The DNA damage checkpoint kinase ATM regulates germination and maintains genome stability in seeds

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Key words: dormancy cycling, soil seed bank, DNA repair, germination, seed vigor, Arabidopsis, DNA damage response, DNA damage checkpoint

Abstract
Genome integrity is crucial for cellular survival and the faithful transmission of genetic information. The eukaryotic cellular response to DNA damage is orchestrated by the DNA damage checkpoint kinases ATAXIA TELANGIECTASIA MUTATED (ATM) and ATM AND RAD3-RELATED (ATR). Here we identify important physiological roles for these sensor kinases in control of seed germination. We demonstrate that double strand breaks (DSBs) are rate-limiting for germination. We identify that desiccation tolerant seeds exhibit a striking transcriptional DSB damage response during germination, indicative of high levels of genotoxic stress, which is induced following maturation drying and quiescence. Mutant *atr* and *atm* seeds are highly resistant to ageing, establishing ATM and ATR as determinants of seed viability. In response to ageing, ATM delays germination, while *atm* mutant seed germinate with extensive chromosomal abnormalities. This reveals ATM as a major factor which controls germination in aged seed, integrating progression through germination with surveillance of genome integrity. Mechanistically ATM functions through control of DNA replication in imbibing seeds. ATM signaling is mediated by transcriptional control of the cell cycle inhibitor SIAMESE-RELATED 5, an essential factor required for the ageing-induced delay to germination. In the soil seed bank, seeds exhibit increased transcript levels of *ATM* and *ATR* with changes in dormancy and germination potential, modulated by environmental signals including temperature and soil moisture. Collectively our findings reveal physiological functions for these sensor kinases in linking genome integrity to germination, thereby influencing seed quality, crucial for plant survival in the natural environment and sustainable crop production.

Significance statement
The DNA checkpoint kinases ATM and ATR play crucial roles in the maintenance of genome stability, safeguarding cellular survival and the faithful transmission of genetic information. Here we show that ATM is an important factor which influences seed quality by linking progression through germination with genome integrity. Our findings provide new insight into the roles of DNA damage responses, identifying their importance in regulating germination, a process critical for plant survival in the natural environment and crop production.
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Introduction

Maintenance of genome integrity is indispensable for cellular survival and transmission of genetic information to the next generation. However, constant exposure of DNA to environmental and cellular oxidative stresses results in damage which can arrest growth and result in mutagenesis or cell death. Consequently organisms have evolved powerful DNA repair and DNA damage signaling mechanisms. In plants, as in other eukaryotes, the cellular response to DNA damage is orchestrated by the phosphoinositide-3-kinase-related protein kinases (PIKKs) ATAXIA TELANGIECTASIA MUTATED (ATM) and ATM AND RAD3-RELATED (ATR) (1). In response to genotoxic stresses, these checkpoint kinases activate DNA repair factors, delay or halt cell cycle progression, and promote endocycles or programmed cell death (PCD) (1-4). ATM is activated by double strand DNA breaks (DSBs), a highly toxic form of DNA damage that results in chromosome fragmentation (1, 5), and plants mutated in *ATM* display hypersensitivity to DSBs induced by gamma radiation or radiomimetics (6). ATM additionally mediates a

strong transcriptional upregulation of hundreds of genes including the SIAMESE/SIAMESE-RELATED cell cycle inhibitors *SMR5* and *SMR7* (1, 7). ATR is activated by single-stranded regions of DNA, arising during DNA replication or processing of DSBs (1, 8). While much of our knowledge of plant DNA damage responses has originated using genotoxins that would not be naturally encountered, the physiological roles of these DNA damage response pathways are less well characterized.

Seeds represent a stage of the life cycle in which plants experience particularly high levels of genotoxic stress (9). Maturing orthodox (desiccation tolerant) seeds enter a period of desiccation during which their moisture content reduces to around 10-15% reducing cellular activity in the embryo to minimal levels, enabling maintenance of a viable but quiescent embryo for prolonged periods (10). Reduced cellular maintenance in the quiescent state in combination with cycle(s) of desiccation and rehydration are associated with high levels of damage by reactive oxygen species resulting in deterioration of proteins, DNA and cellular structures (11). Genome damage is exacerbated by adverse environmental conditions (typically high temperature and relative humidity) that cause increased oxidative damage and seed ageing (12-15). This results in loss of seed vigor, manifest as decreasing rapidity and uniformity of germination leading to significant losses in yield in crop species (16) and ultimately culminates in loss of viability (17). Delayed germination is accompanied by an extended period of pre-germinative DNA repair initiated in the earliest stages of imbibition (17). This is observed as high levels of *de novo* DNA synthesis several hours before entry of cells into S-phase and completion of germination, marked by the emergence of the young root (radicle) from the seed coat (17, 18). Seeds can also remain in a dormant state in the soil seed bank, often experiencing cycles of hydration and dehydration. Germination only occurs when dormancy has declined and seeds become sensitive to environmental conditions favorable for growth (10, 19, 20). DNA repair activities are operative in imbibed dormant seeds enhancing seed longevity by preserving genome integrity during prolonged stasis in the soil seed bank and ensuring optimal germination when environmental conditions are favorable (17, 21).

In agriculture rapid germination (high seed vigor) and high seed viability are important traits ensuring efficient seedling establishment, key to plant survival and crop production. Loss of seed viability is preceded by a progressively increasing lag period in imbibed non-dormant seed prior to germination. Cytological studies established a correlation between loss of vigor and viability upon seed ageing and accumulation of DNA damage, reporting increased frequencies of both chromosome breakage and rearrangements and abnormal, mutated seedlings upon germination of aged seed (11). Significantly, even high quality seeds displayed a background level of chromosomal breaks (DSBs) (12, 22). Consistent with these results, our recent studies identified a strong DSB-specific transcriptional response, indicative of high levels of genotoxic stress, initiated early in seed imbibition even in unaged seeds (23). In addition, increased sensitivity to seed ageing of Arabidopsis mutants deficient in DSB repair suggested that the repair of chromosomal breaks is fundamental to seed germination (23). The observation that DNA repair activity can influence the rate of seed germination indicates that seed vigor and viability are linked to levels of genomic damage.

Here we sought to identify the molecular link between progression of germination and genome integrity in seeds. We show that the persistence of DSBs is sufficient to delay the completion of germination. We identify ATR as a determinant

of seed longevity, and reveal ATM as a key factor which influences seed vigor and viability, integrating genome integrity with progression through germination in response to damage accumulated during the dry quiescent state. In the natural hydrated dormant state of seeds in the soil seed bank, *ATM* and *ATR* transcript levels display dynamic regulation with changes in environmental conditions. Taken together, these results reveal critical functions for DNA damage responses in regulating seed quality, critical for plant survival and crop productivity.

Results

DSBs are rate limiting for germination in Arabidopsis.

Our previous studies identified hypersensitivity to seed ageing in mutants deficient in the DSB repair enzymes DNA LIG6 and LIG4, suggesting that ageing-induced DSBs are a major factor that delays progression to germination (23). To establish the rate-limiting effects of DSBs on germination, we analyzed transgenic Arabidopsis lines in which endonuclease-induced DSBs can be created by treatment of plants with dexamethasone. Induction of the endonuclease I-PpoI, which cleaves the Arabidopsis genome within the conserved 5S rRNA genes *in vitro*, resulted in an increase in cell death in seedling root tips, indicative of DNA damage (3) (SI Appendix Fig. S1A-D). Upon I-PpoI induction in seeds, germination was delayed, with the mean germination time (MGT, a measure of germination vigor) increasing from 3.1 to 4.5 days ($P<0.05$) (Fig. 1A). This demonstrates that accumulation of DSBs in the seed genome is rate-limiting for germination and that their persistence adversely affects germination vigor.

ATM is activated in early imbibition of orthodox seeds

Previously, we identified marked activation of the DNA damage transcriptional response within 3 hours of Arabidopsis seed imbibition, indicating that high levels of genome damage are incurred even under ideal conditions of seed development, storage and imbibition (23). Activation of the plant transcriptional response to DSBs is predominantly dependent on ATM and includes genes involved in DNA metabolism, chromatin structure and repair (1). Consistent with the role of ATM in controlling DNA-damage responsive gene expression, we found no significant induction of *RAD51* recombinase transcripts in *atm* mutant seeds upon imbibition, in contrast to wild type seeds (SI Appendix Fig. S2). Analysis of *HvRAD51* transcript levels during seed imbibition of wild type barley (*Hordeum vulgare)* also showed high levels of gene induction at 12 hours imbibition (Fig. 1B), demonstrating that early induction of the DNA damage response is a conserved component of germination in desiccation tolerant seeds. However, the transcriptional DNA damage response was absent in germination of physiologically mature barley embryos that had not undergone maturation drying on the mother plant (Fig. 1B). This signifies that the cellular response to genome damage in germination is directly activated in response to high levels of DNA damage accumulated by the seed during the cycle of desiccation, embryo quiescence and rehydration.

Regulation of DNA damage responsive genes in seeds

To investigate further the roles of DNA repair in seeds, we compared the transcriptional profiles of the major DNA damage response genes in published seed array data using the Arabidopsis eFP browser (24). This analysis revealed similar patterns in transcript levels of *LIG6*, *ATR* and *ATM* in hydrated seeds undergoing laboratory based dormancy cycling (Table S1) (25, 26). Particularly high expression

levels of these genes were observed in seeds in a prolonged hydrated state of dormancy, from 1-2 months, as would be experienced by seeds over-wintering in the soil. Significantly, transcriptional regulation of *ATM* and *ATR* has not been previously reported in plants and this led us to look at the response of these genes in seeds experiencing the seasonally changing soil seed bank environment. Fluctuations in soil temperature and moisture content elicit transcriptional responses in seeds and influence dormancy and germination potential (19). We identified large seasonal fluctuations in transcript levels of *ATM*, *ATR* and *LIG6* (Figure 2). In spring *ATM* transcript levels in overwintered seeds increased fivefold during prolonged hydration in the soil, declining sharply in May as soil moisture is lost and prior to seedling emergence (Fig. 2A). Seasonal transcript profiles of *ATR* and *LIG6* correlated with each other ($P<0.05$) and with levels of dormancy (AR50, $P<0.01$ and $P<0.05$ respectively), whilst negatively correlating with soil temperature ($P<0.01$ and $P<0.05$ respectively) (Fig. 2B and C). These results are consistent with ATM and ATR regulating genome maintenance during dormancy cycling in the soil seed bank.

