genes (less expressed than in Col-0) in at least five out of the six long-lived accessions, and not differentially-expressed (less expressed than in Col-0) in at least four out of the six short-lived accessions

(a) Transcripts more expressed in seeds of long-lived accessions than in Col-0		(b) Transcripts less expressed in seeds of long-lived accessions than in Col-0	
ID	**Annotation**	**ID**	**Annotation**
AT1G72800	RNA-binding family protein	**AT1G76130**	alpha-amylase-like 2
AT1G10200	GATA type zinc finger transcription factor family protein	**AT3G51420**	strictosidine synthase-like 4
AT1G11210	Protein of unknown function (DUF761)	**AT1G64950**	cytochrome P450, family 89, subfamily A, polypeptide 5
AT1G19565	no_annotation_available	**AT2G25580**	Tetratricopeptide repeat (TPR)-like superfamily protein
AT1G47813	unknown protein	**AT1G31600**	RNA-binding family protein
AT1G49720	abscisic acid responsive element-binding factor 1	**AT2G43570**	chitinase, putative
AT1G58350	Putative serine esterase family protein	**AT1G14930**	Polyketide cyclase/dehydrase
AT1G63210	Transcription elongation factor Spt6	**AT4G18280**	glycine-rich cell wall protein-related
AT1G64900	cytochrome P450, family 89, subfamily A, polypeptide 2	**AT1G24530**	Transducin/WD40 repeat-like superfamily protein
AT1G75730	unknown protein		
AT2G35140	DCD (Development and Cell Death) domain protein		
AT2G35810	unknown protein		
AT2G46070	mitogen-activated protein kinase 12		
AT3G10820	Transcription elongation factor (TFIIS) family protein		
AT3G11600	unknown protein		
AT3G42658	transposable element gene		
AT3G43430	RING/U-box superfamily protein		
AT3G47420	phosphate starvation-induced gene 3		
AT4G10000	Thioredoxin family protein		
AT5G10720	histidine kinase 5		
AT5G20220	zinc knuckle (CCHC-type) family protein		
AT5G24630	double-stranded DNA binding		
AT5G27200	acyl carrier protein 5		
ATCG00380	chloroplast ribosomal protein S4		
ATMG00910	hypothetical protein		

for seed development or longevity, such as the negative regulator of seed dormancy ERF12, the DREB2A-interactor RCD1, the ethylene response factor ERF72, the tocopherol biosynthesis gene VTE1, the SNRK2.3 kinase, and circadian rhythm proteins such as TIME FOR COFFEE or REVEILLE 4. Group 3 includes the category 'protein targeting to chloroplasts', including the import proteins TIC40, TOC159, TOC132 or ALBINO3, important for chloroplast biogenesis. Group 4 includes categories related to seed development, including proteins involved in meristem initiation such as OBE1, OBE2 and TPR1. A higher amount of these transcripts could guarantee a better performance during germination of aged seeds, resulting in enhanced seed longevity. Finally, group 5 includes categories such as 'response to oxidative stress' or 'response to heat' as clearly enriched. Proteins in those categories include 22 heat-shock proteins or chaperone-like proteins (see Table 3), belonging mainly to the HSP20 subfamily, but also containing some HSP70, HSP90 and HSP100 members. HSPs are under the control of heat-shock factors (HSF). Only one of them, HSF1E, was found differentially expressed in dry seeds of these

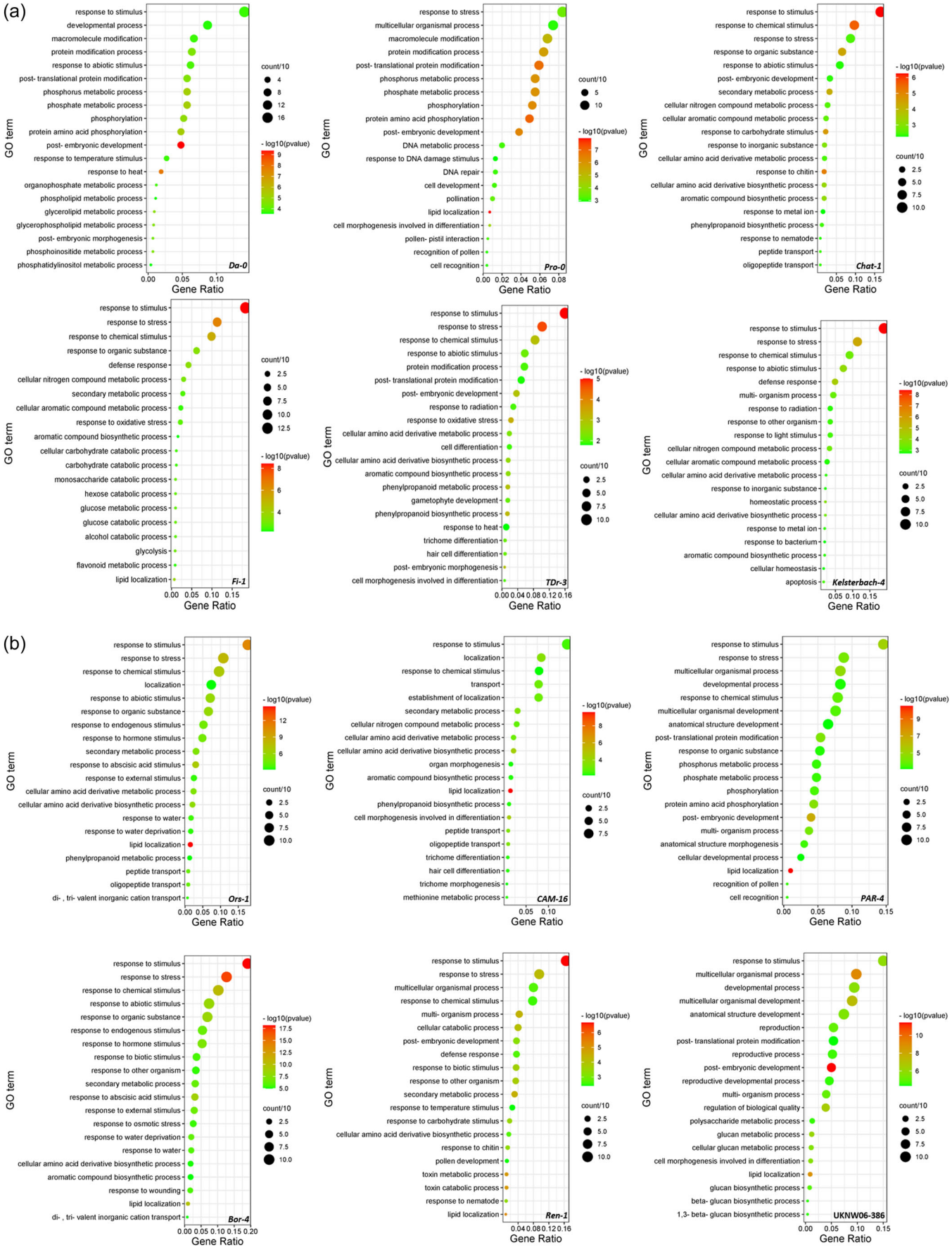

FIGURE 4 (See caption on next page)

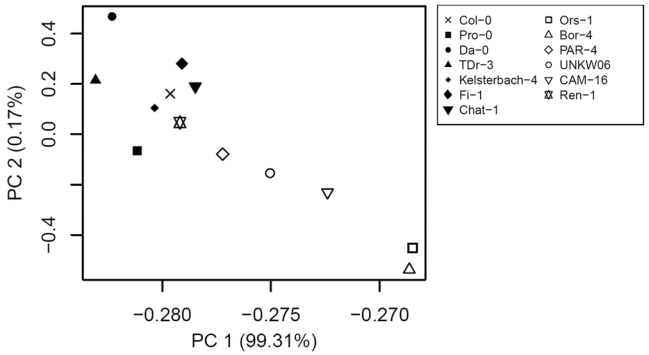

FIGURE 5 Principal component analysis of transcriptome data of long-lived (black symbols) and short-lived (white-symbols) accessions. Col-0. with intermediate seed longevity. is included for reference (grey). Average of read counts was used for every accession. Most variable genes (those showing a standard deviation of count data in all accessions higher than 500) were included in the calculation

accessions differing in longevity (Table 3). However, neither HSFA9, whose overexpression increases seed longevity (Prieto-Dapena et al., 2006) nor HSF1A/HSF1B, expressed during seed development, were found differentially-expressed. In the case of the dry seed-specific AT4G27670 (encoding a small chloroplast HSP) and AT4G10250 (encoding an endomembrane-localised HSP20), the abundance in the long-lived accession Da-0 as compared to the short-lived Ors-1 is 84-fold and 114-fold, respectively. This category also includes the HSP-regulator DREB2A transcription factor, as well as the catalase CAT2. No clear evidence of known hormone regulators of seed longevity, such as auxins or gibberellins, was found in our transcriptional data set. As these hormones participate in the acquisition of seed longevity during seed development, this is not completely unexpected in data from dry seeds. We could neither find big differences in the expression of DNA-damage related genes between these accessions, although their relevance to seed longevity is demonstrated (Waterworth et al., 2019; as a review), but the category was highlighted when comparing the long-lived Pro-0 accession against Col-0.

Conversely, clusters 3–6 (Figure 6c) included genes that were more expressed in the short-lived accessions Ors-1, Bor-4 or both. Among the categories enriched in these clusters of genes, four groups could be distinguished (Figure 6d and Table S3). Those categories include some genes associated with increased seed longevity. Group 1 includes categories related to the degradation of chloroplasts, with genes such as NYC1 or NYE1. Groups 2 and 3 refer to categories related to 'seed development' and 'lipid storage', including genes coding for lipid transfer proteins LTGPs and oleosins. Finally, in Group 4, the category 'response to abscisic acid' marked higher abundance of genes induced by this hormone, including the transcription factor ABI5 and 11 late-embryogenesis-abundant proteins. Overall, this new analysis again indicates that seeds accumulate different transcripts coding for proteins previously associated with increased longevity, although this accumulation does not prevent rapid deterioration in short-lived seeds.