Germination of *atm* and *atr* mutant seed is resistant to accelerated ageing

We tested the hypothesis that ATM and ATR integrate the sensing of DNA integrity with onset of germination by analyzing the germination phenotypes of multiple independent alleles of *atm* and *atr* mutant lines (27). Accelerated ageing is widely used to simulate natural ageing by incubating seeds at elevated temperature and relative humidity (RH) (28). Here we used three different ageing regimes (28ºC and 84.6% RH, 35ºC and 83% RH and 49ºC and 80.6% RH (29)), with the lower temperature and humidity treatments representing conditions typically experienced by seeds in the natural environment (30). In the absence of seed ageing, germination of wild type (Col-0) Arabidopsis seeds and the mutant lines *atm-3* and *atr-6* displayed no significant difference (Fig. 3A). However, *atm* mutant seed germinated markedly more rapidly than wild type after ageing at all temperatures regimes investigated (Fig. 3B-F, SI Appendix, Figs. S3-5). Progressively increased seed ageing resulted in severely delayed germination in Col-0 compared to *atm* mutants, and by 14 days ageing at 35ºC the MGT for *atm* was 2.9 days, whereas for Col-0 the MGT was 6.4 days ($P<0.01$) (SI Appendix, Fig. S3). Our data therefore indicates that ATM controls the time to germination in aged seeds, observed as an extending delay to radicle emergence with increasing seed deterioration. Progressive loss of vigor upon seed ageing eventually culminates in loss in viability. *atm* and *atr* mutants both exhibited elevated germination compared to wild type controls after ageing (Fig. 3E,F; SI Appendix, Fig. S5). While Col-0 seeds display low viability after 14 days of ageing at 35ºC (19%), *atm* and *atr* lines retain significantly higher levels of germination (62% and 77% respectively, $P<0.01$, Fig 3E). Seeds that failed to germinate were resistant to dormancy-breaking treatments including gibberellin, fluridone or cold imbibition at 4ºC. Lack of viability was confirmed by staining with 2,3,5-triphenyltetrazolium chloride (31) (SI Appendix, Fig. S5I,J). The elevated germination of *atm* and *atr* mutants after ageing was confirmed in the independent mutant alleles *atm-2* and *atr-2* (1) (SI Appendix, Fig. S5G,H). Taken together, these results establish both ATM and ATR as molecular determinants of viability and reveal ATM as a key controller of germination in response to the deteriorative effects incurred during seed ageing.

ATM controls cell cycle progression in response to seed ageing

In response to DNA damage, ATM and ATR activate checkpoints that delay or arrest cell cycle progression to promote repair processes, delaying growth (2, 32). We hypothesized that the rapid germination of *atm* mutant seed is due to loss of the checkpoint activity that slows germination in the presence of DNA damage. In Arabidopsis seeds, cells in the embryo are arrested in G1 phase and resumption of cell cycle activity and S-phase (DNA replication) is required for normal germination (33, 34). We investigated whether an ageing-induced delay to cell cycle activation was evident in wild type but not *atm* or *atr* mutant lines. Cell cycle initiation was determined by incorporation of the thymidine nucleoside analog 5-ethynyl-2'-deoxyuridine (EdU) to monitor the onset of DNA replication in wild type, *atm* and *atr* lines. In wild type seeds, few embryos contained cells that were replicating DNA until around the time of seed coat rupture, when labelled nuclei are first visible in root meristem cells (Figure 4A-F; SI Appendix, Fig. S6). We observed no significant difference in the timing of S-phase between unaged wild type, *atm* and *atr* seeds (Fig. 4G). However, using seeds aged for 7 days at 35ºC and 14 days at 35ºC, DNA replication was initiated substantially earlier in *atm* mutant seed than in Col-0 or *atr* lines (Fig. 4G), consistent with the faster germination of the aged *atm* lines. This indicates that ATM, but not ATR, activity delays onset of S-phase in the wild type lines, coinciding with a loss in germination vigor. At 14 days we see increased numbers of S-phase cells in both *atm* and *atr* deficient seed, correlating to the elevated germination observed in these mutants (SI Appendix Fig. S7). We conclude that ATM delays initiation of the cell cycle in aged seed and that both ATM and ATR contribute to inhibition of DNA replication in aged seeds.

Severe chromosomal abnormalities are elevated in *atm* mutant seed

To investigate the hypothesis that ATM integrates germination progression with genome integrity, we determined frequencies of chromosomal abnormalities in aged and unaged Col-0, *atm* and *atr* mutant seed (Fig. 4H-L). Anaphase bridges were scored after commencement of cell division in radicle cells of germinating seeds. These bridges represent inaccurate chromosomal break repair by the host cell recombination machinery. Consistent with the induction of the ATM-dependent DNA damage response, even in high quality seeds, we observed a low frequency (1-2%) of abnormalities in unaged Col-0, *atm* and *atr* seed (Fig. 4H), significantly higher than reported of other stages of the Arabidopsis lifecycle (35). After ageing (7d at 35ºC and 83% RH) this increases to 4% in Col-0 and *atr* seed, indicative both that high levels of genome damage accumulate in the dry quiescent state and can be tolerated by the germinating seed (Fig. 4H). However, substantially higher frequencies (9%) are observed in aged *atm seed*, further supporting function of ATM to control the advancement of germination in order to promote repair of high levels of genome damage sustained by extended periods in the dry quiescent state.

The cell cycle inhibitor SMR5 regulates germination in response to ageing

We further investigated the mechanism by which control of germination is integrated with genome surveillance by analysis of the downstream effectors of ATR and ATM signaling. ATR activates a DNA replication stress checkpoint through transcriptional induction of the cell cycle regulator *WEE1* (8, 36). However, in our analysis of seed ageing, *WEE1* transcript levels did not differ significantly between Col-0, *atm* or *atr* mutant seed and, furthermore, *wee1* mutants did not exhibit the ageing resistant phenotype of *atr* seeds (SI Appendix Fig. S8). This indicates that the effects of seed ageing on germination are not attributable to replication stress (1, 8, 36).

In response to genotoxic stress ATM induces *SMR5* and *SMR7,* members of a family of SIAMESE/SIAMESE-RELATED cell cycle inhibitors. Both genes display ATM-dependent transcriptional induction in response to genotoxic stresses and regulate cell cycle activity and growth under stress conditions (7, 37). In seeds, *SMR5* transcripts rapidly decline during imbibition, reaching low levels by time of germination (SI Appendix, Fig. S9A,C). However, transcript analysis of aged seeds (7d at 35°C) at 48h imbibition reveals significantly higher *SMR5* levels in wild type but not *atm* lines (Figure 5A, B). This is consistent with roles for SMR5 in regulating germination of aged seeds, given the known functions of this cell cycle inhibitor in genotoxic stress responses (7). In the soil seed bank *SMR5* and *ATM* transcript levels were highly correlated ($p<0.05$; SI Appendix Fig. S10). *SMR5* GUS-reporter analysis localized promoter activity to regions of the radicle where S-phase is initiated (Figure 5C-F). As both SMR5 and SMR7 are involved in plant cell cycle control, with SMR5 in particular being required for the reduction in cell division under conditions of elevated oxidative stress (7), we therefore tested single and double *smr5* and *smr7* mutant lines for sensitivity to seed ageing. After accelerated ageing *smr7* mutants were not significantly different to wild type lines, whereas both *smr5* and the *smr5 smr7* lines displayed an ageing-induced delay to germination relative to wild type lines (Figures 5G-H; SI Appendix, Fig. S11). We conclude that SMR5 plays a key role in controlling the delay to germination observed upon seed ageing.

Discussion

The evolution of the desiccation tolerant seed represents a highly successful survival strategy, effectively prolonging embryo viability and providing resistance to adverse environmental stresses until favorable conditions for germination are encountered. However, extended periods in the dry quiescent state are associated with the deterioration of biological macromolecules and a progressive delay to germination which culminates in loss of viability (17). There is considerable intra- and interspecific variation in seed longevity and understanding the genetic basis of seed quality is important to maintain crop production in changing climates (31). Here we identify important functions for DNA checkpoint kinases in linking genome integrity to germination thereby influencing seed quality, crucial for plant survival in the natural environment and for sustainable crop production. We establish that both ATM and ATR are determinants of seed longevity, and demonstrate that ATM controls the progression of seed germination, integrating genome surveillance with cell cycle activation.

Seed ageing is associated with increasing incidence of chromosomal aberrations (Fig. 4 H-L), and even seed lots with high germination rates display levels of genome damage not experienced at other stages of plant development (12, 22). Consistent with this, during the early imbibition phase, seeds display a large and rapid induction of the ATM-mediated transcriptional DSB response (23). We show that activation of the DNA damage response requires prior desiccation and quiescence of the embryo, rather than being a developmentally programmed part of germination *per se*. The quiescent desiccated state is associated with accumulation of high levels of DSBs, as observed in the desert-dwelling bacterium *Deinococcus radiodurans* (38). In seeds, the accumulation of genome damage upon ageing is further compounded by telomere loss (39), a source of chromosomal breaks that may also contribute to loss of vigor. Here, using enzymatic induction of DSBs by the meganuclease I-PpoI during seed imbibition, we provide direct evidence that low levels of chromosomal breaks limit the progression of germination in Arabidopsis.

Plants have evolved robust DNA damage response and repair pathways to counteract the detrimental effects of genotoxic stress, coordinated by the eukaryotic DNA checkpoint kinases ATM and ATR. Here we identify that ATM and ATR function as major factors which influence germination in response to seed ageing. We demonstrate that ATM delays progression of germination by regulating the initiation of DNA replication through up-regulation of the cell cycle inhibitor *SMR5* (7). Previously, chemical inhibition of the cell cycle was shown to be sufficient to slow germination in Arabidopsis (34) and here we establish a physiological role for DNA damage cell cycle checkpoints in seeds. Progressive ageing culminates in loss of seed viability, and a component of this viability loss is attributable to both ATM and ATR. While ATR induces transcription of the cell cycle regulator *WEE1* in response to DNA replication stress (8, 36), *WEE1* is not required for the ATR response to genotoxic stress in plants (1, 2, 40). Here, we establish that ATR function in seeds is not dependent on WEE1, consistent with DNA damage rather than replicative stress limiting germination.

The elevated germination of aged *atm* and *atr* mutant seed relative to wild type is associated with higher levels of S-phase activity, which is consistent with increased DNA replication in the presence of DNA damage and increased genome instability. Here cytological analysis reveals high frequencies of chromosomal aberrations in *atm* mutant seeds, identifying a critical role for ATM in safeguarding the genome of the germinating embryo. Furthermore, seedlings germinated from aged *atm* mutant seed exhibit lower survival and slower development of true leaves in comparison to their aged wild type counterparts (SI Appendix, Fig. S12). This demonstrates the requirement for ATM activation in imbibed seeds to ensure that germination does not proceed until damage to the genome is repaired, thereby promoting successful establishment and growth of the young seedling.

In the natural environment seeds in the soil seed bank typically undergo several wet-dry cycles at or near the soil surface, or remain continually hydrated deeper in the soil. Imbibed dormant seeds display DNA repair processes (21), which are often active over several imbibition and re-drying cycles (41). These seasonal fluctuations in hydration and temperature in the soil seed bank lead to dynamic regulation of *ATM*, *SMR5* and *ATR* transcript levels. Active genome surveillance and DNA damage signaling in hydrated dormant seeds provides highly effective mechanisms for protection of genomic integrity, which is important for long-term stability of plant communities**.**

In conclusion, we show that ATM and ATR are important factors which control germination in plants. We identify major roles for ATM in integrating genome surveillance with germination through regulation of cell cycle activities, providing new insight into the physiological functions of DNA damage response mechanisms in plant development. Our findings further establish critical functions for ATM in safeguarding genome stability in germinating seeds and in promoting seedling growth and establishment, key determinants of crop yield (42). Understanding the mechanisms that regulate germination provides important insight into the molecular basis of seed vigor and viability, traits that are of critical agronomic and ecological importance.