Clustering analysis including additional accessions confirms this observation (Figures S2 and S4). Despite being short-lived, UKNW06 accumulates transcripts more expressed in the long-lived accessions, and the transcripts accumulated in Bor-4 and Ors-1 were not shared by the other short-lived accessions, suggesting that different short-lived seeds express their own sets of genes. These results reinforce the idea that both long-, but also short-lived accessions accumulate different sets of transcripts, that, by themselves, contribute to enhance longevity.

3.5 | Alternative splicing contributes to transcriptome diversity in dry seeds, but no clear feature is associated with seed longevity in overall

Alternative mRNA splicing (AS) is a mechanism for expanding proteomic diversity and regulating gene expression, but the contribution of AS events to the landscape of dry seed transcriptomes has not been studied in detail. We performed a systematic analysis of differential AS events using IsoformSwitchAnalyzeR, a recently developed software that enables identification and analysis of alternative splicing and isoform switches with predicted functional consequences (Vitting-Seerup & Sandelin, 2019). The results showed a non-negligible fraction of differential AS events in the dry seed transcriptome between accessions (Figure 7), adding a new level of diversity beyond the expression analysis based on gene levels, and indicating that the differences in stored mRNAs between wild-type accessions could have been underestimated. We next predicted the functional consequences of these AS events in every accession compared with Col-0. As observed in Figure S3, many genes present important differences with Col-0, including ORF or domain gains, or intron retention differences, reinforcing the idea of a distinct population of alternative-spliced mRNAs among accessions. However, a clear feature associated to long-lived accessions could not be found.

3.6 | Mutant analysis identified novel genes involved in seed longevity

To study in more detail the involvement of proteins affecting mRNA regulation in seed longevity, loss-of-function Arabidopsis lines in

FIGURE 4 Scatter plots of enriched Gene ontology terms statistics. Gene ratio is the ratio of the differentially expressed gene number (DEGs) to the total gene number in a certain pathway. The colour and size of the dots represent the range of the *p* value and the number of DEGs/10 mapped to the indicated pathways, respectively. Top 20 enriched terms are shown in the figure. Plots for the long-lived accessions (upper panel) and (short-lived) accessions are shown

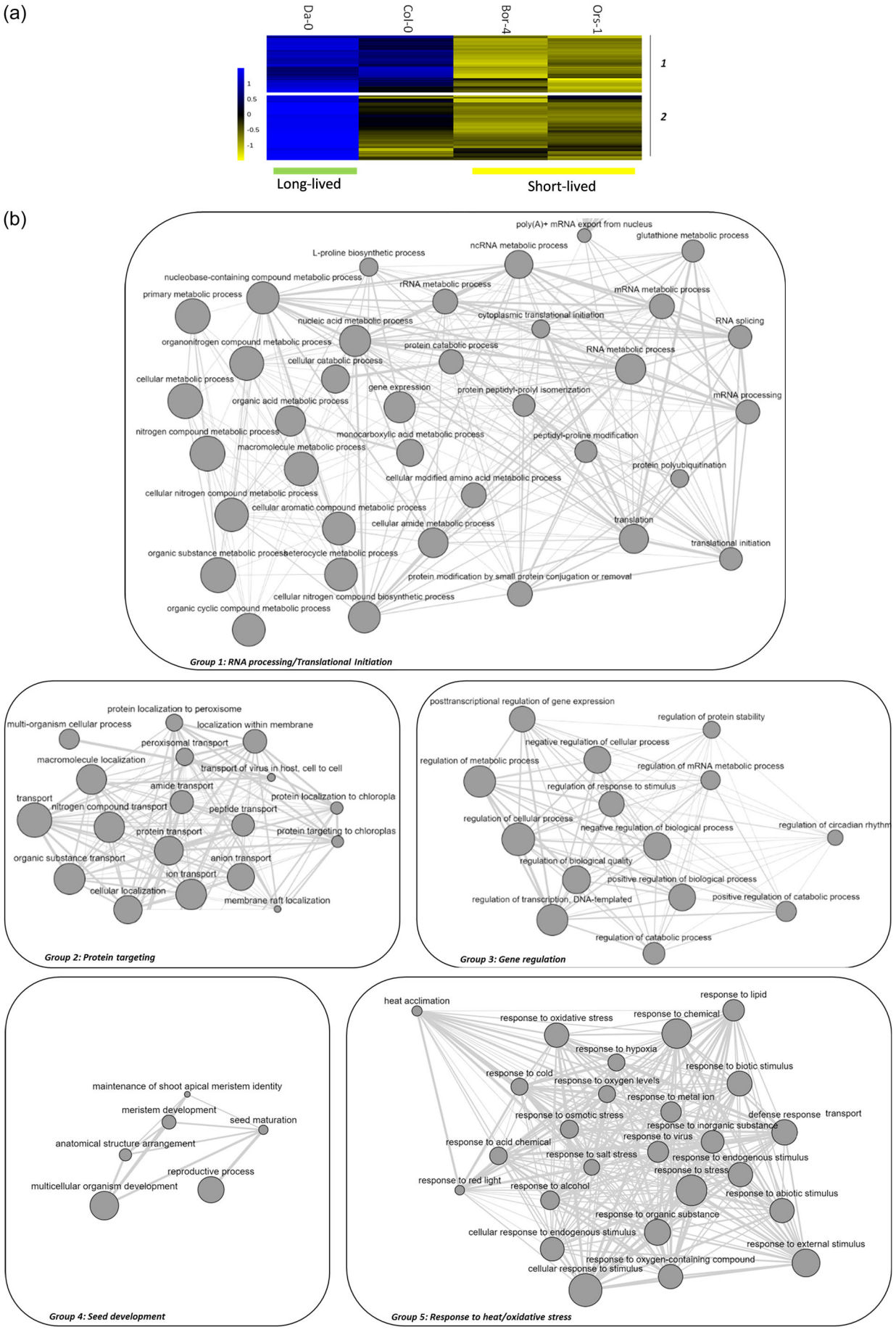

FIGURE 6 (See caption on next page)

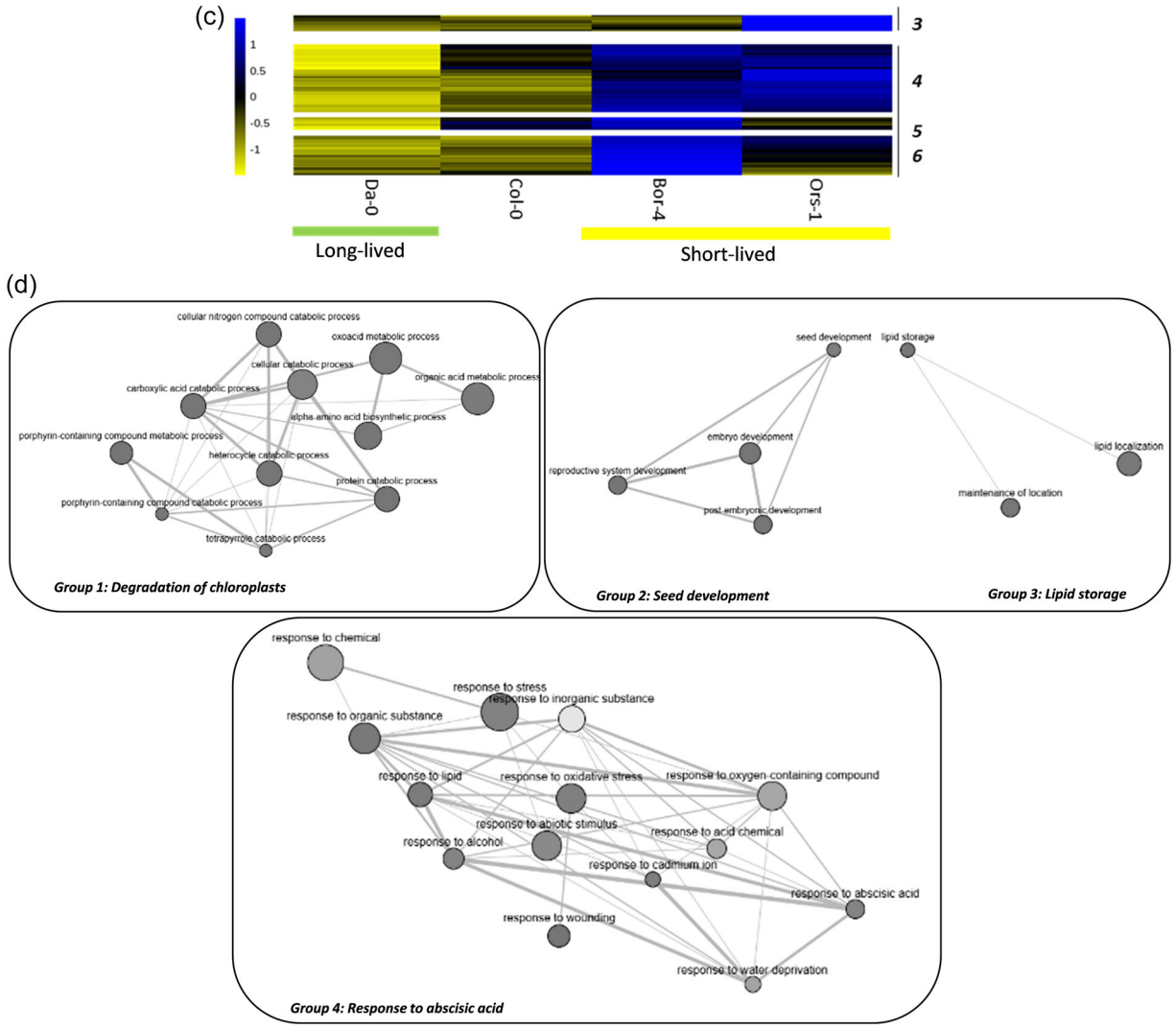

FIGURE 6 Unsupervised UPGMA clustering analysis (average linkage) overexpression data of the long-lived accession Da-0 versus the short-lived accessions Ors-1 and Bor-4. Col-0, with intermediate seed longevity, is included for reference. (a) Heatmap showing clusters (1 and 2) containing genes more expressed in Da-0 or in Da-0 and Col-0. (b) Gene ontology (GO) analysis (biological process) of the genes belonging to clusters 1 and 2 in Figure 4a. GO analysis was performed using AgriGOv2 (Tian et al., 2017). Redundant GO terms were removed and remaining terms were visualised in the network graph according to ReviGO (Supek et al., 2011). (c) Heatmap showing clusters (3–6) containing genes more expressed in Ors-1, Bor-4 or both than in Da-0 and Col-0. (d) GO analysis (biological process) of the genes belonging to clusters 3–6 in Figure 4c. GO analysis was performed using AgriGOv2 (Tian et al., 2017). Redundant GO terms were removed and remaining terms were visualised in the network graph according to ReviGO (Supek et al., 2011)