Materials and Methods

Seed propagation and germination

Arabidopsis plants were raised in growth chambers under constant humidity (30%), with 16h light and 8h dark cycles at 23°C. Col-0, *atr-6* (SALK_054383) and *atm-3*

mutants (SALK_089805) were obtained from the NASC. For each experimental replicate, seeds from all lines were harvested simultaneously and stored at 15°C and 15% humidity for 2 months to allow after-ripening. Germination tests and accelerated ageing were performed as described previously (23, 43) and mean time to germination was calculated as described in ref (44). Arabidopsis lines were as previously described *atm-3* (27)*, lig6 lig4* (23), *atr-2* and *atm-2* (1), *wee1* (36) and p*SMR5:GUS, smr5, smr7 and smr5 smr7* (7). Viability staining performed as previously reported (31). The *atr-6* mutant allele is presented in SI Appendix, Fig. S13.

Nucleic acid purification and cloning
DNA procedures and bacterial manipulations were by established protocols (36). Real-time RT-PCR analysis was performed on a CFX96 thermocycler (Bio-Rad), as described previously (45), using SYBR Green Supermix (Bio-Rad). A plant codon-optimized I-PpoI gene was synthesized (Genscript, SI Appendix, Fig S14) and cloned into pBI-ΔGR and the expression cassette subcloned into pCB1300 carrying a I-PpoI recognition site to create pPPOΔGR (SI Appendix, Fig S15). Propidium iodide (PI) staining and EdU labeling was performed as described previously (46). Full methods are described in SI Appendix.

Dormancy analysis
Seeds production, harvest, storage and details of seed burial in, and recovery from field soils and post-recovery seed handling are as described previously (19)(20)(25, 26, 47). Further details are provided in the SI Appendix.

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Figure Legends

Figure 1. Seed desiccation is required for induction of the plant DNA damage response and DSBs are rate limiting for germination. (A) Germination of two independent lines holding the I-PpoI construct was analyzed with or without the induction of DSBs by dexamethasone. Seeds were plated onto germination paper pre-wetted with 10 µM dexamethasone or 0.5% DMSO (control) and stratified at 4°C for 48h before transfer to 20°C. Error bars show the standard error of the mean of 3 replicates of 50 seeds. Significant differences in mean values are indicated *: P<0.05, **: P<0.01 (T-Test). **(B)** Embryos were isolated from desiccated barley grain cv Maris Otter or from undesiccated barley grain (30 dpa) capable of germination but which had not undergone maturation drying and placed on 1% agar plates. Q-PCR analysis of *HvRAD51* expression using cDNA synthesized from RNA isolated from

imbibing embryos. Control Q-PCR was performed using primers specific to *HvACTIN2*

Figure 2. Transcript profiles of *ATM*, *ATR* and *LIG6* in relation to seasonal changes in the soil seed bank environment. (A) *ATM* transcript levels in seeds recovered from the soil over 12 months from October 2007. Soil moisture (%) profile at seed depth (5 cm). Also seedling emergence (% of total emerged) in the field following monthly soil disturbance (n=4). (**B)** *ATR* transcript levels and soil temperature (°C) at seed depth. (**C**) *LIG6* transcript levels and dormancy level measured as the dry after ripening time (days) required to achieve 50% germination at 20°C in the light (AR50). Error bars indicate the standard error of the mean, n=3. Soil temperature and moisture content; AR50 and emergence data are from Footitt et al., 2011 (19).

Figure 3. Germination of *atm* and *atr* mutant seed is resistant to accelerated ageing. Analysis of *atm-3*, *atr-6* and Col-0 seed germination after accelerated ageing at 28°C or 35°C over a saturated solution of KCl. Germination of (**A**) unaged seeds, (**B-C**) after accelerated ageing at 28°C, (**D-F**) accelerated ageing at 35°C. Seeds were stratified at 4°C for 48h before transfer to 22°C/Light and seeds scored for radicle emergence each day post-imbibition. Error bars show the standard error of the mean of 3 replicates of 50 seeds.

Figure 4. ATM induces a delay in DNA replication upon seed ageing.
The timing of DNA replication (S-phase) in germination of aged and unaged seeds. **(A-F)** The timing of S-phase in unaged wild-type seeds was determined using EdU labelling and fluorescence microscopy. **(A,D)** Prior to seed coat rupture, little EdU labelling is observed. **(B,E)** Rupture of the seed coat occurs around 24h, coincident with onset of S-phase, with several EdU positive nuclei evident in embryos. **(C,F)** As seeds germinate, progressively increasing numbers of EdU-positive nuclei are observed in cells of the expanding radicle. Top left panel: DAPI stained nuclei. Top right panel: EdU labeled nuclei. Bottom left panel: image merge. Scale bar: 10µm. Bottom right panel: representative stage of germination. **(G)** Analysis of S-phase initiation in aged and unaged wild type, *atm* and *atr* mutant seed. Quantification of numbers of embryos containing one or more EdU-positive (S-phase) cells in unaged seeds (broken lines) and seeds aged for seven days at 35°C, 83% RH (continuous lines). Aged seeds displayed loss of vigor but retain high viability, as shown in Fig 3D. **(H)** Frequency of anaphase bridges in germinating seeds. Seeds were either unaged or aged for 7d at 35°C and 83% RH. Error bars show the standard error of 3 replicates of 50 anaphases. *: P<0.05 (T-Test). **(I-L)** Airy scanning confocal images showing examples of anaphases: **(I)** normal anaphase from unaged Col-0. **(J-L)** anaphase bridges from **(J)** aged Col-0; **(K)** aged *atm-3*; **(L)** aged *atr-6*. Scale bar is 1µm.

Figure 5 ATM controls germination in an SMR5-dependent pathway.
SMR5 and *SMR7* transcript levels in seeds at 48h imbibition in **(A)** unaged Col-0, *atm-3* and *atr-6* seeds and **(B)** Col-0, *atm-3* and *atr-6* seed aged at 35°C, 83% RH for 7 days to provide vigor loss but 100% viability **(C-F)** GUS-reporter analysis of *SMR5* promoter activity in wild type unaged seeds and seeds aged at 35°C, 83% RH for 5 days **(G-H)** Analysis of *smr5, smr7*, *smr5 smr7* and wild type seed germination performance after accelerated ageing. Seed viability and vigor of unaged seeds **(G)**

and after 10 days accelerated ageing at 35°C, 83% RH **(H)**. Seeds were plated and stratified at 4°C for 48h before transfer to an environmental growth chamber at 22°C and seeds scored for radicle emergence each day post-imbibition. Error bars show the standard error of the mean of 3 replicates of 50 seeds. **: P<0.01 (T-Test).

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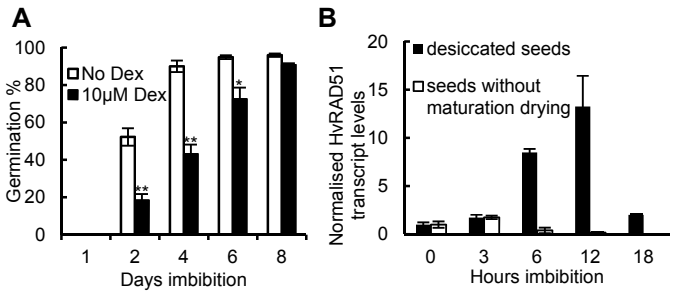
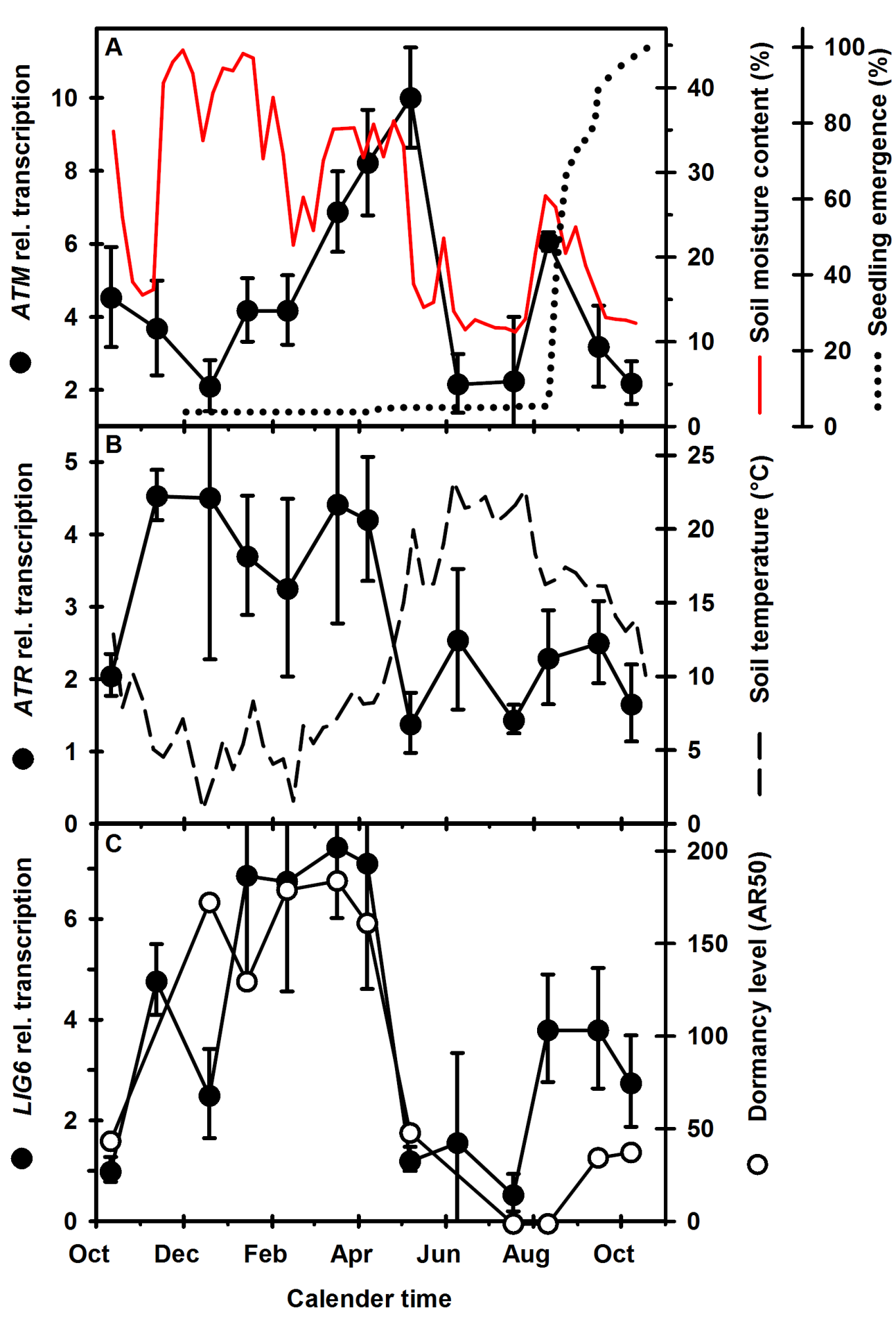
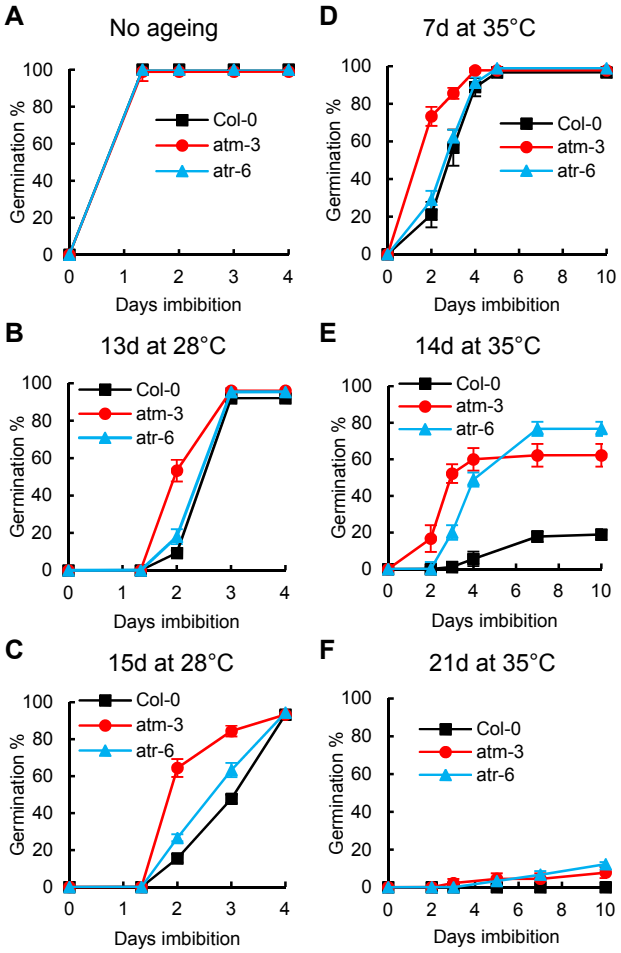
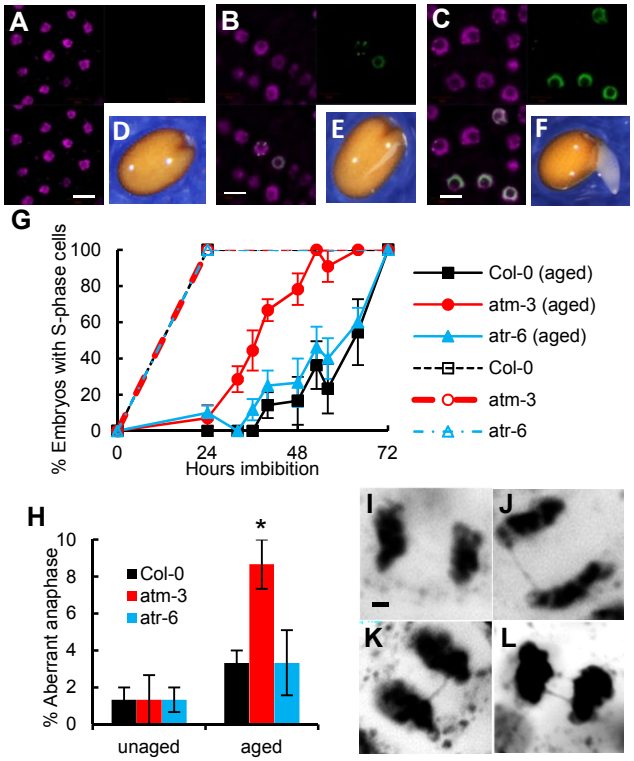
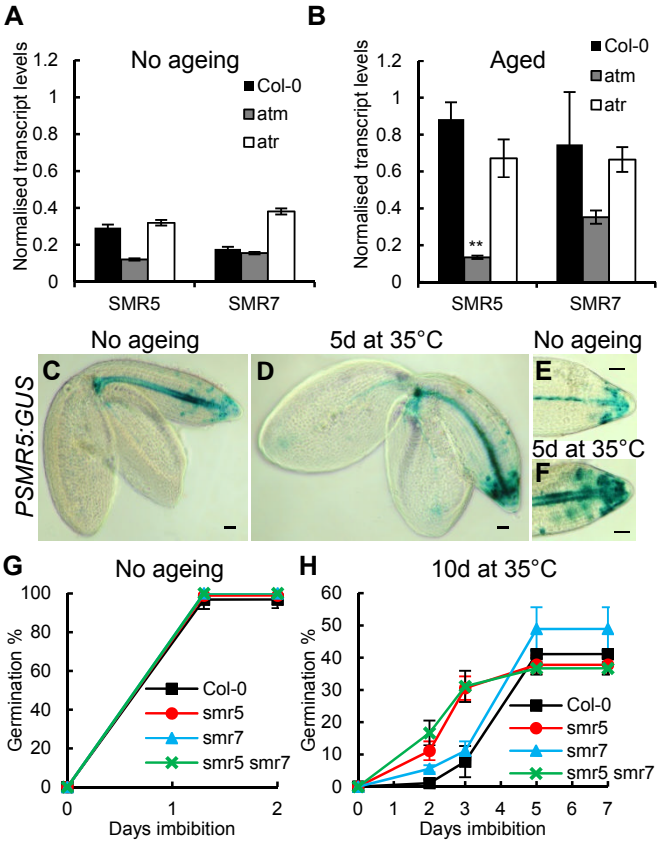