selected genes were assayed for seed longevity phenotype. Tandem zinc finger protein 9 (TZF9) is an RNA-binding protein involved in the assembly of RNA granules, which harbour transcripts excluded from the translationally active pool (Chantarachot & Bailey-Serres, 2018; Standart & Weil, 2018) and influences the number of mRNAs associated with ribosomes. TZF9 interacts with poly(A)-binding protein 2 (PAB2), a hallmark constituent of RNA granules (Tabassum et al., 2020). We found PAB2 expression 1.6-fold higher in the long-lived Da-0 (6460 RPKMs) than in the short-lived Ors-1 (3950 RPKMs) or Bor-4 (4135 RPKMs) (Table S4). Given the redundancy of PAB proteins in Arabidopsis, and the lethality of the multiple mutants (Zhao et al., 2019), we explore the putative involvement of RNA granules in seed longevity using the surrogate PAB2-interacting protein TZF9. Seeds of *tzf9* resisted the CDT (40°C, 75% RH) better than wild-type seeds. After 21 days of treatment, 22% of *tzf9* seeds, but only 2.6% of wild-type seeds, were able to germinate (Figure 8a). This highlights the relevance of mRNA-packing regulation in seed longevity. Similarly, expression of the MAC3B gene was 2.6-fold and 2.2-fold higher in Da-0 than in Ors-1 and Bor-4, respectively. MAC3B is redundant to MAC3A, and both are subunits of the

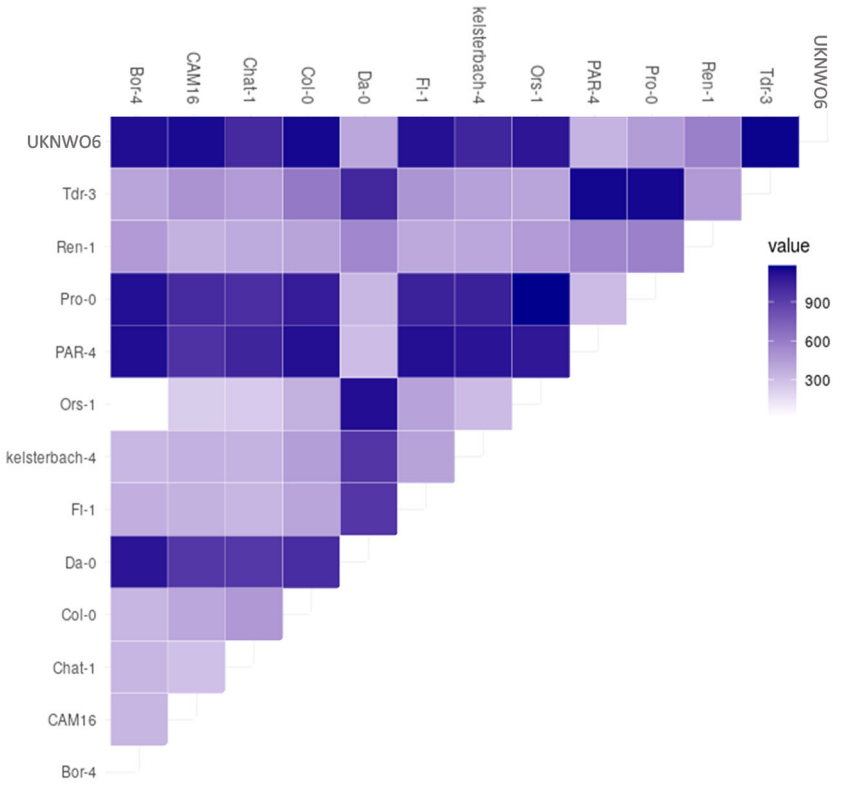

FIGURE 7 Alternative splicing analysis of the transcriptomes of dry seeds of wild-type accessions. Heatmap showing the number of alternative splicing events between accessions according to IsoformSwitchAnalyzeR [Color figure can be viewed at wileyonlinelibrary.com]

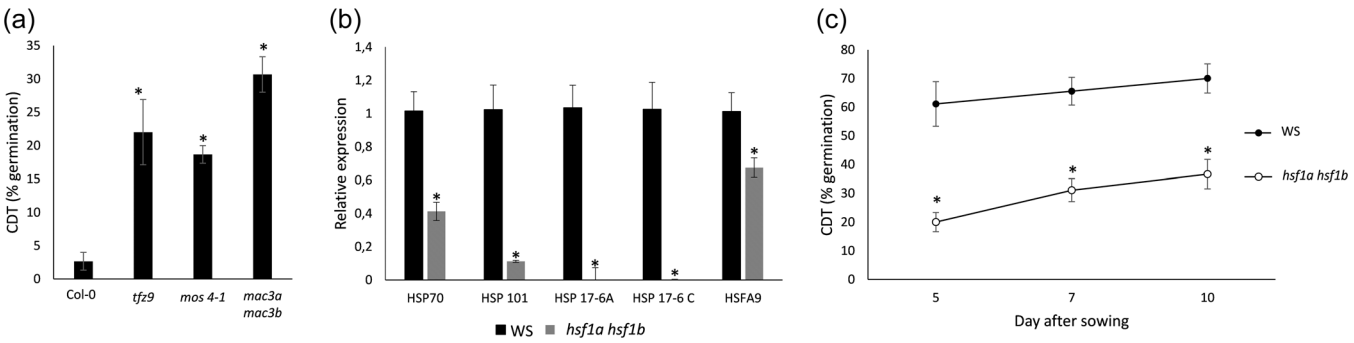

FIGURE 8 Controlled-deterioration treatment on RNA regulation loss-of-function mutant lines. HSPs expression levels and controlled-deterioration treatment of seeds of the *hsf1a hsf1b* mutant line. (a) Percentage of germination of wild type and *tzf*, *mac3a mac3b* and *mos4-1* seeds aged for 21 days at 75% R.H and 40°C. *Significantly differing from control (Col-0) at $p < 0.05$ (Student's *t* test). (b) Real-time quantitative PCR of HSP70. HSP101. HSP17-6.A and HSP 17-6.C from seeds of the *hsf1a hsf1b* Arabidopsis mutant line. Data are the mean of three replicates. Expression for every HSP in wild type is set to 1 in the y-axis and relative expression in *hsf1a hsf1b* is shown. (c) Percentage of germination at 5, 7 and 10 days after sowing of wild-type and *hsf1a hsf1b* seeds aged for 14 days at 75% R.H and 40°C. *Significantly differing from control (WS) at $p < 0.05$ (Student's *t* test)

MOS4-associated complex (MAC), involved in pre-mRNA splicing (Jia et al., 2017). This difference in expression in MAC3B prompt us to investigate whether the impairment of this complex could affect seed longevity. To avoid redundancy, we use double mutant *mac3a mac3b* seeds. Whereas 30% of *mac3a mac3b* seeds were able to germinate after 21 days of CDT, only 2.6% of wild types did (Figure 8a). The involvement of this complex in seed longevity was confirmed using a mutant in MOS4, another subunit of the same complex. Similarly, *mos4-1* was able to resist deterioration better than wild-type seeds, with 18.6% of seeds germinating versus only 2.6% of wild type (Figure 8a). These results uncover an unexpected new role for RNA splicing in shortening seed longevity.

Stored mRNAs of many HSPs particularly accumulated in dry seeds of the long-lived accessions Da-0, Pro-0 and TDr-3 (Table 3 and Table S4). RT-PCR data for the highly permeable accession Bs-2 showed that at least HSP70 and HSP17.6A are also more expressed in dry seeds of this accession than in the short-lived Ors-1 (Figure S4), indicating that this could be a shared mechanism

TABLE 3 Ratio of Da-0 RPKMs (reads per kilobase per million reads) to Bor-4 RPKMs (left) or to Ors-1 RPKMs (right) for the heat shock-related proteins in clusters I and II

		RPKMs ratio	
ID	Annotation	Da-0/ Bor-4	Da-0/ Ors-1
AT4G27670	heat shock protein 21	68.9	84.9
AT4G10250	HSP20-like chaperones superfamily protein	38.9	114.4
AT5G12020	17.6 kDa class II heat shock protein	19.0	19.5
AT1G53540	heat shock protein 17.6C	18.9	6.0
AT2G29500	HSP20-like chaperones superfamily protein	17.4	8.1
AT4G25200	mitochondrion-localized small heat shock protein 23.6	13.5	15.6
AT5G52640	heat shock protein 90.1	6.3	6.6
AT1G52560	HSP20-like chaperones superfamily protein	6.3	7.7
AT4G12400	stress-inducible protein. putative	5.3	5.6
AT5G48570	FKBP-type peptidyl-prolyl cis-trans isomerase family protein	4.2	8.9
AT1G65280	DNAJ heat shock N-terminal domain-containing protein	4.0	3.7
AT5G56030	heat shock protein 81-2	3.9	2.5
AT1G74310	heat shock protein 101	3.1	4.6
AT1G79920	Heat shock protein 70 (Hsp 70) family protein	3.1	2.6
AT3G02990	heat shock transcription factor A1E	2.9	2.1
AT5G02500	heat shock cognate protein 70-1	2.9	1.9
AT1G54050	HSP20-like chaperones superfamily protein	2.6	3.6
AT1G16030	heat shock protein 70B	2.5	2.0
AT2G20560	DNAJ heat shock family protein	2.2	2.8
AT4G24280	chloroplast heat shock protein 70-1	2.2	2.3
AT3G44110	DNAJ homologue 3	1.9	2.0
AT5G12030	heat shock protein 17.6A	1.7	1.7
AT3G12580	heat shock protein 70	1.3	1.5

Note: In grey, genes known to be under the control of HSF1A/HSF1B (Busch et al., 2005 ; Lohmann et al., 2004).

supporting longevity in highly-permeable seeds. As indicated, only HSF1E, but not HSFA9, involved in seed longevity, or HSF1A/HSF1B, expressed during seed development, were found differentially-expressed in dry seeds of these accessions differing in longevity (Table 3), likely to regulate later expression of HSPs. In fact, some of these differentially-expressed HSPs of Table 2 are under the control of HSF1A/HSF1B (Busch et al., 2005; Lohmann et al., 2004), two synergistic transcription factors which accumulate during seed maturation in Arabidopsis (Kotak et al., 2007). Impairment of the HSFA9 expression programme in transgenic tobacco leads to reduced seed longevity (Prieto-Dapena et al., 2006), but the involvement of HSF1A/HSF1B, and the possible redundancy with other HSFs is unknown. We found that HSP101, HSP70, HSP17.6.A and HSP17.6.C expression in dry seeds was reduced (for HSP70), or practically abolished (for HSP101, HSP17.6.A and HSP17.6.C) in dry seeds of *hsf1a hsf1b*, (Figure 8b). These results indicate an absence of redundancy between HSFs for the expression of these particular HSPs during seed maturation that are dependent on HSF1A/HSF1B for expression. Moreover, expression of HSF9A was reduced in *hsf1a hsf1b*, indicating that these two transcription factors partially control the HSFA9 expression programme. Ageing assays on *hsf1a/hsf1b* seeds revealed a clear sensitivity in the mutant: After 14 days of CDT, *hsf1a hsf1b* dropped to 37%, whereas 70% of wild-type seeds germinated (Figure 8c), confirming the importance of HSF1A/HSF1B on HSP provision in dry seeds to counteract seed deterioration.

3.7 | Glutathione concentrations in dry seeds correlated with longevity

Glutathione is considered a key antioxidant in seeds, and the GSSG/GSH redox couple has been correlated with seed viability. To investigate the role of this antioxidant in more detail, glutathione concentrations were measured. The accessions investigated here, selected for differences in seed longevity, had clear differences in GSH concentrations and $E_{GSSG/2GSH}$ values before ageing, and these differences correlated with longevity. For example, long-lived accessions (with >40% TG after 30 months ambient ageing or >25% after the CDT (Figure 1) had >0.3 GSH µmol g^{-1} DW (Figure 9a) and $E_{GSSG}/_{2GSH}$ values more negative than −188 mV (Figure 9b), whereas shorter-lived accessions (with <5% TG after 30 months ambient ageing or <10% after controlled deterioration) had <0.3 GSH µmol g^{-1} DW and $E_{GSSG/2GSH}$ values more positive than −188 mV. Correlating TG after ambient storage with either pre-storage GSH concentrations, or $E_{GSSG/2GSH}$ values, in all accessions, resulted in Pearson R correlations of 0.79 and −0.78 (Figure 9c,d), respectively, and a trend could also be found when considering TG after CDT ($R = 0.64$ and −0.80, respectively) (Figure S5a,b). In summary, across a range of Arabidopsis wild-type accessions, concentrations of GSH and $E_{GSSG/2GSH}$ values before ageing provided a predictive indicator of seed longevity.

4 | DISCUSSION

Natural conditions that slow down the process of seed ageing, such as deeper soil or cold environments, generate less selective pressure on resistance to ageing than those that accelerate this process, such as shallow burial and warmer climates (Mondoni et al., 2011; Pierre et al., 2015; Saatkamp et al., 2011). Thus, variations in seed longevity of widely dispersed species, such as *Arabidopsis thaliana*, may reflect

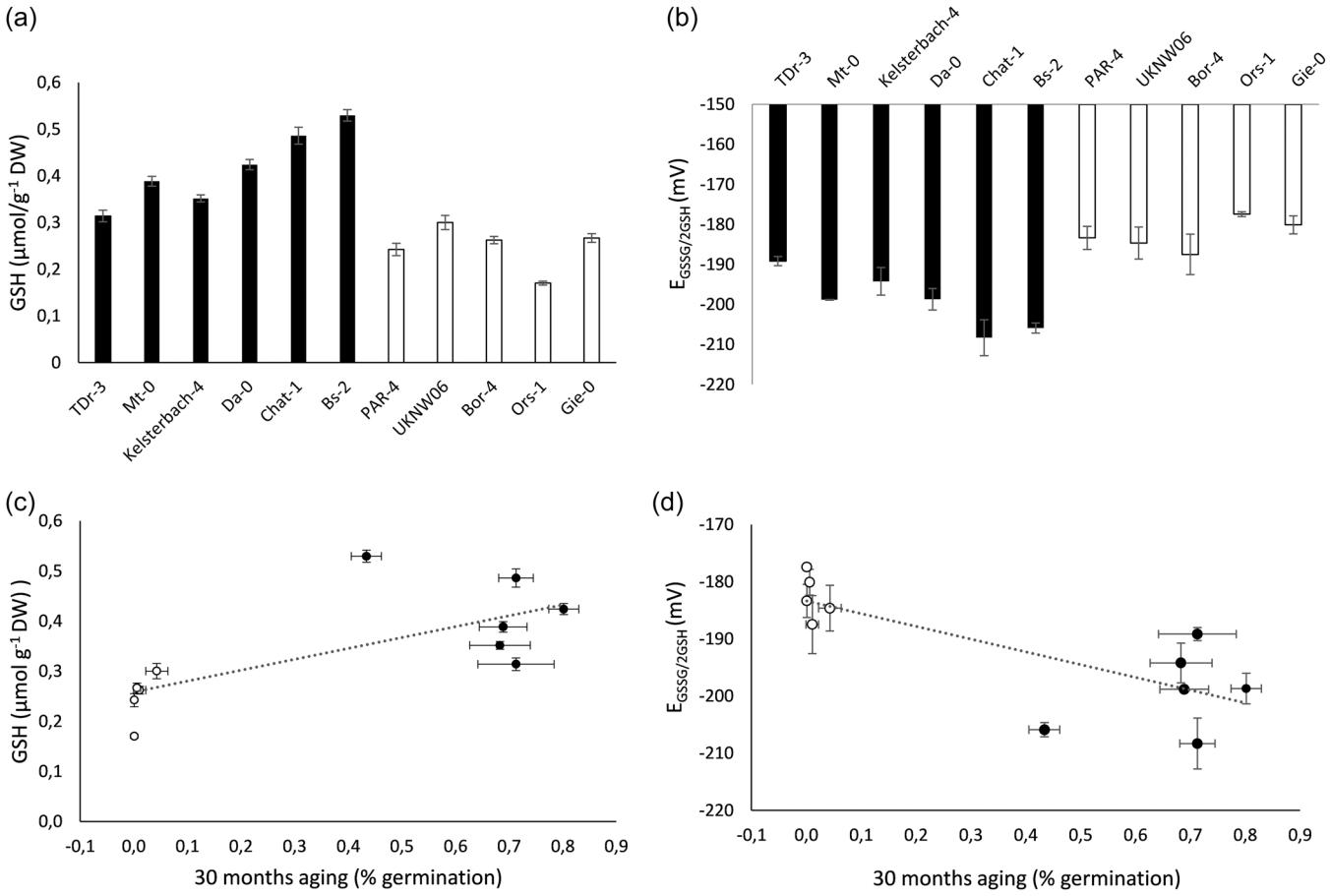

FIGURE 9 Relationship between glutathione and seed longevity in Arabidopsis wild-type accessions. (a) Concentrations of glutathione (GSH) and (b) glutathione half-cell reduction potentials ($E_{GSSG/2GSH}$). Reflecting cellular redox state in dry seeds. Data are means of four replicates ± *SD*. (c, d) Correlation of data shown in (a) and (b), respectively, with total germination after 30 months of ambient ageing

adaptive differences to localised habitats. In addition, longevity coexists with related traits, such as dormancy or germination speed, and a delicate balance between them is required to assure the most adequate timing for germination, without penalty to cellular components affecting seed competitiveness (Shen et al., 2011). Adaptation starts the fixation of an advantageous allele for a specific trait, but entails a secondary phase of changes suppressing the pleiotropic effects caused by the first, sometimes detrimental to another trait. Differential gene expression is a mechanism used to compensate these pleiotropic effects (Maisnier-Patin & Andersson, 2004; Pavlicev & Wagner, 2012). When seed properties in natural variations were assessed, positive and negative aspects for seed longevity were found both in long- and short-lived accessions, as one might intuitively expect, as seeds are complex structures with multiple components affecting different traits.