A
100
80
60
40
20
0
Germination %
No Dex
10μM Dex
**
**
*
1
2
4
6
8
Days imbibition
B
20
15
10
5
0
Normalised HvRAD51 transcript levels
desiccated seeds
seeds without maturation drying
0
3
6
12
18
Hours imbibition


A
B
C
ATM rel. transcription
ATR rel. transcription
LIG6 rel. transcription
Soil moisture content (%)
Seedling emergence (%)
Soil temperature (°C)
Dormancy level (AR50)
Oct
Dec
Feb
Apr
Jun
Aug
Oct
Calender time


A
No ageing
B
13d at 28°C
C
15d at 28°C
D
7d at 35°C
E
14d at 35°C
F
21d at 35°C
Germination %
Days imbibition
Col-0
atm-3
atr-6


A
B
C
D
E
F
G
% Embryos with S-phase cells
100
80
60
40
20
0
0
24
48
72
Hours imbibition
Col-0 (aged)
atm-3 (aged)
atr-6 (aged)
Col-0
atm-3
atr-6
H
% Aberrant anaphase
10
8
6
4
2
0
Col-0
atm-3
atr-6
*
unaged
aged
I
J
K
L


A
No ageing
Normalised transcript levels
1.2
1
0.8
0.6
0.4
0.2
0
Col-0
atm
atr
SMR5
SMR7
B
Aged
**
C
No ageing
D
5d at 35°C
E
No ageing
5d at 35°C
F
PSMR5:GUS
G
No ageing
Germination %
100
80
60
40
20
0
Col-0
smr5
smr7
smr5 smr7
Days imbibition
H
10d at 35°C
60
50
40
30
20
10

Supplementary data

Figure S1: Induction of I-PpoI by dexamethosone results in cell death in Arabidopsis roots of 7 d seedlings.

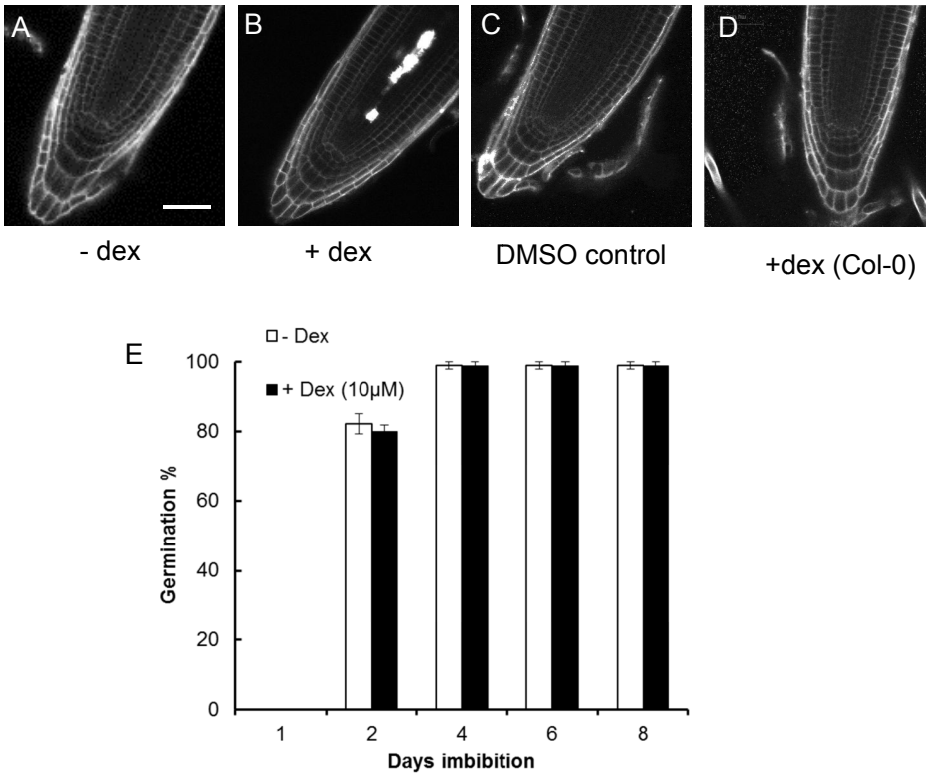


Figure S1 Induction of I-PpoI by dexamethosone results in cell death in Arabidopsis roots of 7 d seedlings.
Cell death in lines expressing inducible I-PpoI was visualized by propidium iodide staining and fluorescence microscopy **(A-D).** I-PpoI lines - dex **(A)**, + dex **(B),** DMSO control **(C)**, wild type col0 +Dex **(D)**. PI stains cell walls and in the presence of dexamethasone, dead root meristem cells can also be observed. Scale bar is 50µm. Dexamethasone does not affect germination performance of wild type col0 seeds **(E).** Seeds were plated onto germination paper pre-wetted with 10 µM dexamethasone or 0.5% DMSO (control) and stratified at 4°C for 48h before transfer to 20°C. Error bars show the standard error of the mean of 3 replicates of 50 seeds.

Figure S2: *RAD51* transcript levels in imbibing Col-0 and *atm-3* seeds.

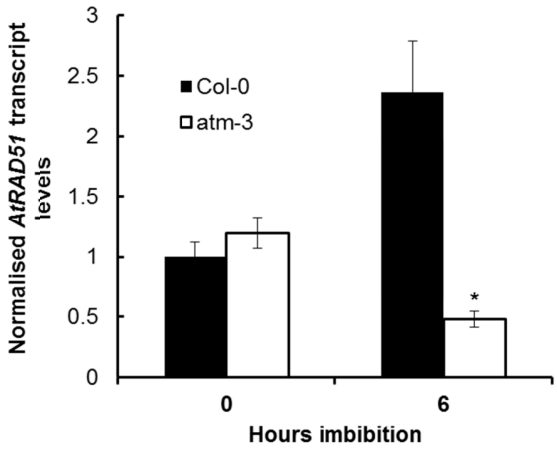


Figure S2. *RAD51* transcript levels during imbibition.
Q-PCR analysis of *AtRAD51* expression using cDNA synthesized from RNA isolated from imbibing Col-0 or *atm3* mutant Arabidopsis seed. Control Q-PCR was performed using primers specific to *AtACTIN2*. Significant differences in mean values are indicated *: P<0.05 (T-Test).

Table S1: Heat map of DNA repair gene transcript levels in dormant seeds.

Magnitude of change	0	40	80	120	160	200	240	280	320	360	>400

* For *SMR5* maximum colour intensity set at 4000

		PDD	DDL	PD24h	PD48h	PD30d	SD1	SD2	PDC	PDL	ND	PDN	PDLN	LIG	Imbibed seeds Max	Min	Fold change
AT3G48190	*ATM*	295	185	304	445	728	515	323	165	171	115	217	128	159	728	115	6
AT5G40820	*ATR*	92	88	76	79	132	172	166	48	53	57	57	46	91	172	46	4
AT1G66730	*LIG6*	56	44	79	142	397	252	149	72	59	50	61	44	47	397	44	9
AT1G07500	*SMR5*	3958	5101	2610	3657	4675	2637	1815	1394	1677	103	1235	205	208	4675	103	45
AT2G31970	*RAD50*	152	171	142	137	395	274	343	90	142	83	207	153	139	395	83	5

Data are mean absolute transcript values obtained from Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) based on published microarray experiments (1-4).

Abbreviation	Treatment
PDD	Primary dormant: seeds dry (will not complete germinate when imbibed in Light or dark).
DDL	Primary dormant seeds, dry after-ripened: seeds dry (will germinate when imbibed in the light).
PD24h	Primary dormant seeds imbibed for 24 h (will not complete germination).
PD48h	Primary dormant: seeds imbibed for 48 h in the dark (will not complete germination).
PD30d	Primary dormant: seeds imbibed for 30 days in the dark (will not complete germination).
SD1	Secondary dormant: DL seeds imbibed in the dark for a further 24 days (sensitive to nitrate).
SD2	Secondary dormant: SD1 seeds imbibed at 3°C in the dark for 20 days (insensitive to nitrate).
PDC	Primary dormant seeds after-ripened for 117 days and then imbibed for 4 days at 3°C (will not complete germination unless exposed to light).
PDL	Primary dormant seeds after-ripened for 91 days and then imbibed for 24 h in the light (will not complete germination).
ND	Dry after-ripened seeds imbibed for 24 h (will germinate if placed in the light).
PDN	Primary dormant seeds after-ripened for 91 days and then imbibed for 24 h on a 10 mM KNO_3 solution (will not complete germination unless exposed to light).
PDLN	Primary dormant seeds after-ripened for 91 days and then imbibed in white light for 24 h on a solution 10 mM KNO_3 (will complete germination).
LIG	Dry after-ripened seeds imbibed for 20 h in the dark and then 4 h in red light (will complete germination).

Figure S3: Mean germination time of *atm* and *atr* mutant seed and wild type lines after accelerated ageing.

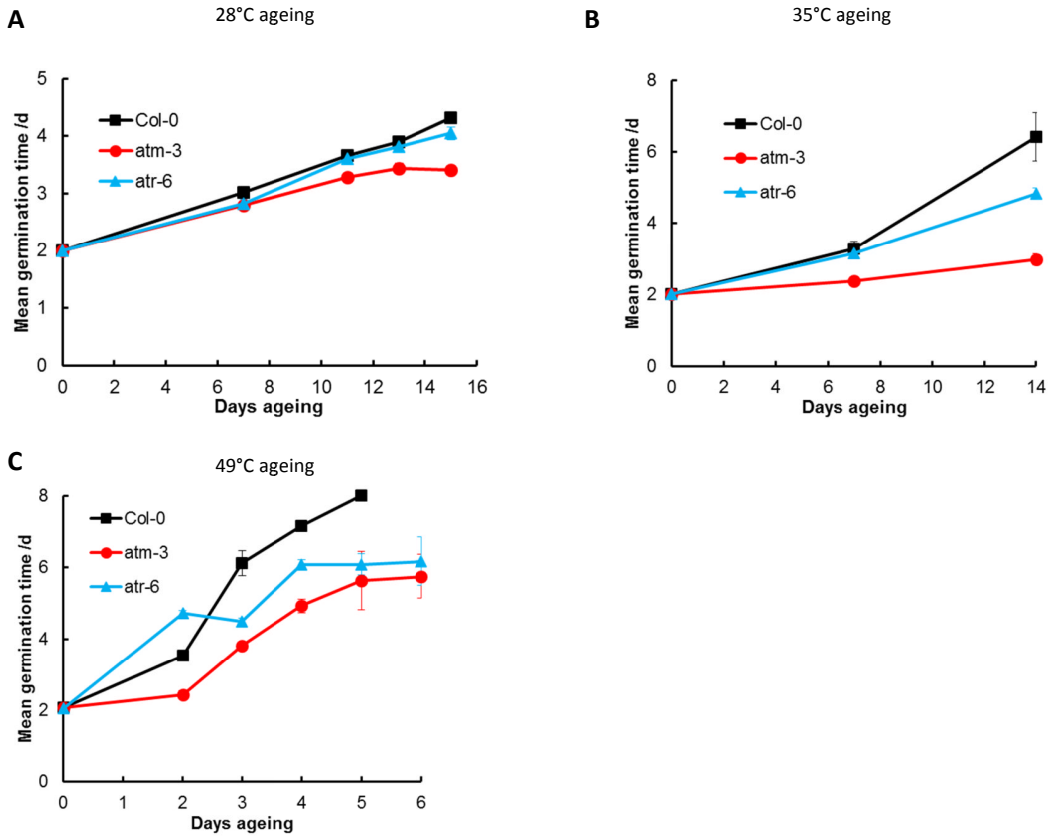


Figure S3. Mean germination time (MGT) of *atm* and *atr* mutant seed and wild type lines after accelerated ageing

Analysis of *atm-3, atr-6* and WT mean germination time after accelerated ageing at **(A)** 28°C, **(B)** 35°C and **(C)** 49°C. Accelerated ageing was performed for up to 15 days over a saturated solution of KCl. Seeds were plated and stratified at 4°C for 48h before transfer to an environmental growth chamber at 22°C and seeds scored for radicle emergence each day post-imbibition. Error bars show the standard error of the mean of 3 replicates of 50 seeds.

Figure S4: Germination of *atm* and *atr* mutant seed is resistant to accelerated ageing at 28°C, 84.6% RH.

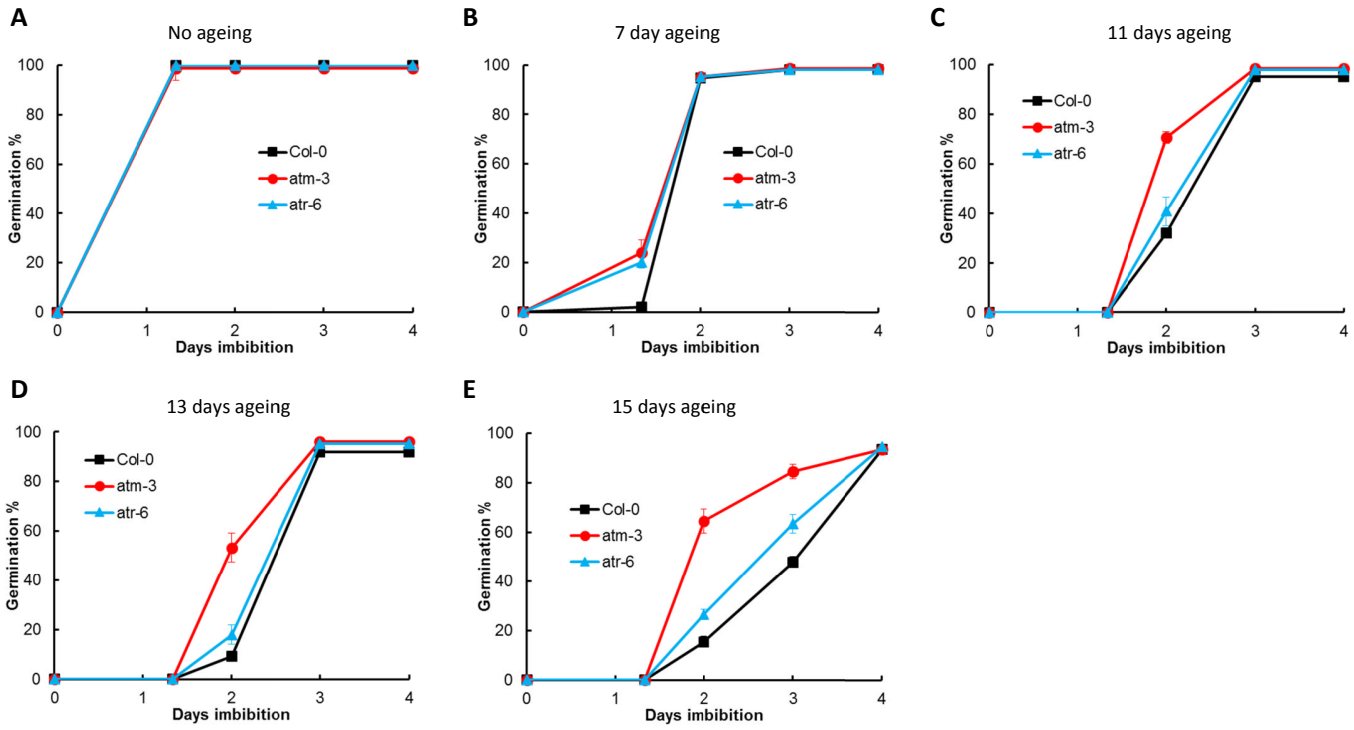


Figure S4. Germination of atm and atr mutant seed is resistant to accelerated ageing at 28°C, 84.6%. Analysis of *atm-3*, *atr-6* and WT seed germination performance after accelerated ageing. Seed viability and vigor after **(A)** 0 days accelerated ageing **(B)** 7 days accelerated ageing **(C)** 11 days accelerated ageing **(D)** 13 days ageing **(E)** 15 days ageing. Accelerated ageing was performed at 28°C for between 0 to 7 days over a saturated solution of KCl. Seeds were plated and stratified at 4°C for 48h before transfer to an environmental growth chamber at 22°C and seeds scored for radicle emergence each day post-imbibition. Error bars show the standard error of the mean of 3 replicates of 50 seeds.

Figure S5: Germination of *atm* and *atr* mutant seed is resistant to accelerated ageing at 49°C, 80.6%RH.