Prevention of germination by dormancy can be alleviated by initial ageing associated with an oxidative shift in the thiol-based cellular redox state (Morscher et al., 2015). Oxidation of seed macromolecules, including nucleotides (mRNA) and redox-active groups of proteins, may be involved in promoting germination (El-Maarouf-Bouteau et al., 2013; Sano et al., 2020). By enabling O_2 diffusion, permeability of the seed coat could be beneficial for germination success if low dormancy suits the local conditions, but would also translate into low longevity. However, an inverse correlation between dormancy and longevity has been observed in various Arabidopsis mutants and natural accessions (He et al., 2014; Nguyen et al., 2012). Some of the long-lived accessions studied here showed a much higher permeability than Col-0 (or even higher than some of the shorter-lived accessions, see Figure 2a). This indicates that the detrimental effects on longevity of these permeable seed coats are compensated by other components of the seeds. We found a great number of categories of stored mRNAs enriched upon transcripts more expressed in long-lived accessions. The higher abundance of HSPs in Da-0 and other highly permeable long-lived accessions, such as TDr-3 or Bs-2, could clearly be one of the compensating adaptive changes to this high permeability, and would explain its unexpected longevity. The relevance of these proteins on longevity will be discussed below. Similarly, the enrichment in DNA repair genes in Pro-0, the accumulation of transcripts of the catalase CAT2 in Da-0 and TDr-3, the proline biosynthesis pyrroline-5-carboxylate (P5C) reductase or the vitamin E biosynthesis-related VTE1 could also alleviate the higher oxidative stress of highly

permeable seeds, at least when seeds were sufficiently hydrated. DNA repair has been demonstrated to be essential for seed longevity (Waterworth et al., 2019). Catalase could be necessary following seed imbibition, when the reactivation of metabolism generates ROS (Bailly et al.,2001; Gallardo et al., 2001). Moreover, catalase inhibition reduced seed repair after ageing in primed sunflower seeds (Kibinza et al., 2011), highlighting the importance of antioxidant defence in the repair of the damage incurred during ageing. Proline content and P5C reductase levels increase during storage of oat seeds at 28% RH, and has been suggested to play a main role in adaptation to oxidative stress in seeds at high humidity (Kong et al., 2015). Interestingly, genes belonging to proline metabolism were also found in a genome-wide association study to identify seed longevity-related markers and loci in common wheat (Zuo et al., 2019), where similarity analysis on the flanking sequences of significantly associated SNP markers revealed a gene encoding the delta-1-pyrroline-5-carboxylate synthase (P5CS), reinforcing the role of proline metabolism in seed longevity. Loss-of-function of *VTE1* compromises seed longevity (Sattler et al., 2004). Moreover, a high accumulation of transcripts of translational initiation factors in Da-0 could also assure a higher provision of these proteins in the seed, which will guarantee translation efficacy at the time of germination (Dirk & Downie, 2018).

The existence of positive components for longevity in short-lived seeds, such as Lea proteins (Hundertmark et al., 2011), the ABI5 transcription factor (Zinsmeister et al., 2016), lipid transfer proteins, chloroplast degradation-related proteins (Li et al., 2017; Nakajima et al., 2012) or oleosins (Shao et al., 2019) may be difficult to interpret, as we catalogued these accessions as having low resistance to deterioration. Accumulation of these transcripts could, however, restrain an otherwise harsher phenotype after ageing in nature. Positive components for longevity in natural accessions presenting short-lived phenotypes were also reported by Sugliani et al., 2009 using introgression lines from the Sei-0 and Sha accessions of Arabidopsis in the *abi3-5* and *lec1-3* backgrounds, highly impaired in seed longevity. These two accessions were described as sensitive to deterioration among more than 250 accessions (Renard et al., 2020b), but they could partially re-establish the seed developmental programmes controlled by LEC1 and restore the accumulation of seed storage proteins and Lea proteins that were reduced in *abi3-5* and *lec1-3*.

Overall, the differences found in mRNA provision between the accessions, both at the level of genes and at the level of spliced variants, could, together with the differences in protein and metabolite provisions (Chibani et al., 2006; Joshi et al., 2012; Kliebenstein et al., 2001; Knoch et al., 2017), provide the seed with efficient mechanisms to tune not only its tolerance to deterioration, but other aspects of seed performance, adapting its potential to its particular requirements in nature. This seems reasonable, as the observed natural variability in seed properties such as seed dormancy, germination speed or seed longevity is very high (Alonso-Blanco et al., 2003; Atwell et al., 2010; Renard et al., 2020a). Our analysis of stored mRNAs and alternative splicing switches in 13 different wild-type accessions grown under identical environmental conditions clearly indicate that the diversity between accessions is wider than previously assumed (Kimura & Nambara, 2010). From 1600 to 3100 transcripts, and from 360 to 1120 alternative splicing isoforms, depending on the accession, were differentially accumulated with Col-0, suggesting major differences in seed biochemical potential between them. New components involved in seed longevity have been suggested thanks to the comparison of accessions with differences in transcript composition correlating with differences in seed longevity (Figure 6). Interestingly, some of the processes highlighted in this study complement some results obtained in previous genome-wide association studies. For example, QTL analyses for seed longevity in Arabidopsis identified the vitamin E (VTE1) locus as a candidate (Nguyen et al., 2012), confirming previous findings using mutants in tocopherol biosynthesis (Sattler et al., 2004). The increased expression of this gene in the long-lived accession Da-0 corroborates the importance of these compounds to limit lipid oxidation during seed storage. Similarly, DOG1 was also found by Nguyen et al., 2012 in a genome-wide association study as controlling seed longevity and seed dormancy. We found also DOG1 more expressed in the long-lived Da-0 accession. Levels of DOG1 protein correlates with dormancy (Nakabayashi et al., 2012), but *dog1* mutants also present reduced seed longevity (Bentsink et al., 2006), and interfere with the abscisic acid pathway and the expression of heat-shock proteins (Dekkers et al., 2016).

Other observations found here match with previous results at the level of biological processes. One example is the abovementioned role of proline metabolism. Another example is the finding, in another GWAS study, of dehydroascorbate reductase, a key enzyme involved in the recycling of ascorbate, as a genetic component in seed longevity, highlighting the importance of oxidative stress in this trait (Renard et al., 2020a). The same process is marked here with the increased expression of the catalase gene in the Da-0 long-lived accession. Other published marker studies do not reach the level of candidate genes (Agacka-Modoch et al., 2015; Arif & Börner, 2019; Nagel et al., 2015; Schwember & Bradford, 2010) making difficult to compare the information provided by comparative transcriptomics and association studies.

Alternative splicing can affect the amplitude of expression, the stability and translational efficiency of the mRNA, or it can result in protein isoforms. Alternative transcription start site leads to great differences in translation activity (Rojas-Duran & Gilbert, 2012; Ebina et al., 2015; Kurihara et al., 2018). Similarly, alternative termination sites do not seem to increase proteome complexity, but are involved in posttranscriptional regulation (Reyes & Huber, 2018). Interestingly, DOG1 is alternative spliced, and the different isoforms present differences in self-binding affinities (Nakabayashi et al., 2015). Natural variation in binding efficiency was observed among Arabidopsis accessions and contributes to variation in seed dormancy, and this could also be the case for seed longevity. The mechanisms by which dry seeds coordinate stored mRNAs translation stalling and protection with priming for efficient translation during imbibition could be mediated by a fine regulation of alternative splicing and by

other aspects of RNA regulation, given the differences observed in alternative splicing switches and in expression of genes involved in RNA regulation, but the data using wild-type accessions did not give a clear view of the impact of these processes. However, mutant analysis uncovers novel roles in longevity for specific genes. Half-life of mRNAs ranges from minutes to hours, suggesting that protective mechanisms to safeguard stored mRNAs during seed life span should exist. Stress granules (SG) (Chantarachot & Bailey-Serres, 2018; Davies et al., 2012) have recently been suggested as one of these protective structures (Sajeev et al., 2019). We found PAB2, a well-stablished marker of SGs, more expressed in the long-lived accession Da-0, Pro-0 or TDr-3 than in the short-lived accessions Ors-1 or Bor-4, suggesting higher accumulation of stress granules that could protect mRNAs from degradation. TZF9 interacts with PAB2 in RNA granules and is important for stalling mRNAs from translation, probably by recruitment in SGs (Tabassum et al., 2020). Unexpectedly, we found *tzf9* seeds more resistant to ageing. If SG formation was indeed impaired in *tzf9* mutants, this would indicate that RNA protection in SGs is less relevant to longevity than expected. By contrast, ribosome-bound mRNAs were shown to increase in *tzf9* (Tabassum et al., 2020). Perhaps this also means that a higher proportion of stored mRNAs is bound to ribosomes in dry seeds. Recently, Bai et al., 2020 showed that approximately 60% of stored mRNA in dry seeds was associated to monosomes, suggesting a priming mechanism during seed maturation for specific fates during germination, and an alternative protective mechanism mediated by ribosome protection. This would explain the response obtained in the *tzf9* mutant. During germination, protein synthesis requires rapid and selective translation from stored mRNAs, so a ribosome-protected and primed population of mRNAs could confer an advantage to *tzf9* seeds after ageing. Similarly, we found in the Da-0 long-lived accession a higher expression of MAC3B, a subunit of the MAC complex, involved in the splicing machinery (Jia et al., 2017). Only a small proportion of genes are under the control of the MAC complex for splicing regulation (Jia et al., 2017), discarding a global effect of splicing on the whole population of mRNAs that could impact on seed properties. Seeds of both *mac3a mac3b* and *mos4-1* mutants performed better than wild-type seeds after CDT (see Figure 8), which could indicate that a differentially spliced MAC-regulated gene could confer an advantage to the seeds. The observed increased transcription in the group of long-lived seeds of genes whose loss-of-function seems beneficial to longevity could indicate, in the case of MOS4 and MAC3A/MAC3B, that higher expression of these genes could cause alternative splicing switches in downstream genes that would be beneficial for the seeds in aspects of seed quality or germination other than longevity.