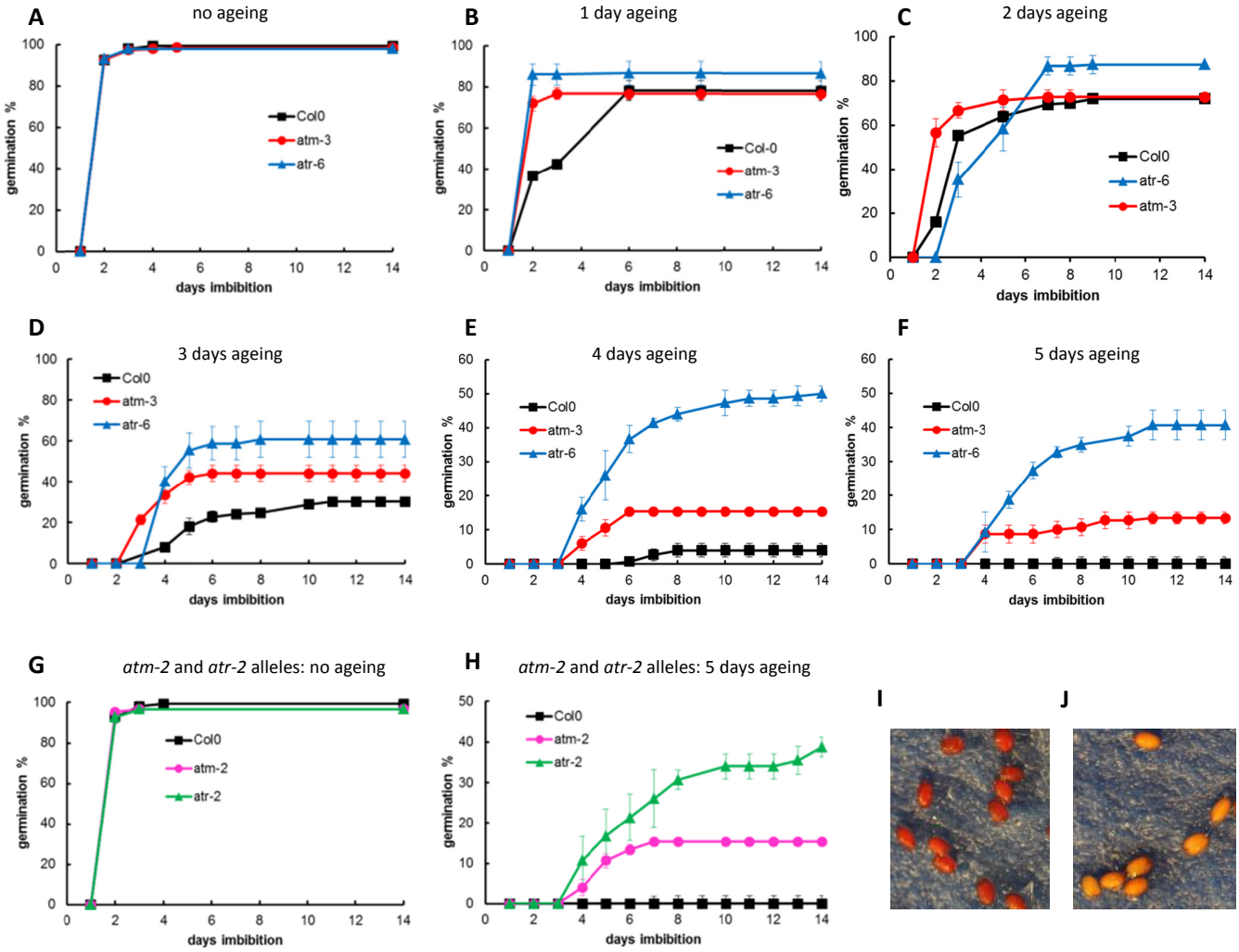


Figure S5 Germination of *atm* and *atr* mutant seed is resistant to accelerated ageing at 49°C, 80.6%.
Analysis of *atm-3*, *atr*-6 and WT seed germination performance after accelerated ageing. Seed viability and vigor after **(A)** 0 days accelerated ageing **(B)** 1 day accelerated ageing **(C)** 2 days accelerated ageing **(D)** 3 days ageing **(E)** 4 days accelerated ageing **(F)** 5 days accelerated ageing **(G)** Germination of wild type, *atm-2* and *atr-2* mutant seed after 0d ageing **(H)** Germination of wild type, atm-2 and atr-2 mutant seed after no ageing. Analysis of *atm-2*, *atr-2* and WT seed germination performance after 5 days accelerated ageing. Accelerated ageing was performed at 49°C for between 0 to 7 days over a saturated solution of KCl. Seeds were plated and stratified at 4°C for 48h before transfer to an environmental growth chamber at 22°C and seeds scored for radicle emergence each day post-imbibition. Error bars show the standard error of the mean of 3 replicates of 50 seeds. Example of viability staining with 2,3,5-triphenyltetrazolium chloride **(I)** Unaged seeds imbibed for 20h **(J)** Aged seeds that failed to germinate.

Figure S6: Initiation of S-phase in germination

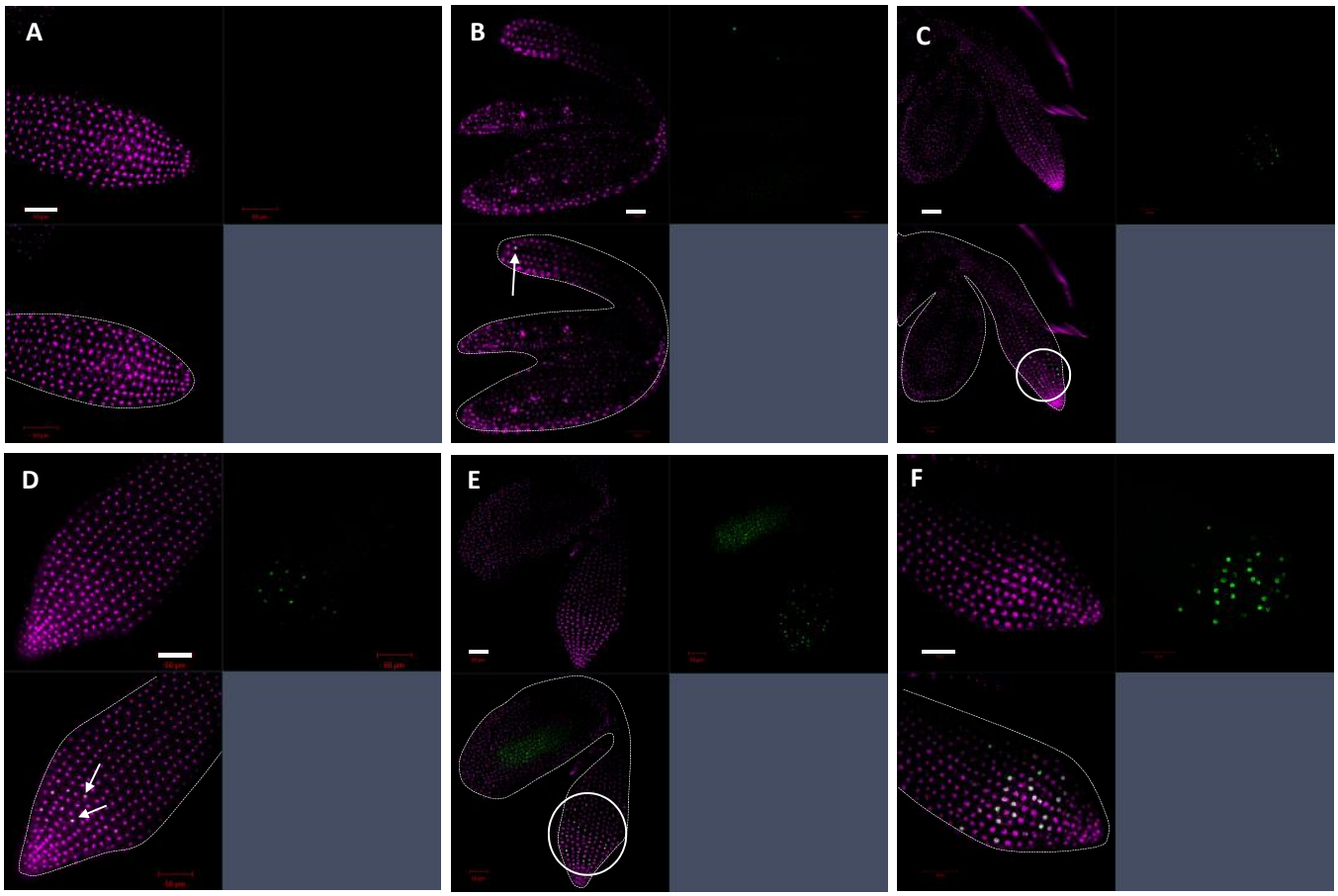


Figure S6: Initiation of S-phase in germination
EdU labelling of unaged wild type seeds reveals S-phase cells in the tip of the embryonic root and spreading up the root axis as germination progresses. Autofluorescence is observed in cotyledons. DAPI staining of nuclear DNA is shown as magenta and newly synthesized EdU labeled DNA is colored green and indicated by arrows in panel B and D and circled in panels C and E. The outline of the embryo is inducted in the merged images by a dotted white line. Panels A to F are seeds at progressively further stages though germination. **(A)** ungerminated **(B-D)** seed coat split **(E-F)** germinated with the radicle fully emerged from the seed coat. Bar is 50µm.

Figure S7: Initiation of S-phase in seeds aged for 14d .

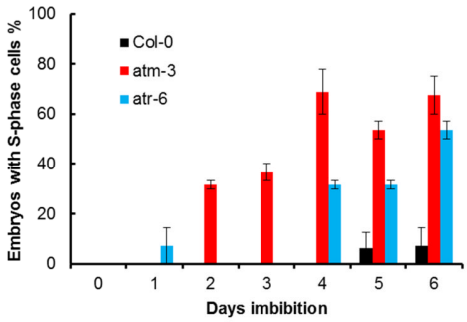


Figure S7. DNA replication in seeds aged for 14 days at 35°C, 83% RH. Embryos were scored for the presence of cells in S-phase as described in Fig.4. Both *atr* and *atm* mutants display greater number of embryos with S-phase cells relative to wild type controls, correlating with increased viability observed in the mutant lines (Fig. 3).

Figure S8: Analysis of WEE1 in seed germination.

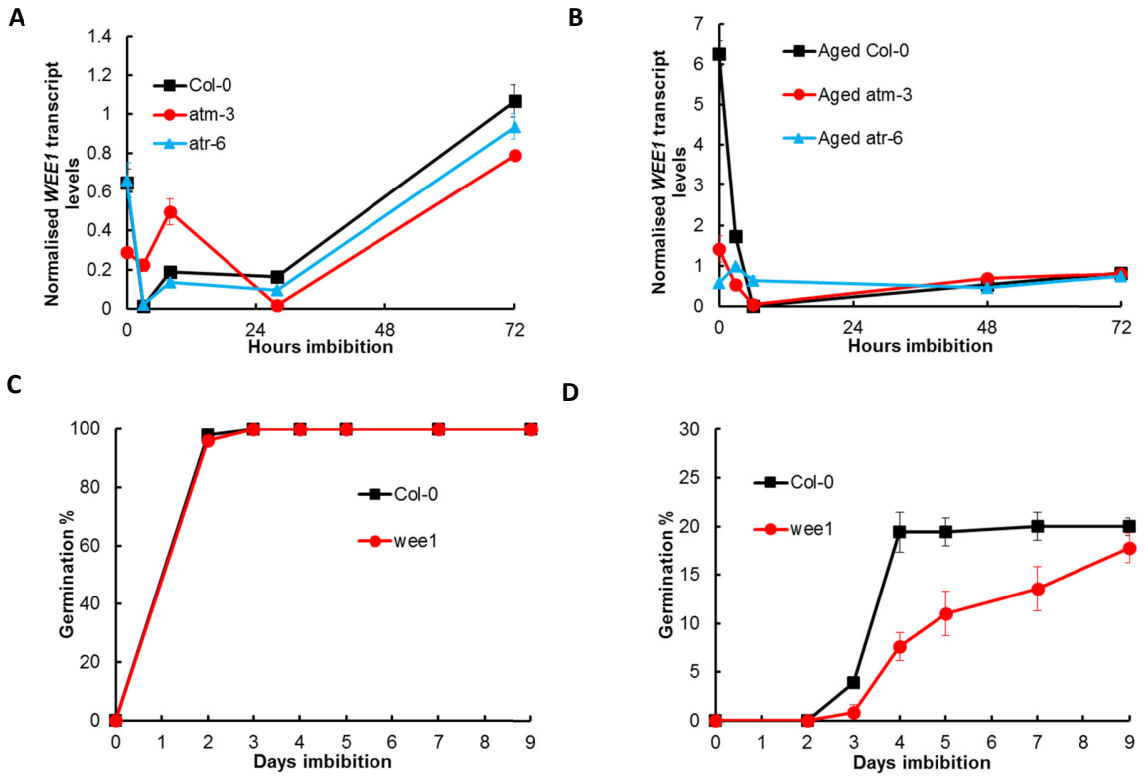


Figure S8 Analysis of WEE1 in seed germination

Transcript levels of *WEE1* in germinating seeds is independent of ATM and ATR **(A-B).** *WEE1* transcript levels in seeds at 0-48h imbibition in **(A)** unaged Col-0 and **(B)** *wee1* seed aged at 35°C, 83% RH for 7 days. Seeds deficient in the cell cycle regulator WEE1 show slowed germination relative to Col-0 **(C-D)**. Analysis of *wee1* and wild type seed germination performance after accelerated ageing. Seed viability and vigor of unaged seeds **(C)** and after 14 days **(D)** accelerated ageing at 35°C, 83% RH. Seeds were plated and stratified at 4°C for 48h before transfer to an environmental growth chamber at 22°C and seeds scored for radicle emergence each day post-imbibition. Error bars show the standard error of the mean of 3 replicates of 50 seeds.