The overabundance of HSP transcripts was highlighted in the highly permeable, long-lived accessions Da-0, Pro-0, TDr-3 or Bs-2. HSP expression is associated with desiccation tolerance, by preventing aggregation of proteins in the low-water content environment of the seed (Gallardo et al., 2001; Wang et al., 2014). In addition, HSPs are important during germination. The cotton GhHSP24.7 modulates cytochrome C/C1 production to induce ROS generation, which accelerates endosperm rupture and promotes seed germination (Ma et al., 2019). Transgenic tobacco plants ectopically expressing small HSPs were able to germinate in the dark, indicating that HSPs are part of the light-dependent programme during germination (Koo et al., 2015). Moreover, HSPs are ageing-responsive proteins whose abundance increases after natural or artificial ageing treatments (Kaur et al., 2015; Rajjou et al., 2008), and are more expressed in long-lived near isogenic *Hordeum vulgare* lines (Wozny et al., 2018). A better provision of HSPs in the seed could help refold misfolded proteins during seed desiccation and to foster germination in long-lived aged seeds. In *Helianthus annuus*, seeds overexpressing the HSF HSFA9, which over-accumulate HSP101 and small HSPs, showed increased resistance to controlled CDT (Prieto-Dapena et al., 2006), and loss-of-function of HSF9A in tobacco was associated with reduced resistance to artificial ageing (Tejedor-Cano et al., 2010). We show a similar response in the *hsf1a hsf1b* double mutant line. Arabidopsis has four members of the HSF1A subfamily, and three of them (HSF1A, 1B and 1E) are expressed during seed development (Kotak et al., 2007), setting up a not well understood multistep mechanism for HSP regulation in the dry seed, similar to the one deployed in heat response (Schöffl et al., 1998) and raising the question of genetic redundancy. The fact that the levels of HSP70, HSP101, HSP 17.6A and 6C dropped dramatically in *hsf1a hsf1b* seeds indicates that HSF1A or HSF1B expression is essential for activation of these HSPs. HSP101 is induced by HSF9A in Arabidopsis (Kotak et al., 2007), and repressed in a loss-of-function mutant of HSF9A in tobacco (Tejedor-Cano et al., 2010). HSFA9 expression was reduced in *hsf1a hsf1b* seeds (Figure 9a), suggesting that HSF9A is acting in seed development downstream of HSF1A/HSF1B regulating HSPs expression. Interestingly, in the early response to heat stress, HSF1A/HSF1B also mediate the activation of HSF4 (Lohmann et al., 2004), which is also expressed during seed development. In all, these results suggest that the regulation of HSP synthesis during seed maturation, mediated by the HSF1A and 1B transcription factors, could be a novel mechanism to tune seed deterioration in nature.

Seed ageing is associated with the oxidation of macromolecules (Bailly, 2004), and efficient antioxidant systems are deployed by the seed to prevent excessive oxidation. The GSSG/GSH redox couple is the main cellular redox buffer in seeds and plays a major role in the regulation of the redox environment. Loss of seed viability was correlated with a shift in the $E_{GSSG/2GSH}$ towards more positive values (more oxidising), whereby half of the seed population had lost viability when $E_{GSSG/2GSH}$ shifted into a zone of $E_{GSSG/2GSH}$ between −180 and −160 mV in *Pisum sativum*, *Cytisus scoparius and Lathyrus pratensis* (Kranner et al., 2006). This was also observed in barley using 26 genotypes (Nagel et al., 2015) and it is confirmed here for Arabidopsis. Moreover, the data obtained for GSH and $E_{GSSG/2GSH}$ in non-aged seeds differing in viability indicate that GSH concentration and $E_{GSSG/2GSH}$ in the dry seed are correlated with seed viability (Figure S10c,d and Figure S5a,b). This suggests that glutathione biosynthesis, and the ability to maintain a minimum reservoir of GSH before seed drying (>0.3 GSH μmol g^{-1} DW) is a major determinant of viability in wild-type accessions of Arabidopsis. Interestingly, this

difference in glutathione metabolism does not seem to be directed by transcriptional changes, as expression of genes involved in glutathione biosynthesis of recycling was similar in the long-lived Da-0 accessions and in the short-lived Ors-1 and Bor-4 (Table S4).

In summary, the genetic complexity of seed longevity in wild-type accessions, previously evidenced by the multiple loci identified in numerous genome-association studies (Nagel et al., 2015; Renard et al., 2020a; Zuo et al., 2019) is complemented by this study, showing how multiple molecular and cellular factors contribute positively, but also negatively, to this trait, determining the adaptive responses of every accession in nature. Moreover, our results support recent discussions about the impact on seed longevity of the regulation of mRNAs that are stored during seed development, but that will be translated years later.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GEO Omnibus database at https://www.ncbi.nlm.nih.gov/geo/, reference number GSE179008.

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REFERENCES

Agacka-Modoch, M., Nagel, M., Doroszewska, T., Lewis, R.S. & Börner, A. (2015) Mapping quantitative trait loci determining seed longevity in tobacco (*Nicotiana tabacum* L.). *Euphytica*, 202, 479–486.

Alonso-Blanco, C., Bentsink, L., Hanhart, C.J., Blankenstijn-de Vries, H. & Koornneef, M. (2003) Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana*. *Genetics*, 164, 711–729.

Anders, S., Pyl, P.T. & Huber, W. (2015) HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics*, 31(2), 166–169.

Andrews, S. FastQC: a Quality Control Tool for High Throughput Sequence Data [Online]. 2010. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Angelovici, R., Galili, G., Fernie, A.R. & Fait, A. (2010) Seed desiccation: a bridge between maturation and germination. *Trends in Plant Science*, 15(4), 211–218.

Arif, M.A.R. & Börner, A. (2019) Mapping of QTL associated with seed longevity in durum wheat (*Triticum durum* Desf.). *J Appl Genetics*, 60, 33–36.

Atwell, S., Huang, Y.S., Vilhjálmsson, B.J., Willems, G., Horton, M., Li, Y. et al. (2010) Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. *Nature*, 465(7298), 627–631.

Bai, B., van der Horst, S., Cordewener, J., America, T., Hanson, J. & Bentsink, L. (2020) Seed-stored mRNAs that are specifically associated to monosomes are translationally regulated during germination. *Plant Physiology*, 182(1), 378–392.

Bailly, C. (2004) Active oxygen species and antioxidants in seed biology. *Seed Science Research*, 14, 93–107.

Bailly, C., Audigier, C., Ladonne, F., Wagner, M.H., Coste, F., Corbineau, F. et al. (2001) Changes in oligosaccharide content and antioxidant enzyme activities in developing bean seeds as related to acquisition of drying tolerance and seed quality. *Journal of Experimental Botany*, 52(357), 701–708.

Bentsink, L., Jowett, J., Hanhart, C.J. & Koornneef, M. (2006) Cloning of DOG1, a quantitative trait locus controlling seed dormancy in arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, 103(45), 17042–17047.

Bissoli, G., Niñoles, R., Fresquet, S., Palombieri, S., Bueso, E., Rubio, L. et al. (2012) Peptidyl-prolyl cis-trans isomerase ROF2 modulates intracellular pH homeostasis in arabidopsis. *The Plant Journal*, 70(4), 704–716.

Bueso, E., Muñoz-Bertomeu, J., Campos, F., Brunaud, V., Martínez, L., Sayas, E. et al. (2014) *Arabidopsis thaliana* HOMEOBOX 25 uncovers a role for Gibberellins in seed longevity. *Plant Physiology*, 164(2), 999–1010.

Bueso, E., Muñoz-Bertomeu, J., Campos, F., Martínez, C., Tello, C., Martínez-Almonacid, I. et al. (2016) Arabidopsis COGWHEEL1 links light perception and gibberellins with seed tolerance to deterioration. *The Plant Journal*, 87(6), 583–596. Available at https://doi.org/10.1111/tpj.13220

Buitink, J. & Leprince, O. (2008) Intracellular glasses and seed survival in the dry state. *Comptes Rendus Biologies*, 331, 788–795.

Busch, W., Wunderlich, M. & Schöffl, F. (2005) Identification of novel heat shock factor-dependent genes and biochemical pathways in *Arabidopsis thaliana*. *The Plant Journal*, 41(1), 1–14.

Chantarachot, T. & Bailey-Serres, J. (2018) Polysomes, stress granules, and processing bodies: a dynamic triumvirate controlling cytoplasmic mRNA fate and function. *Plant Physiology*, 176(1), 254–269.

Chen, X., Yin, G., Börner, A., Xin, X., He, J., Nagel, M. et al. (2018) Comparative physiology and proteomics of two wheat genotypes differing in seed storage tolerance. *Plant Physiology and Biochemistry: PPB/Société française de physiologie végétale*, 130, 455–463.

Chibani, K., Ali-Rachedi, S., Job, C., Job, D., Jullien, M. & Grappin, P. (2006) Proteomic analysis of seed dormancy in arabidopsis. *Plant Physiology*, 142(4), 1493–1510.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K. & Scheible, W.R. (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in arabidopsis. *Plant Physiology*, 139(1), 5–17.

Davies, E., Stankovic, B., Vian, A. & Wood, A.J. (2012) Where has all the message gone? *Plant Science*, 185-186, 23–32.

Debeaujon, I., Léon-Kloosterziel, K.M. & Koornneef, M. (2000) Influence of the testa on seed dormancy, germination, and longevity in arabidopsis. *Plant Physiology*, 122(2), 403–414.

Dekkers, B.J., He, H., Hanson, J., Willems, L.A., Jamar, D.C., Cueff, G. et al. (2016) The Arabidopsis DELAY OF GERMINATION 1 gene affects ABSCISIC ACID INSENSITIVE 5 (ABI5) expression and genetically interacts with ABI3 during Arabidopsis seed development. *The Plant Journal*, 85(4), 451–465.

Demonsais, L., Utz-Pugin, A., Loubéry, S. & Lopez-Molina, L. (2020) Identification of tannic cell walls at the outer surface of the endosperm upon Arabidopsis seed coat rupture. *The Plant Journal*, 104(3), 567–580.

Dirk, L.M.A. & Downie, A.B. (2018) An examination of job's rule: protection and repair of the proteins of the translational apparatus in seeds. *Seed Science Research*, 8, 168–181.

Ebina, I., Takemoto-Tsutsumi, M., Watanabe, S., Koyama, H., Endo, Y., Kimata, K. et al. (2015) Identification of novel *Arabidopsis thaliana* upstream open reading frames that control expression of the main

coding sequences in a peptide sequence-dependent manner. *Nucleic Acids Research*, 43(3), 1562–1576.