Figure S9: Transcript levels of *SMR5*, *SMR7* and *RAD51* in Arabidopsis seeds.

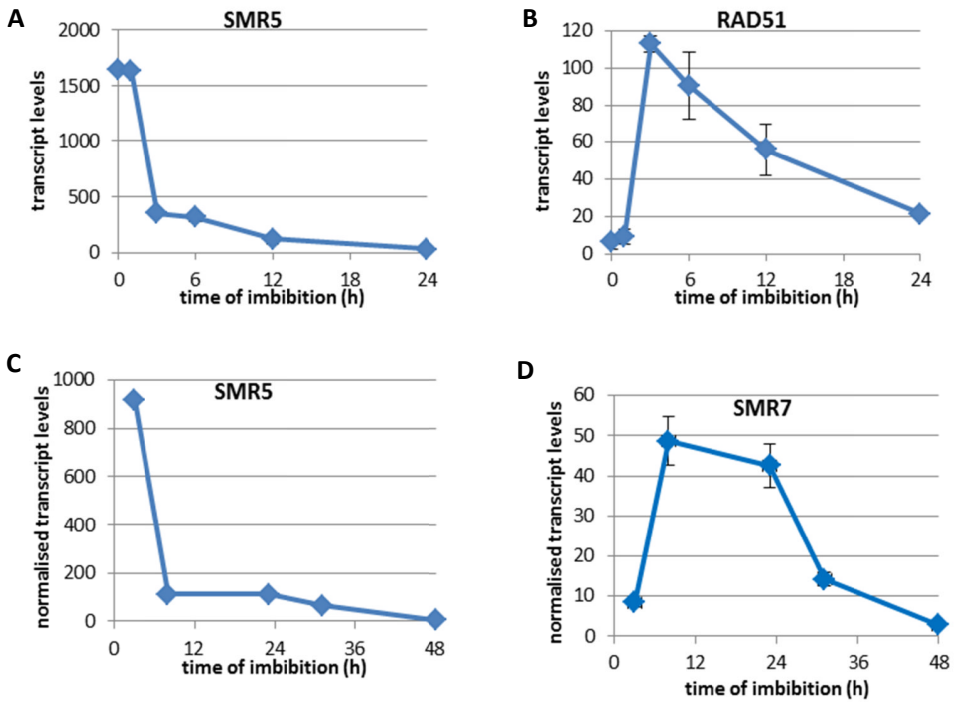


Figure S9: Transcript levels of SMR5, SMR7 and RAD51 in wild type Arabidopsis seeds. Transcript levels of *SMR5* **(A)** and *RAD51* **(B)** in imbibing seeds were analyzed using publically available microarray data (1). *SMR7* is not represented on microarrays used in the study by Nakabayashi et al (2005) (1). Real-time PCR data normalized to ACTIN7 expression (AT5G09810) shown for *SMR5* **(C)** and *SMR7* **(D)**.

Figure S10: Transcript levels of *SMR5* Arabidopsis seeds during dormancy cycling in the seed soil bank.

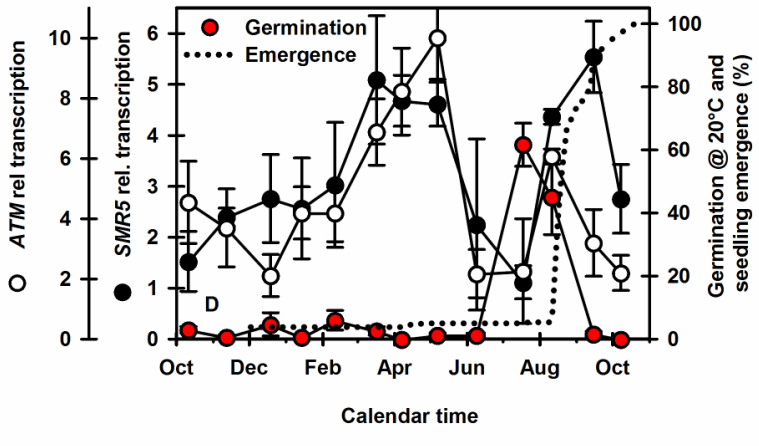


Figure S10: Transcription of *ATM* and *SMR5* and germination potential of seeds in the soil seed bank. Transcription profiles of *ATM* and *SMR5* in seeds recovered from the soil over 12 months from October 2007.Germination at 20°C in the light of seeds recovered from the soil at monthly intervals. Seedling emergence (% of total emerged) in the field following monthly soil disturbance (n=4). Error bars indicate the standard error of the mean, n=3. Germination and emergence data are from Footitt et al., 2011 (19) and with *ATM* transcript levels are also displayed in Figure 2 of the main text.

Figure S11: Mean germination time of *smr5, smr7, smr5 smr7* mutant seed after accelerated ageing.

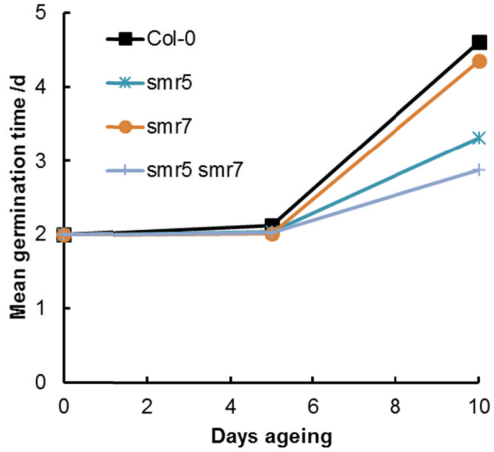


Figure S11: Mean germination time of *smr5, smr7, smr5 smr7* mutant seed after accelerated ageing
Analysis of *smr5*, *smr7*, *smr5 smr7* and WT mean germination time after accelerated ageing. MGT after accelerated ageing of *smr5*, *smr7*, *smr5 smr7* and WT at 35°C. Accelerated ageing was performed for up to 14d days over a saturated solution of KCl. Seeds were plated and stratified at 4°C for 48h before transfer to an environmental growth chamber at 22°C and seeds scored for radicle emergence each day post-imbibition. Error bars show the standard error of the mean of 3 replicates of 50 seeds.

Figure S12: Development of true leaves is delayed in seedlings germinated from viable *atm-3* and *atr-6* mutant seeds relative to Col-0 after accelerated ageing.

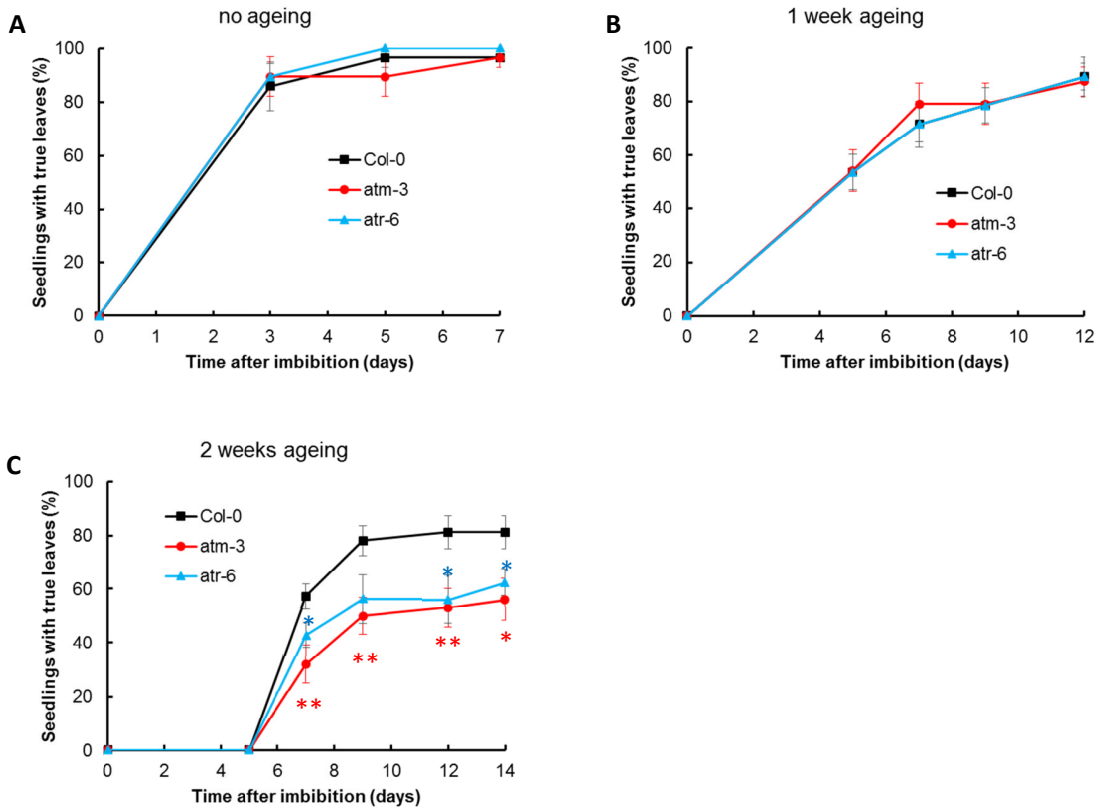


Figure S12 Development of true leaves is delayed in seedlings germinated from viable aged *atm* and *atr* mutant seeds relative to Col-0. Analysis of development in those plants which germinated from *atm-3*, *atr-6* and Col-0 seed after accelerated ageing at 35°C over a saturated solution of KCl for 0 day **(A)**, 7 day **(B)** and 14 day **(C)**. Seeds were stratified at 4°C for 48h before transfer to 22°C/Light. After 7 days seedlings germinated from viable seed were transferred to soil and plants subsequently scored for development of true leaves. Error bars show the standard error of the mean of 10 replicates of 4 plants. Significant differences in mean values are indicated *: P<0.05, **: P<0.01 (T-Test).

Figure S13: Isolation of the *atr-6* mutant line (SALK_054383).