Edstam, M.M. & Edqvist, J. (2014) Involvement of GPI-anchored lipid transfer proteins in the development of seed coats and pollen in *Arabidopsis thaliana*. *Physiologia Plantarum*, 152(1), 32–42.

El-Maarouf-Bouteau, H., Meimoun, P., Job, C., Job, D. & Bailly, C. (2013) Role of protein and mRNA oxidation in seed dormancy and germination. *Frontiers of Plant Science*, 4, 77.

Gallardo, K., Job, C., Groot, S.P., Puype, M., Demol, H., Vandekerckhove, J. et al. (2001) Proteomic analysis of arabidopsis seed germination and priming. *Plant Physiology*, 126(2), 835–848.

He, H., de Souza Vidigal, D., Snoek, L.B., Schnabel, S., Nijveen, H., Hilhorst, H. et al. (2014) Interaction between parental environment and genotype affects plant and seed performance in arabidopsis. *Journal of Experimental Botany*, 65(22), 6603–6615.

Hundertmark, M., Buitink, J., Leprince, O. & Hincha, D.K. (2011) The reduction of seed-specific dehydrins reduces seed longevity in *Arabidopsis thaliana*. *Seed Science Research*, 21, 165–173.

Jia, T., Zhang, B., You, C., Zhang, Y., Zeng, L., Li, S. et al. (2017) The arabidopsis MOS4-associated complex promotes MicroRNA biogenesis and precursor messenger RNA splicing. *The Plant Cell*, 29(10), 2626–2643.

Joshi, H.J., Christiansen, K.M., Fitz, J., Cao, J., Lipzen, A., Martin, J. et al. (2012) 1001 proteomes: a functional proteomics portal for the analysis of *Arabidopsis thaliana* accessions. *Bioinformatics*, 28(10), 1303–1306.

Kaur, H., Petla, B.P., Kamble, N.U., Singh, A., Rao, V., Salvi, P. et al. (2015) Differentially expressed seed ageing responsive heat shock protein OsHSP18.2 implicates in seed vigor, longevity and improves germination and seedling establishment under abiotic stress. *Frontiers of Plant Science*, 6, 713.

Khan, A. & Mathelier, A. (2017) Intervene: a tool for intersection and visualization of multiple gene or genomic region sets. *BMC Bioinformatics*, 18, 287.

Kibinza, S., Bazin, J., Bailly, C., Farrant, J.M., Corbineau, F. & El-Maarouf-Bouteau, H. (2011) Catalase is a key enzyme in seed recovery from ageing during priming. *Plant Science*, 181(3), 309–315.

Kim, D., Langmead, B. & Salzberg, S.L. (2015) HISAT: a fast-spliced aligner with low memory requirements. *Nature Methods*, 12, 357–360.

Kimura, M. & Nambara, E. (2010) Stored and neosynthesized mRNA in arabidopsis seeds: effects of cycloheximide and controlled deterioration treatment on the resumption of transcription during imbibition. *Plant Molecular Biology*, 73(1-2), 119–129.

Kliebenstein, D.J., Kroymann, J., Brown, P., Figuth, A., Pedersen, D., Gershenzon, J. et al. (2001) Genetic control of natural variation in arabidopsis glucosinolate accumulation. *Plant Physiology*, 126(2), 811–825.

Knoch, D., Riewe, D., Meyer, R.C., Boudichevskaia, A., Schmidt, R. & Altmann, T. (2017) Genetic dissection of metabolite variation in arabidopsis seeds: evidence for mQTL hotspots and a master regulatory locus of seed metabolism. *Journal of Experimental Botany*, 68(7), 1655–1667.

Kong, L., Huo, H. & Mao, P. (2015) Antioxidant response and related gene expression in aged oat seed. *Frontiers of Plant Science*, 19, 6, 158.

Koo, H.J., Park, S.M., Kim, K.P., Suh, M.C., Lee, M.O., Lee, S.K. et al. (2015) Small heat shock proteins can release light dependence of tobacco seed during germination. *Plant Physiology*, 167(3), 1030–1038.

Kotak, S., Vierling, E., Bäumlein, H. & von Koskull-Döring, P. (2007) A novel transcriptional cascade regulating expression of heat stress proteins during seed development of arabidopsis. *The Plant Cell*, 19(1), 182–195.

Kranner, I. (1998). Determination of Glutathione, Glutathione Disulphide and Two Related Enzymes,Glutathione Reductase and Glucose-6-Phosphate Dehydrogenase, in Fungal and Plant Cells. In: Varma A, ed. Springer Lab Manual. Mycorrhiza Manual. Berlin, Heidelberg: Springer, 227–241.

Kranner, I. & Birtic, S. (2005) A modulating role for antioxidants in desiccation tolerance. *Integrative and comparative biology*, 45(5), 734–740.

Kranner, I., Birtić, S., Anderson, K.M. & Pritchard, H.W. (2006) Glutathione half-cell reduction potential: a universal stress marker and modulator of programmed cell death? *Free Radical Biology and Medicine*, 15 40(12), 2155–2165.

Kurihara, Y., Makita, Y., Kawashima, M., Fujita, T., Iwasaki, S. & Matsui, M. (2018) Transcripts from downstream alternative transcription start sites evade uORF-mediated inhibition of gene expression in arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, 115(30), 7831–7836.

Kusunoki, K., Nakano, Y., Tanaka, K., Sakata, Y., Koyama, H. & Kobayashi, Y. (2017) Transcriptomic variation among six *Arabidopsis thaliana* accessions identified several novel genes controlling aluminium tolerance. *Plant, Cell and Environment*, 40(2), 249–263.

Leon-Kloosterziel, K.M., Keijzer, C.J. & Koornneef, M. (1994) A seed shape mutant of arabidopsis that is affected in integument development. *The Plant Cell*, 6(3), 385–392.

Li, Z., Wu, S., Chen, J., Wang, X., Gao, J., Ren, G. et al. (2017) NYEs/SGRs-mediated chlorophyll degradation is critical for detoxification during seed maturation in arabidopsis. *The Plant Journal*, 92(4), 650–661.

Lohmann, C., Eggers-Schumacher, G., Wunderlich, M. & Schöffl, F. (2004) Two different heat shock transcription factors regulate immediate early expression of stress genes in arabidopsis. *Molecular Genetics and Genomics*, 271(1), 11–21.

Love, M.I., Huber, W. & Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq. 2. *Genome Biology*, 15(12), 550.

Ma, W., Guan, X., Li, J., Pan, R., Wang, L., Liu, F. et al. (2019) Mitochondrial small heat shock protein mediates seed germination via thermal sensing. *Proceedings of the National Academy of Sciences of the United States of America*, 116(10), 4716–4721.

Maisnier-Patin, S. & Andersson, D.I. (2004) Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. *Research in Microbiology*, 155(5), 360–369. Available at https://doi.org/10.1016/j.resmic.2004.01.019

Maldonado-Bonilla, L.D., Eschen-Lippold, L., Gago-Zachert, S., Tabassum, N., Bauer, N., Scheel, D. et al. (2014) The arabidopsis tandem zinc finger 9 protein binds RNA and mediates pathogen-associated molecular pattern-triggered immune responses. *Plant and Cell Physiology*, 55(2), 412–425.

Martin, M. (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, 17(1), 10–12.

Molina, I., Ohlrogge, J.B. & Pollard, M. (2008) Deposition and localization of lipid polyester in developing seeds of *Brassica napus* and *Arabidopsis thaliana*. *The Plant Journal*, 53(3), 437–449.

Mondoni, A., Probert, R.J., Rossi, G., Vegini, E. & Hay, F.R. (2011) Seeds of alpine plants are short lived: implications for long-term conservation. *Annali di Botanica*, 107(1), 171–179.

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. *Annali di Botanica*, 116, 669–678.

Nagel, M., Kranner, I., Neumann, K., Rolletschek, H., Seal, C.E., 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 and Environment*, 38(6), 1011–1022.

Nakabayashi, K., Bartsch, M., Ding, J. & Soppe, W.J. (2015) Seed dormancy in arabidopsis requires Self-Binding ability of DOG1

protein and the presence of multiple isoforms generated by alternative splicing. *PLoS Genetics*, 11(12), e1005737.

Nakabayashi, K., Bartsch, M., Xiang, Y., Miatton, E., Pellengahr, S., Yano, R. et al. (2012) The time required for dormancy release in arabidopsis is determined by DELAY OF GERMINATION1 protein levels in freshly harvested seeds. *The Plant Cell*, 24(7), 2826–2838.

Nakabayashi, K., Okamoto, M., Koshiba, T., Kamiya, Y. & Nambara, E. (2005) Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed. *The Plant Journal*, 41(5), 697–709.

Nakajima, S., Ito, H., Tanaka, R. & Tanaka, A. (2012) Chlorophyll b reductase plays an essential role in maturation and storability of arabidopsis seeds. *Plant Physiology*, 160(1), 261–273.

Nguyen, T.P., Cueff, G., Hegedus, D.D., Rajjou, L. & Bentsink, L. (2015) A role for seed storage proteins in arabidopsis seed longevity. *Journal of Experimental Botany*, 66(20), 6399–6413.

Nguyen, T.P., Keizer, P., van Eeuwijk, F., Smeekens, S. & Bentsink, L. (2012) Natural variation for seed longevity and seed dormancy are negatively correlated in arabidopsis. *Plant Physiology*, 160(4), 2083–2092.

Oñate-Sánchez, L. & Vicente-Carbajosa, J. (2008) DNA-free RNA isolation protocols for *Arabidopsis thaliana*, including seeds and siliques. *BMC Research Notes*, 1, 93.

Patro, R., Duggal, G., Love, M.I., Irizarry, R.A. & Kingsford, C. (2017) Salmon provides fast and bias-aware quantification of transcript expression. *Nature Methods*, 14(4), 417–419.

Pavlicev, M. & Wagner, G.P. (2012) A model of developmental evolution: selection, pleiotropy and compensation. *Trends in Ecology and Evolution (Personal Edition)*, 27(6), 316–322.

Pellizzaro, A., Neveu, M., Lalanne, D., Ly Vu, B., Kanno, Y., Seo, M. et al. (2020) A role for auxin signalling in the acquisition of longevity during seed maturation. *New Phytologist*, 225(1), 284–296.

Pierre, J.S., Perroux, J., Whan, A., Rae, A.L. & Bonnett, G.D. (2015) Poor fertility, short longevity, and low abundance in the soil seed bank limit volunteer sugarcane from seed. *Frontiers in Bioengineering and Biotechnology*, 3, 83.

Prieto-Dapena, P., Castaño, R., Almoguera, C. & Jordano, J. (2006) Improved resistance to controlled deterioration in transgenic seeds. *Plant Physiology*, 142(3), 1102–1112.

Probert, R., Adams, J., Coneybeer, J., Crawford, A. & Hay, F. (2007) Seed quality for conservation is critically affected by pre-storage factors. *Australian Journal of Botany*, 55, 326–335.

Rajjou, L., Lovigny, Y., Groot, S.P., Belghazi, M., Job, C. & Job, D. (2008) Proteome-wide characterization of seed ageing in arabidopsis: a comparison between artificial and natural ageing protocols. *Plant Physiology*, 148(1), 620–641.

Renard, J., Martínez-Almonacid, I., Queralta Castillo, I., Sonntag, A., Hashim, A., Bissoli, G. et al. (2021) Apoplastic lipid barriers regulated by conserved homeobox transcription factors extend seed longevity in multiple plant species. *New Phytologist*, 231(2), 679–694.

Renard, J., Martínez-Almonacid, I., Sonntag, A., Molina, I., Moya-Cuevas, J., Bissoli, G. et al. (2020b) and PRX25, peroxidases regulated by COG1, are involved in seed longevity in arabidopsis. *Plant, Cell and Environment*, 43(2), 315–326.

Renard, J., Niñoles, R., Martínez-Almonacid, I., Gayubas, B., Mateos-Fernández, R., Bissoli, G. et al. (2020a) Identification of novel seed longevity genes related to oxidative stress and seed coat by genome-wide association studies and reverse genetics. *Plant, Cell and Environment*, 43(10), 2523–2539.

Reyes, A. & Huber, W. (2018) Alternative start and termination sites of transcription drive most transcript isoform differences across human tissues. *Nucleic Acids Research*, 46(2), 582–592.

Righetti, K., Vu, J.L., Pelletier, S., Vu, B.L., Glaab, E., Lalanne, D. et al. (2015) Inference of Longevity-related genes from a robust coexpression network of seed maturation identifies regulators linking seed storability to biotic defense-related pathways. *The Plant Cell*, 27(10), 2692–2708.

Roach, T., Beckett, R.P., Minibayeva, F.V., 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 and Environment*, 33(1), 59–75.

Rojas-Duran, M.F. & Gilbert, W.V. (2012) Alternative transcription start site selection leads to large differences in translation activity in yeast. *RNA*, 18(12), 2299–2305.

Saatkamp, A., Affre, L., Baumberger, T., Dumas, P.J., Gasmi, A., Gachet, S. et al. (2011) Soil depth detection by seeds and diurnally fluctuating temperatures: different dynamics in 10 annual plants. *Plant and Soil*, 349, 331–340.

Sajeev, N., Bai, B. & Bentsink, L. (2019) Seeds: a unique system to study translational regulation. *Trends in Plant Science*, 24(6), 487–495.

Sano, N., Kim, J.S., Onda, Y., Nomura, T., Mochida, K., Okamoto, M. et al. (2017) RNA-Seq using bulked recombinant inbred line populations uncovers the importance of brassinosteroid for seed longevity after priming treatments. *Scientific Reports*, 7(1), 8095.

Sano, N., Rajjou, L. & North, H.M. (2020) Lost in translation: physiological roles of stored mRNAs in seed germination. *Plants (Basel)*, 9(3), 347.

Sano, N., Rajjou, L., North, H.M., Debeaujon, I., Marion-Poll, A. & Seo, M. (2016) Staying alive: molecular aspects of seed longevity. *Plant and Cell Physiology*, 57(4), 660–674.

Sattler, S.E., Gilliland, L.U., Magallanes-Lundback, M., Pollard, M., Della & Penna, D. (2004) Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. *The Plant Cell*, 16(6), 1419–1432.

Schafer, F.Q. & Buettner, G.R. (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biology and Medicine*, 30(11), 1191–1212.

Schöffl, F., Prändl, R. & Reindl, A. (1998) Regulation of the heat-shock response. *Plant Physiology*, 117(4), 1135–1141.

Schwember, A.R. & Bradford, K.J. (2010) Quantitative trait loci associated with longevity of lettuce seeds under conventional and controlled deterioration storage conditions. *Journal of Experimental Botany*, 61(15), 4423–4436.

Shao, Q., Liu, X., Su, T., Ma, C. & Wang, P. (2019) New insights into the role of seed oil body proteins in metabolism and plant development. *Frontiers of Plant Science*, 10, 1568.

Shen, Y., Zhao, C. & Liu, W. (2011) Seed vigor and plant competitiveness resulting from seeds of *Eupatorium adenophorum* in a persistent soil seed bank, flora - morphology, distribution. *Functional Ecology of Plants*, 206(11), 935–942.

Standart, N. & Weil, D. (2018) P-bodies: cytosolic droplets for coordinated mRNA storage. *Trends in Genetics*, 34(8), 612–626.

Sugliani, M., Rajjou, L., Clerkx, E.J., Koornneef, M. & Soppe, W.J. (2009) Natural modifiers of seed longevity in the arabidopsis mutants abscisic acid insensitive3-5 (*abi3-5*) and leafy cotyledon1-3 (*lec1-3*). *New Phytologist*, 184(4), 898–908.

Supek, F., Bošnjak, M., Škunca, N. & Šmuc, T. (2011) REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS One*, 6(7), e21800.

Tabassum, N., Eschen-Lippold, L., Athmer, B., Baruah, M., Brode, M., Maldonado-Bonilla, L.D. et al. (2020) Phosphorylation-dependent control of an RNA granule-localized protein that fine-tunes defence gene expression at a post-transcriptional level. *The Plant Journal*, 101(5), 1023–1039.

Tejedor-Cano, J., Prieto-Dapena, P., Almoguera, C., Carranco, R., Hiratsu, K., Ohme-Takagi, M. et al. (2010) Loss of function of the HSFA9 seed longevity program. *Plant, Cell and Environment*, 33(8), 1408–1417.

Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z. et al. (2017) agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. *Nucleic Acids Research*, 45(W1), W122–W129.

van Veen, H., Vashisht, D., Akman, M., Girke, T., Mustroph, A., Reinen, E. et al. (2016) Transcriptomes of eight *Arabidopsis thaliana* accessions reveal core conserved, genotype- and organ-specific responses to flooding stress. *Plant Physiology*, 172(2), 668–689.

Vitting-Seerup, K. & Sandelin, A. (2019) IsoformSwitchAnalyzeR: analysis of changes in genome-wide patterns of alternative splicing and its functional consequences. *Bioinformatics*, 35(21), 4469–4471.

Walters, C., Wheeler, L. & Stanwood, P.C. (2004) Longevity of cryogenically stored seeds. *Cryobiology*, 48(3), 229–244.

Wang, W., He, A., Peng, S., Huang, J., Cui, K. & Nie, L. (2018) The effect of storage condition and duration on the deterioration of primed rice seeds. *Frontiers of Plant Science*, 9, 172.

Wang, W.Q., Ye, J.Q., Rogowska-Wrzesinska, A., Wojdyla, K.I., Jensen, O.N., Møller, I.M. et al. (2014) Proteomic comparison between maturation drying and prematurely imposed drying of *Zea mays* seeds reveals a potential role of maturation drying in preparing proteins for seed germination, seedling vigor, and pathogen resistance. *Journal of Proteome Research*, 13(2), 606–626.

Waterworth, W.M., Bray, C.M. & West, C.E. (2019) Seeds and the art of genome maintenance. *Frontiers of Plant Science*, 10, 706.

Waterworth, W.M., Footitt, S., Bray, C.M., Finch-Savage, W.E. & West, C.E. (2016) DNA damage checkpoint kinase ATM regulates germination and maintains genome stability in seeds. *Proceedings of the National Academy of Sciences of the United States of America*, 113(34), 9647–9652.

Wozny, D., Kramer, K., Finkemeier, I., Acosta, I.F. & Koornneef, M. (2018) Genes for seed longevity in barley identified by genomic analysis on near isogenic lines. *Plant, Cell and Environment*, 41(8), 1895–1911.

Zhang, R., Calixto, C.P.G., Marquez, Y., Venhuizen, P., Tzioutziou, N.A., Guo, W. et al. (2017) A high-quality arabidopsis transcriptome for accurate transcript-level analysis of alternative splicing. *Nucleic Acids Research*, 45(9), 5061–5073.

Zhao, T., Huan, Q., Sun, J., Liu, C., Hou, X., Yu, X. et al. (2019) Impact of poly(A)-tail G-content on arabidopsis PAB binding and their role in enhancing translational efficiency. *Genome Biology*, 20(1), 189.

Zinsmeister, J., Lalanne, D., Terrasson, E., Chatelain, E., Vandecasteele, C., Vu, B.L. et al. (2016) ABI5 is a regulator of seed maturation and longevity in legumes. *The Plant Cell*, 28(11), 2735–2754.

Zuo, J.H., Chen, F.Y., Li, X.Y., Xia, X.C., Cao, H. & Liu, J.D. et al. (2019) Genome-wide association study reveals loci associated with seed longevity in common wheat (*Triticum aestivum* L.). *Plant Breed*, 139(2), 295–303.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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