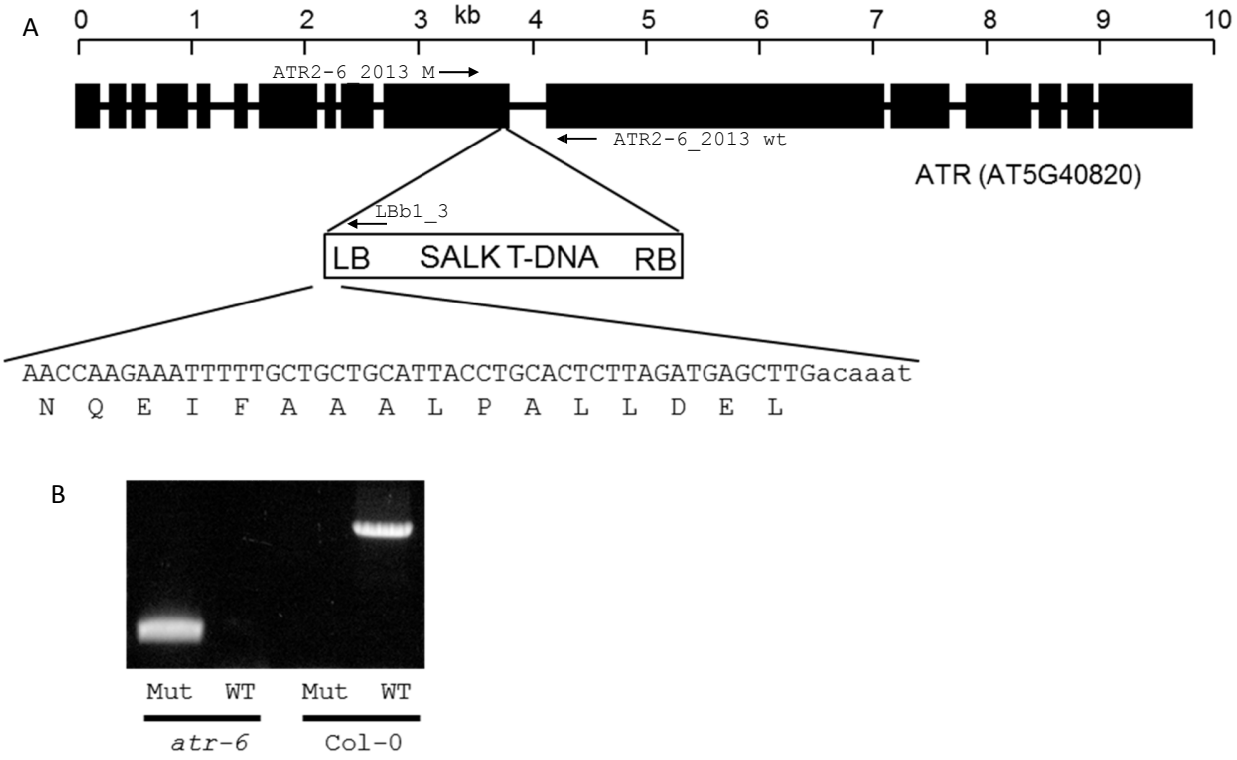


Figure S13. Isolation of the *atr-6* mutant line. A) The T-DNA insertion (SALK_054383) is located in the 10th exon. T-DNA sequence is in lower case. B) PCR confirmed the *atr-6* homozygous mutant had no wild type *ATR* allele. Mutant (Mut) primers: ATR-6_2013 M CAGAGTTCTTGCATTTGCTCTGA LBb1_3_SALK ATTTTGCCGATTTCGGAAC. Wild type (WT) primers: ATR-6_2013 M CAGAGTTCTTGCATTTGCTCTGA ATR-6_2013 wt AGTTCGAGATTCTTCTGCACACC

Figure S14: Construction of the pPPOΔGR vector: Codon optimized I-PpoI.

```
atggctaagtctaatcaagctagagttaacggaggatctaattatgattctcttactcct
 M  A  K  S  N  Q  A  R  V  N  G  G  S  N  Y  D  S  L  T  P
cttaatatggctcttactaatgctcaaattcttgctgttattgattcttgggaagaaact
 L  N  M  A  L  T  N  A  Q  I  L  A  V  I  D  S  W  E  E  T
gttggacaatttcctgttattactcatcatgttcctcttggaggaggacttcaaggaact
 V  G  Q  F  P  V  I  T  H  H  V  P  L  G  G  G  L  Q  G  T
cttcattgttatgaaattcctcttgctgctccttatggagttggatttgctaagaacgga
 L  H  C  Y  E  I  P  L  A  A  P  Y  G  V  G  F  A  K  N  G
cctactagatggcaatataagagaactattaatcaagttgttcatagatggggatctcat
 P  T  R  W  Q  Y  K  R  T  I  N  Q  V  V  H  R  W  G  S  H
actgttccttttcttcttgaacctgataatatcaacggaaagacttgtactgcttctcat
 T  V  P  F  L  L  E  P  D  N  I  N  G  K  T  C  T  A  S  H
ctttgtcataatactagatgtcataatcctcttcatctttgttgggaatctcttgatgat
 L  C  H  N  T  R  C  H  N  P  L  H  L  C  W  E  S  L  D  D
aataagggaagaaattggtgtcctggacctaacggaggatgtgttcatgctgttgtttgt
 N  K  G  R  N  W  C  P  G  P  N  G  G  C  V  H  A  V  V  C
cttagacaaggacctctttatggacctggagctactgttgctggacctcaacaaagagga
 L  R  Q  G  P  L  Y  G  P  G  A  T  V  A  G  P  Q  Q  R  G
tctcattttgttgtt
 S  H  F  V  V
```

Figure S15: Map of pPPOΔGR.

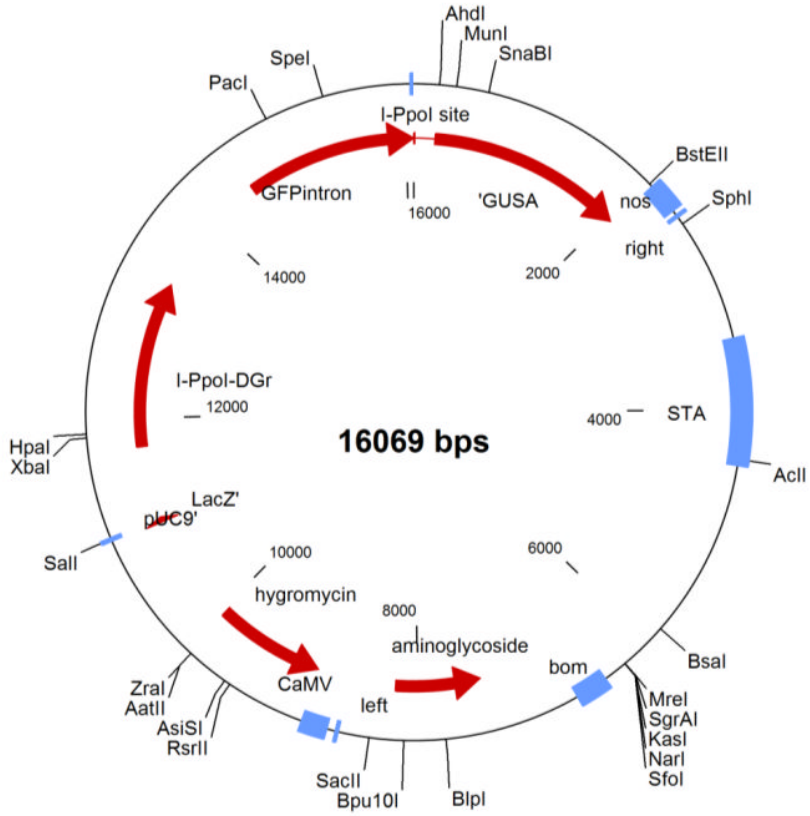


Table S2: QPCR primers

		Forward	Reverse
At4g34270	*Tip 41-like*	gtgaaaactgttggagagaagcaa	tcaactggatacccttcgca
At4g12590		gagatgaaaatgccattgatgac	gcacccagactctttgatg
AT3g48190	*ATM*	gatggccatgaggcattatt	tctcctttgaggaggttaccaa
AT5g40820	*ATR*	ctggagaagcctgagttggt	atgcccaaaccatcaatcat
AT1g66730	*LIG6*	cagaaagctgtttcagggaga	aggaacaaccacgtccagag
AT3G18780	Actin	tccctcagcacattccagcagat	aacgaattctggacctgcctcatc
AT1G49250	AtLIG4	ctttagtttcgaaaagcgaaatg	cttgtagtggatcctcatgg
AT5G20850	RAD51	gttcttgagaagtcttcagaagttag	gctgaaccatctacttgcgcaactac
At5g09810	ACT7	ctttaggatgcttgtgatgatgct	gcgccaatataacaatcgacaata
At1g07500	SMR5	tacggtgacggttgatgatg	gctgctaccaccgagaagaa
At3g27630	SMR7	ccggtgaagacgaaactcat	caccaactcgaaatctgaagg

Supplementary methods

Nucleic acid purification and cloning

DNA procedures and bacterial manipulations were by established protocols (36). RNA was isolated from above ground tissues of flowering Arabidopsis using the SV total RNA isolation kit (Promega) according to the manufacturer's instructions. Plasmid DNA was prepared using QIAGEN columns according to the manufacturer's instructions (Qiagen). cDNA synthesis was performed with Superscript II reverse transcriptase (Invitrogen) and followed by amplification with iProof DNA polymerase (Bio-Rad). Analytical PCR was performed using PCR Reddymix (ABGene) and analyzed by agarose gel electrophoresis. Real-time RT-PCR analysis was performed on a CFX96 thermocycler (Bio-Rad), as described previously (5), using SYBR Green Supermix (Bio-Rad). A plant codon-optimized I-PpoI gene was synthesized (Genscript, SI Appendix, Fig S14) and cloned into pBI-ΔGR and the expression cassette subcloned into pCB1300 carrying a I-PpoI recognition site to create pPPOΔGR (SI Appendix, Fig S15). Propidium iodide (PI) staining was performed using 10µg.ml^{-1} PI in water and EdU labeling was performed as described previously (6) and analyzed on a Zeiss LSM700 Inverted confocal microscope. Quantification of anaphase figures was performed on radicle tissue isolated 6h into the 16h light phase of a 16/8h light dark cycle and analyzed using a Zeiss LSM880 Upright confocal laser scanning Airyscan deconvolution microscope.

Dormancy analysis

Seeds production, harvest, storage and details of seed burial in, and recovery from field soils and post-recovery seed handling are as described previously (7). RNA was extracted from seeds as described elsewhere (8). cDNA synthesis and Quantitative PCR was performed in triplicate on each of three independent biological samples as in ref 13 using a 1/25 dilution of cDNA and the following touchdown PCR thermal cycle: one cycle at 95°C for 10 min followed by 50 cycles at 95 °C for 30s, 70°C (decreasing by 0.2°C/cycle to a target temperature of 67°C) for 30s, and 72 °C for 30s. Data was analyzed using LightCycler® 480 software (version 1.5; Roche Diagnostics). Gene expression levels were determined using a cDNA dilution series of the primer pairs of each gene (SI Appendix, Table S2) of interest with normalization against the combined mean at each time point of the reference genes *At4g34270* (Tip 41-like) and *At4g12590*. These reference genes have highly stable transcripts in Arabidopsis seeds in both microarray and QPCR studies (2, 3, 9).

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